Networking in the endoplasmic reticulum

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Abstract
The network of the ER (endoplasmic reticulum) is set up by cytoskeletal control of the movement and remodelling of polygonal rings of tubules, bundles of tubules and cisternal regions. We have developed a new image analysis tool, persistency mapping, to understand the framework upon which the plant ER remodels. With this new tool, we have explored the network nodes, called anchor/growth sites, that may anchor the network by attachment to the plasma membrane. We have determined how the polygonal ring structure depends on myosin XI-K for ‘opening’ and ‘closing’. With latrunculin B treatment, we have investigated the involvement of actin in the elongation and persistency of the tubules. We also show how the cytoskeleton is involved in directed diffusion within the membrane. This observation may lead to an answer to the question of what function this network structure serves in the cell. We propose that the ER acts as a trafficking network, delivering lipid, protein, calcium and signalling molecules to different regions of the cell. It does so by directional reduced dimensional diffusion. The ER network of tubules restricts the dimensionality of diffusion to near one-dimensional, whereas the cisternae reduce it to near two-dimensional. The cytoskeleton does not drive the diffusion, but participates by providing directionality to the diffusion.

Introduction
The present review focuses on the network aspects of the ER (endoplasmic reticulum), its structure as a network of membranes and its function as delivery network. Although there is much known about its appearance as a network of membranes and the role it serves as the source of the phospholipids and proteins for the endomembrane pathway, how the network arrangement of the ER serves the many functions of the ER is not generally understood. Our work [1] shows that ER ‘movement through remodelling’ in non-dividing plant cells depends on the actomyosin cytoskeleton. It also shows that this cytoskeleton determines the directionality of diffusion in the network. These observations are placed in the context of our current understanding of how traffic within the ER may allow the ER to efficiently network with other organelles.

The ER network and its movement through remodelling
The basic underlying organization of the ER in eukaryotes is a polygonal network of tubules connecting to, and morphing into, sheet-like cisternae (Figure 1A). We start with the simple observation that tubules can form cisternae and cisternae can form tubules (Figure 1B), based on the pioneering work using fluorescently dyed ER and confocal microscopy [2,3] and video-enhanced techniques using unstained samples [4,5]. Our recent work [1] builds on these observations, showing that the ER network in tobacco leaf epidermal cells does not move by global shifting of cisternal or tubular polygons that make up the network. This stands in contrast with the picture generated by the work of Kachar and Reese [6] in Characean cells, where the ER network is proposed to move en masse, motorized by myosins moving along the organized bed of cortical filamentous actin, as shown in standard cell biology texts [7]. In higher plants, there is no bed of organized filamentous actin, but a dynamic network of actin bundles and randomly oriented individual filaments [8]. Likewise, the higher-plant ER does not move en masse. Instead, movement of the ER is achieved through network remodelling, but is, as in Characean cells, dependent on the actomyosin network [1].

What is ‘movement through remodelling’? Tubules can dynamically change their polygonal network by changing branching patterns and moving network nodes (Figure 1B). This is characteristic of generalized network movement, where movement within the structure occurs through the formation or movement of network nodes and branches [9]. Cisternae can generate tubules by producing tubules at their edges or by extensive fenestration (Figure 1B). All of these activities are called remodelling. One way to appreciate the level of remodelling, and hence ER network movement, is with time-lapse movies (see Supplementary Movie S1 at http://www.biochemsoctrans.org/bst/038/bst0380747add.htm). The plant ER is much more dynamic than the animal ER, perhaps reflecting the fact that they are driven by different cytoskeletal systems, as discussed below. Nevertheless, the structural similarity of animal and plant ER networks is remarkable and they may have similar rules for remodelling. Lee and Chen [10] identified three basic events that remodel the network in animal cells: (i) tubules branch

Key words: cytoskeleton, directional diffusion, endoplasmic reticulum, microtubule, persistency map, reduced dimensionality.
Abbreviations used: 1D, one-dimensional; 2D, two-dimensional; 3D, three-dimensional; ER, endoplasmic reticulum; ERNS, endoplasmic reticulum exit site; GFP, green fluorescent protein; paGFP, photoactivatable GFP; RTN, reticulon; RTNLB, reticulon-like protein, plant subfamily B; STED, stimulated emission depletion; STIM1, stromal-interaction molecule 1; YFP, yellow fluorescent protein.

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Figure 1 | The network of tubules and cisternae comprising the ER of plants and its formation

(A) 3D reconstruction using isosurfaces of a BY2 cell expressing HDEL–GFP. The bottom is the view from the inside-out. The top is the view from the outside-in. The upper view clearly shows the network of the cortical reticulum. The lower view shows the nucleus, which in this cell has transnuclear channels and transvacuolar strands connecting with the cell cortex.

(B) Mechanisms of tubule and cisterna formation and removal. See the text for more details.

producing three-way junctions (and tubules extend to form a new polygon); (ii) junctions at the branch points slide; and (iii) when junctions slide so much that they return on themselves, polygonal rings are closed and polygons are eliminated from the network. As shown in Figure 1(B), however, several additional activities are quite common in plants: extended ‘blind-end’ tubules shrink, tubules kink and deform, small cisternae (punctae) are often present at some kinks and three-way junctions, and polygons fill with membrane, generating cisternal structures.

To know the exact extent of cisternalization of a moving network of tubules and cisternae requires super-resolution microscopy because ER tubules are 60 nm in diameter, well below the diffraction limit of light. A recent paper demonstrating subdiffraction limit microscopy of animal cells examined the ER in cells transfected with ER-targeted HDEL–citrine [11]. As shown in Figure 2, regions that could be classified as cisternal regions when viewed with conventional confocal microscopy are bundles of clearly resolvable 60 nm tubules with super-resolution STED (stimulated emission depletion) microscopy.

The underlying idea behind movement through remodeling is that ER network structure, and perhaps, ultimately, function of the ER, is a consequence of the balance between tubule cycles of growth and shrinkage (events 1 and 2 in Figure 1B), tubule and cisternal persistence (events 4 and 5 in Figure 1B, stabilized tubules and stabilized anchor sites), and tubule–cisternal transitions (event 7 in Figure 1B) and cisternal–tubule transitions (event 8 in Figure 1B). Those elements which control one part of the cycle (e.g. tubule growth) do not necessarily control the other (e.g. tubule shrinkage). However, blocking any one part of the cycle could negatively feed back on another and reveal itself as network of tubules that can neither grow nor shrink.

How is the ER network structure formed?

Animal ER is capable of self-assembly into a network of tubules. In vitro experiments using animal cell extracts reveal that a polygonal network of tubules can be generated without any cytoskeletal involvement [12]. The formation of tubules is thought to be mediated by reticulons (RTNs), a family of membrane proteins necessary for in vitro reassembly of Xenopus ER membranes into tubules [13]. In yeast and mammalian cells [13, 14], there are few reticulons, and those that occur, e.g. RTN1 or RTN4a/NogoA, co-localize with ER markers [sec61–GFP (green fluorescent protein) or sec63–GFP] and are only found in tubular domains of the ER. In Arabidopsis, there is a more complicated story. There are 21 genes identified as reticulons [15,16], named RTNLB (reticulon-like protein, plant subfamily B) 1–21. Although fluorescent protein fusions have not been made of all of the reticulons in plants, of those that have been made, several locate to the ER (RTNLB2 and RTNLB4 [15], and RTNLB13 [17]), but only RTNLB2 is reported to be exclusively co-extensive with tubular regions of the ER [15]. Another labels punctae provisionally identified as Golgi (RTNLB4 [15]).
Figure 2 | Subdiffraction-resolution imaging of the ER in a living mammalian cell
Confocal (A) and simultaneously recorded (B) STED (x, y) images from the ER marked by the fluorescent protein citrine targeted to the ER (raw data: 16.3 mW STED focal intensity). Scale bar, 1 mm. The arrowheads indicate rings formed by the tubular network of the ER, which are visible only in the STED image. (C) Confocal and corresponding STED image revealing features of 52 nm full-width at half-maximum (FWHM) as indicated by the profile below (raw data: 35.7 mW STED focal intensity), indicating that the lateral resolution in the STED image is approx. 50 nm. Scale bar, 500 nm. Reproduced from Hein et al. [11] with permission. © 2008 The National Academy of Sciences of the USA.

The model for the mode of action of reticulons is that the two C-terminal hydrophobic domains [RHDs (reticulon homology domains)] insert into the membrane on the cytoplasmic side, occupying more space in the cytoplasmic leaflet than in the inner leaflet, causing the membrane to curve because hydrophobic interactions between the two leaflets tend to keep them coupled together [18]. Overexpression of reticulons in yeast and animal cells never labels their ER cisternal compartment, the nuclear envelope [13], and does not change the tubulation of the ER [18]. During division, reticulons appear to be removed from the ER tubules as they associate with the chromatin, thereby opening the way to cisternalization and formation of the nuclear envelope [19].

Overexpression of reticulons does produce higher degrees of curvature in existing tubules [22]. In line with these observations are those of Tolley et al. [17] who have shown that overexpression of plant RTNLB13 interferes with luminal traffic, perhaps by producing more severe curvature of tubules, resulting in decreased diffusion coefficients as measured using FRAP (fluorescence recovery after photobleaching). This treatment also causes aggregation of luminal GFP retained in the ER with an HDEL tag (HDEL–GFP), as shown in Figure 3. These discrete aggregates, or boluses, move through the ER tubule network co-labelled with reticulin [RTNLB13–YFP (yellow fluorescent protein)].

Cytoskeletal regulation of ER remodelling
The cytoskeleton is involved in remodelling the ER. Animal cells primarily use the microtubule cytoskeleton to guide remodelling of the network in non-dividing cells [21–23], and may switch to actin-mediated movement of the ER during mitosis [24]. Actin-mediated movement may also be involved in the switch to reticular ER at the end of mitosis in *Caenorhabditis elegans* [25]. Non-dividing yeast [26] and most plants [1–3,16] use the actin cytoskeleton to remodel the ER. Oryzalin, a potent antimicrotubule agent for plants, causes the formation of boluses in the ER, but does not...
otherwise influence ER motility in non-dividing cells [1]. Dividing plant cells, on the other hand, move the ER, and components of the nuclear envelope, to the spindle poles in a microtubule-dependent microfilament-independent manner [27]. There is extensive ultrastructural evidence for the association of the ER network with microtubules in dividing plant cells [28]. Consequently, there appears to be cell-cycle-dependent switching of ER motility between the actin-based cytoskeleton and the microtubule-based cytoskeleton in plants and animals.

The movement of cortical ER in Characean algae can be microtubule-mediated as well as microfilament-regulated in elongating cells [29]. Foissner et al. [29] make the interesting distinction between endoplasmic and cortical ER; oryzalin has a larger effect on remodelling the cortical ER, the ER directly beneath the plasma membrane and in the same plane as cortical microtubules, than on the endoplasmic ER that resides deeper in the cytoplasm in these elongating cells. A similar distinction is made for the ER by Orci et al. [30] who induced the formation of cortical ER, directly beneath the plasma membrane, by a treatment that activates SOCE (store-operated calcium entry). This change in ER form is a consequence of the relocalization of STIM1 (stromal-interaction molecule 1), an integral ER membrane protein, to the peripheral ER where it binds to, and activates, Orai1, a plasma membrane calcium channel [31]. As described below, STIM1 is also associated with the tips of growing ER tubules, binding to the growing (plus) ends of microtubules.

Recent work in animal cells provides some insight into possible control mechanisms for ER remodelling. Motors, both kinesin and dynein, play a role in ER tubule movement [21]. The motors confer directionality on the process, with dynein-driven movements directed towards the centre of the cell and kinesin-1-directed movement driving extension towards the cell periphery. In this way, the polarity of the cytoskeleton may provide the polarity of remodelling activity, with more remodelling occurring near the centre of the interphase cell than at the periphery. Growth and shrinkage of ER tubules (events 1 and 2 in Figure 1B) at the periphery may be mediated by the plus-end microtubule-binding protein STIM1, an ER-resident transmembrane protein [22]. STIM1 binds to the microtubule via a TAC (tip-attachment complex) and as the plus end grows or shrinks, so does the ER tubule (event 1 in Figure 1B). Other microtubule-binding proteins on the ER, such as CLIMP-63 (cytoskeleton-linking membrane protein of 63 kDa), might stabilize the ER translocon complexes, giving rise to cisternal rough ER domains [23]. Other scaffolding proteins, such as ribosome-binding protein p180 [32] and polyribosomes [33] may also give rise to cisternal or lamellar peripheral ER (events 4 and 7 in Figure 1B), lending support to the notion that cisternal ER is rough ER, or ER with polyribosomes bound to it. Patterns emerge from this work: both motors and cytoskeletal polymerization are involved in tubule growth, and the polarity of the cytoskeleton translates into polarized distribution of ER remodelling activities in the cell. Can we find these patterns in plant cells?

### Revealing the underlying nature of the ER network by persistency mapping

As the cortical ER changes shape and becomes remodelled in a streaming plant cell, the elements that do not change in this interconnected network may be of organizational importance, being the organizing ‘theme’ upon which variations are played. This initial hypothesis has prompted us to look for and classify spatial domains in cortical ER on the basis of their shape and degree of persistence. In animal and yeast cells, some shape analysis has been done [33], but much of the analysis of ER shape relies upon identification of the nuclear envelope as the cisternal compartment and everything else as tubules. Sparkes et al. [1] extend this shape classification by using a set of image analysis routines that we call persistency mapping. This procedure dissects the cortical ER (not usually containing the nuclear envelope) into domains containing tubular, cisternal or punctate shapes, and spatially maps the persistence of those domains that remain in the network at or near the same place for longer than 8 s, the approximate time for a 4 μm tubule to extend and shrink back (tubule rates of growth and shrinkage being approx. 1.5–2 μm/s).

Figure 4 shows a persistency map of the ER tubular domains in the basal cells of a hypocotyl of a 3-week-old *Arabidopsis* seedling constitutively expressing HDEL–GFP in a genetic background that eliminates the protein bodies of the ER [34]. The persistent tubular domains can be spatially compared with those domains that are rapidly changing or remodelling. Distinctive features of the ER network emerge from these persistency maps of ER tubules. In Figure 4(A), the streaming ER network in basal hypocotyl cells of *Arabidopsis* can be seen in green and the persistent tubules (Figure 4B) are superimposed in black. Bundles of ER tubules rapidly remodel along major transport tracks (red arrows in Figure 4). Some of the most persistent tubules, the blackest tubules in Figures 4(A) and 4(B) and the green tubules in Figure 4(C), map across these regions of active remodelling, but most are parallel to them. Note that the green tubules in Figure 4(C) do not form a linked system of polygonal rings, indicating that the closure and formation of rings in the network is one of the more transient events. Also, note that persistent nodes emerge (black arrows in Figure 4C), which are sometimes found at the blunt-ends of persistent tubules and at three-way junctions of less persistent remodelling tubules. These punctae may be the anchor points in ER networks that have been hypothesized to attach the ER to the plasma membrane [2,3]. From a comprehensive set of experiments that assessed the role of the cytoskeleton in the form and movement of the ER of non-dividing cells [1], the punctae or nodes are called anchor/growth sites because movement relating to the nodes indicates that they are not only persistent, but may also be the site of ER tubule and actin cytoskeleton growth.

Sparkes et al. [1] use two approaches to test the role of the actomyosin system in ER remodelling in tobacco leaf epidermal cells. First, co-expression of HDEL–GFP and the
truncated tail domain of myosin XI-K, the main myosin involved in cytoplasmic streaming of Golgi, mitochondria and peroxisomes [35,36], produces a dominant-negative phenotype, inhibiting the motion of the streaming organelles and producing cells with many more and longer persistent tubules than controls. Furthermore, the persistent tubules retain their polygonal configuration. Secondly, treatment of leaves expressing HDEL–GFP with latrunculin B (which quickly leads to the breakdown of filamentous actin in these cells) also produces more and longer persistent tubules that likewise retain a polygonal arrangement. Both truncated myosin XI-K expression and latrunculin B treatment produce more and larger persistent cisternae. However, the two treatments differ in their effect on the smaller punctae (anchor/growth sites): latrunculin B treatment increases their number, whereas truncated myosin XI-K expression has no effect. The model that arises from this work is that tubules are stabilized in the absence of actin (event 4 in Figure 1B), but actin polymerization may be involved in tubule growth (event 1 in Figure 1B) and the movement or destruction of anchor/growth sites. Myosin XI-K, on the other hand, is involved in tubule shrinkage (event 2 in Figure 1B) and polygon ring closure (event 6 in Figure 1B). For this model to work in the context of the known polar movement of myosin along the actin filament towards the plus, or growing, end, the outgrowth of the tubule would have to be associated with the minus end of the actin filament, whereas myosin-mediated shrinkage would occur towards the plus end, at the site of the anchor point. This would also explain myosin-mediated ring closure, as the sliding tubule would move towards the anchoring triple junction in the plus-end direction (event 6 in Figure 1B). Hence, the patterns found in ER remodelling in animal cells also apply to plants: both motors and cytoskeletal polymerization are probably involved in tubule growth and there may be local polarity determinants in the cytoskeleton that translate into polarized distribution of ER.

Differentiation within the network and distribution to different sites within the network

The ER in plants and animals varies in form with cellular differentiation. For example, as plant cells develop from relatively cytoplasm-rich meristematic cells to elongated vacuolated cells, the ER form changes from predominantly cisternal to predominantly tubular [37]. These may be related to cell-cycle regulation and cell maturation, because the ER network changes with the cell cycle (see above). Certainly, as cells differentiate, different physiological demands are placed on them, requiring new or enhanced functions of the ER, e.g. more secretory protein synthesis [32] or changes in calcium regulation [31]. These activities rely on ER subdomains in plant cells, some of which also occur in animal cells, e.g. the ER–nuclear envelope, the ER–plasma membrane and the ERES (ER exit site) domains. Other ER subdomains are unique to plants, e.g. the ER–oil body, the ER–plastid and the ER–plasmodesmata domains. It is remarkable that all of these domains remain connected by a network of tubules and cisternae, extending from the nuclear envelope outward to the plasma membrane, and, in the case of plasmodesmata, past the plasma membrane to adjacent cells. Does this connectedness have a function?

The functions for associations of the ER with other organelles are protein delivery to the plasma membrane through non-conventional protein secretion routes [38], calcium transport and regulation (ER–mitochondria [39]; ER–plasma membrane [30]), transport of ER-synthesized lipids and sterols to other organelles (ER–Golgi [40];
ER–plasma membrane [41]) and, potentially, transcellular traffic of viral proteins (ER–plasmodesmata [42]) and transcription factors (ER–plasmodesmata [43]). Most of these ER–organelle associations function to deliver ER contents from their site of synthesis to their site of use by diffusion, and it is the purpose of the next section to show that the connectedness of the ER network provides an efficiency of delivery through the reduction of dimensionality and through providing directionality to the diffusion.

**Directional, reduced-dimensional, diffusive movement through the ER network**

The ER network anastomoses throughout the vacuolated plant cell; it not only is in the cell cortex, but also extends throughout the cell via transvacuolar strands. Although this network brings the ER into the vicinity of other organelles, there would be little reason for it to form such a network if it were only to act as a biosynthetic source of membrane or as a regulator of a signalling molecule. A biosynthetically competent vesicle or tubule bound to the organelle of interest would serve just as well. Instead, the ER provides a way to restrict the dimensionality of diffusion [44,45]. Diffusion to a particular target occurs more rapidly if it occurs within a 2D (two-dimensional) sheet of membrane or near-2D cisterna, rather than within a 3D (three-dimensional) sphere. Diffusion occurs even more rapidly along a 1D (one-dimensional) strand of DNA or in a near-1D tubule. Transcription factors that combine 3D diffusion, which explores the local 3D environment, with 1D diffusion along the DNA strand bind to targets at an accelerated rate of two to three orders of magnitude [46] over those that use only 3D diffusion. Reducing the dimensionality of vesicle traffic to a near-2D lamellopod or to a near-1D axon also accelerates the rate by which vesicles find their target membranes [46,47]. The ER cisternae provide near-2D spaces for diffusion and the tubules provide a near-1D space for their luminal and membrane contents. For example, secretory proteins are delivered to the ER by diffusion [48] and that transport is made more efficient by the reduced dimensionality of the ER. Once delivered to the ER, sorting into it is slow and not limited by diffusion [48].

Previous work also shows that diffusion in the ER is directional [1,49], using calnexin–paGFP (photoactivatable GFP). The initial work [49] indicated that movement was non-diffusive and directional, but became diffusive and non-directional when the actin cytoskeleton was disrupted with latrunculin B. Upon re-examination of these data, which photoactivated entire small cisternal regions of ER, a new approach was taken to quickly photoactivate the calnexin–paGFP, thereby eliminating the possibility that the diffusion of activated paGFP could be restricted by diffusion through small connecting tubules. Smaller regions were photoactivated more quickly and it was found that movement was diffusive and directional [1]. In photoactivation experiments examining the effect of truncated myosin XI-K, latrunculin B and brefeldin A on the movement of calnexin–paGFP, the diffusive nature of the movement was not changed by treatment with latrunculin B and truncated myosin XI-K tail co-expression [1]. However, both treatments result in altered directionality of the movement, with the protein diffusing uniformly on either side of the activation spot. Interestingly, brefeldin A does not change the directional nature of the movement, with photoactivated protein becoming directionally displaced from the site of activation.

Providing cytoskeletal-dependent directional pathways in a diffusive network is important because it improves the efficiency of that network [46]. Directional diffusion may be a property of other small tubular systems in plants; it occurs in stromules of chloroplasts [50]. The demands of the cell for directed diffusion of ER materials could include local traffic of sterols, lipids and proteins for secretion, and signalling agents such as flavonoids and auxins, providing exciting possibilities for future research.

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**References**


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