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# The Role of Endocytosis in the Creation of the Cortical Division Zone in Plants

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Ichirou Karahara, L. Andrew Staehelin and Yoshinobu Mineyuki

Additional information is available at the end of the chapter

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## 1. Introduction

Control of the pattern of cell division is essential for the proper development of multicellular organisms. In animal cells, cytokinesis is mediated by a contractile ring in which the cleavage force is produced by an acto-myosin system. Furthermore, the future site of cell division in animal cells (the site where contraction starts in the cell cortex) is determined by the position of the aster during the later stages of mitosis. In contrast, plant cytokinesis involves the assembly of a cell plate from Golgi-derived vesicles. The division site in plants (the cell cortex where the cell plate fuses with the parental cell walls) is defined by a band of cortical microtubules (MTs) – the preprophase band (PPB) of MTs – that mark the division site during prophase. The PPB MTs subsequently disassemble when the cells enter prometaphase. However, some positional information, or positional memory, is retained in the cell cortex/plasma membrane where the PPB of MTs was located, and the cell plate edge grows towards and fuses with this predetermined division site. Thus, how MTs demarcate the future division site during PPB development, and how the division site memory is created and maintained in the PPB region until the end of cytokinesis, are important questions related to the regulation of division plane positioning in plants.

Several potential cell division plane-positioning molecules have been identified, and these have been classified into “positive memory” and “negative memory” types of molecules. However, how these molecules contribute to the creation of positional memory information has yet to be determined. Early electron microscopists reported the presence of vesicles in the forming PPB regions, and it was suggested that these vesicles might contribute to the creation of a PPB memory site (cortical division zone) either via exocytosis or endocytosis. By using high-pressure freezing to preserve the cells for electron microscopical analysis, we have been able to demonstrate that the vesicles are generated by endocytosis, and with the

help of electron tomography, we have been able to quantify the distribution of vesicles in the cell cortex. The latter analysis has demonstrated that clathrin-mediated endocytosis is enhanced in the PPB region compared to the cell cortex outside the PPB or in the cell cortex of interphase cells. Thus, creation of the cortical division zone appears to involve increased rates of clathrin-mediated endocytosis in the PPB region. Based on these results, we propose that removal of membrane proteins by endocytosis at the division site plays a critical role in the formation of PPB "memory" structures. In this chapter, we will discuss in greater detail how endocytosis at the future site of cell division contributes to the regulation of the plane of cell division in plants.

## 2. Creation and demarcation of the cortical division zone<sup>1</sup> in plants

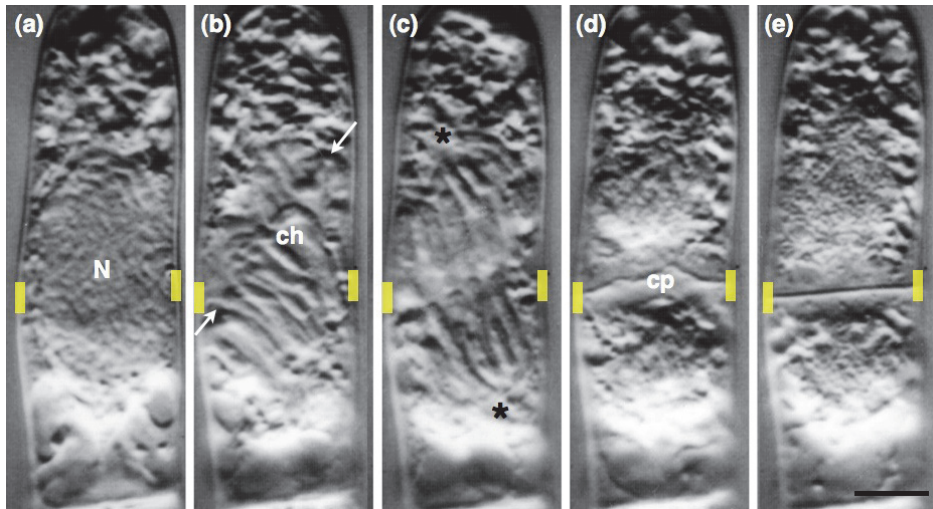
The division site is defined as the region where a new division plane is inserted into a cell at the end of cell division (Gunning, 1982). Since the division plane in animal cells is inserted centripetally from the cell cortex using a contractile ring, the cortical division site corresponds to a region where the cleavage furrow is initiated in the cell cortex. In plant cells, cell plate formation starts with the accumulation of Golgi-derived, cell plate-forming vesicles in the midplane of the phragmoplast MT array in the central region of the cell (Seguí-Simarro et al., 2004). Upon fusion of these vesicles, the cell plate starts to grow centrifugally until it reaches and then fuses with the plasma membrane at the cell division site, the PPB memory site. In the majority of plant cells, the final division plane is inserted in the plane defined by the equatorial plane (the plane where metaphase chromosomes arrange) existed in metaphase, and where the cell plate is initiated at the beginning of cell plate formation. This is not always the case. Figure 1 shows the process of cell division in a *Tradescantia* stamen hair cell where the equatorial plane developed in an oblique orientation (Fig. 1b). Subsequently, however, the cell plate was inserted transversely (Fig. 1e). When the mitotic apparatus of a *Tradescantia* stamen hair cell is displaced experimentally towards the distal end of the cell by centrifugation, the initially formed cell plate develops between the displaced daughter nuclei, but then gradually extends towards the cortical site where it would have been inserted if there had been no centrifugal treatment (Ôta, 1961). This experiment clearly demonstrated that the plant division site is determined prior to the separation of the chromosomes, and that the memory site, where the cell plate fuses to the parental cell wall, is maintained during and after the centrifugal treatment. When and how this cortical division site is established, and how it influences the positioning of the cell plate during cytokinesis remains to be elucidated.

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<sup>1</sup> Normally the PPB is a few micrometer wide and thus this cortical band region is broader than the exact site where the cell plate attaches. Because of this, Van Damme et al. (2011) have proposed the term cortical division zone to distinguish the cortical division site, the exact region where the cell plate attaches to the cell cortex. Here we use this term if we need to distinguish the former PPB region and the exact attachment site of the cell plate.

## 2.1. Proteins involved in preprophase band (PPB) formation and maturation

The most prominent structural change in the region of the future cortical division site is the assembly of a PPB during the G2 and prophase stages of the cell cycle. The PPB is a band of MTs associated with vesicles in the cell cortex (Mineyuki, 1999). Pickett-Heaps & Northcote (1966a, b) provided the first description of the PPB, but did not provide an answer to the question whether the PPB predicts the division site or the position of the equatorial plane in metaphase. This problem was solved in a study of the PPB in onion guard mother cells. Onion guard mother cells are relatively small cells and the equatorial plane in metaphase orients obliquely, but the cell plate is inserted longitudinally (Miehe, 1899). In these cells, the PPB orients longitudinally, thereby predicting the future division site and not the orientation of the equatorial plane (Palevitz & Hepler, 1974). Misorientation of the division planes occurs in cells in which the PPBs are prevented from forming by experimental manipulation (Mineyuki et al., 1991a), as well as in mutant cells that cannot form PPBs (Traas et al., 1995).

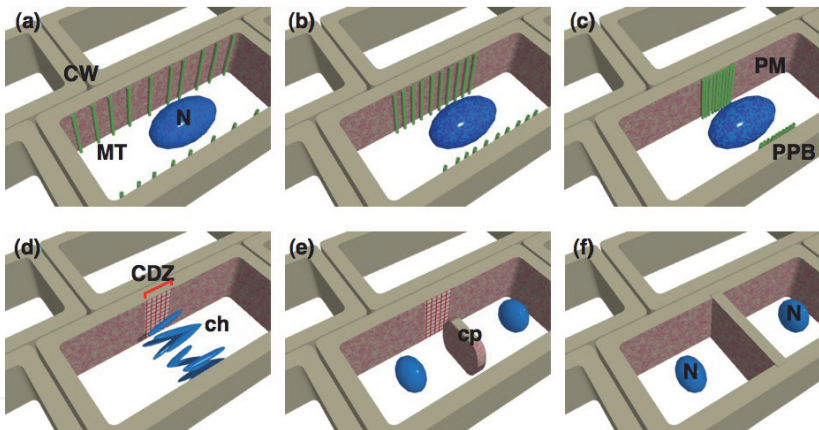


**Figure 1.** Cell division of a stamen hair cell of *Tradescantia virginiana*. (a) prophase, (b) metaphase, (c) anaphase, (d) just after the cell plate has reached the cell wall, (e) 18 min after (d), the cell plate becomes flatten. This cell is the same cell used in the experiment of Fig. 2 in Mineyuki & Gunning (1990). White arrows in (b) show the position of the equatorial plane. Stars (\*) in (c) mark the spindle pole region. Rectangles colored yellow show the cortical division zone. N, nucleus; ch, chromosomes; cp, cell plate. Bar = 10  $\mu$ m.

PPB MTs originate during the G2 phase in the form of a broad band (Fig. 2b), which narrows during prophase. The narrow MT band localizes to the region of the ultimate division site (Fig. 2c). This MT band disappears when the nucleus enters prometaphase but leaves behind positional information that aids in the subsequent orientation and function of the cell plate with the plasma membrane (Fig. 2d, e). Some MT associated proteins (MAPs)

have been shown to be associated with PPBs, and studies on loss-of-function mutants have demonstrated that the MICROTUBULE ORGANIZATION 1 (MOR1) and CLIP-associated proteins (CLASP) are involved in the organization of the MTs in PPBs (Ambrose et al., 2007; Kawamura et al., 2006; Whittington et al., 2001). FASS/TONNEAU2 (TON2), a putative regulatory B" subunit of the Thr/Ser protein phosphatase 2A, and TONNEAU1 (TON1), a protein that interacts with centrin (CEN1) and is related to a human centrosomal protein, are also essential proteins for PPB formation (Camilleri et al., 2002; Traas et al., 1995). As discussed below in greater detail, actin plays a critical role in PPB formation, and the actin-depolymerising drug cytochalasin inhibits the narrowing of the MTs (Eleftheriou & Palevitz, 1992; Mineyuki & Palevitz, 1990).

Besides guiding the cell plate towards the cortical division zone, molecules associated with the PPB memory site also have the ability to induce cell plate flattening. For example, during cell division in *Tradescantia* stamen hair cells, the cell plate tends to be fluid and wrinkled (Fig. 1d) when the cell plate edges attach to the cortical division site, but flattens thereafter (Fig. 1e). The flattening process is delayed or stops when a cell plate fails to reach the correct cortical division zone (Mineyuki & Gunning, 1990).



**Figure 2.** Schematic view of PPB development and the division plane insertion in plants. (a) interphase, (b) early PPB stage (G2~prophase), (c) late PPB stage (late prophase), (d) metaphase, (e) telophase, (f) after cell division. MT (green), microtubule; CW (light brown), cell wall; N (blue), nucleus; PM (pink), plasma membrane; CDZ (red), cortical division zone; ch, chromosome; cp, cell plate.

## 2.2. Candidate proteins of division site memory molecules

The PPB is considered to predict the future site of cell division in plant cells and to generate positional memory information that demarcates the cortical division zone after disappearance of the MTs. Several candidate molecules for the memory function of the cortical division zone have been described (Table 1). The first candidate molecule identified was actin. Although actin serves multiple functions during PPB development, actin

filaments disappear from the PPB zone in late prophase, thereby generating an actin-depleted zone (ADZ) adjacent to the plasma membrane (Cleary et al., 1992; Liu & Palevitz, 1992). Since the ADZ remains after the disappearance of the MT band, the ADZ has been thought of as a kind of "negative memory". Another "negative memory" candidate is the kinesin-like molecule KCA1 (Vanstraelen et al., 2006), which also forms a KCA1 depleted zone (KDZ) in the same area as the ADZ. How these molecules are excluded from the cortical division zone, and how the ADZ and the KDZ are maintained during cell division remains to be determined.

Molecules such as TANGLED (TAN), a highly basic protein that can directly bind to MTs, and RanGAP1, a negative regulator of the small GTPase Ran, are accumulated in the PPB and remain there after the disappearance of the PPB MTs (Rasmussen et al., 2011; Walker et al., 2007; Xu et al., 2008). These are candidates of "positive memory" molecules. Together, the "positive" and "negative" memory molecules may be key players for guiding the cell plate to the predicted cortical division site. PHRAGMOPLAST ORIENTING KINESINs 1 and 2 (POK1, POK2), originally identified as potential partner of TAN in a yeast two-hybrid screen, are required for the correct localization of TAN and RanGAP1 to the PPB region (Müller et al., 2006), and the functional relationship between POK1/POK2 and TAN/RanGAP1 has been examined (Walker et al., 2007; Xu et al., 2008). TAN-interacting proteins DISCORDIA1 (DCD1) and ALTERNATIVE DISCORDIA1 (ADD1), maize homologs of *Arabidopsis* FASS/TON2, are two other proteins that persist at the cortical division zone after disappearance of the PPB MTs. Although DCD1/ADD1 are detectable in the cortical division zone of metaphase cells, they cannot be observed in the cortical division zone in anaphase (Wright et al., 2009).

Molecules, that appear in the cortical division zone just before the cell plate edges reach the plasma membrane, have also been identified. Adaptin-like protein, TPLATE and clathrin reappear in the cortical division zone when the cell plate edge almost reaches the cortical division site (Van Damme et al., 2006, 2011). Whether these molecules are associated with the edge region of the maturing cell plate (Seguí-Simarro et al., 2004) or with structures in the cortical division zone remains to be determined.

Based on the observation of cell plate flattening in *Tradescantia* stamen hair cells, Mineyuki and Gunning (1990) proposed that the PPB leaves behind factors involved in cell plate maturation. A MT-associated protein, AUXIN-INDUCED IN ROOT CULTURES 9 (AIR9) decorates the PPB and phragmoplast MTs and reappears at the cortical division site when the outwardly growing phragmoplast contacts the cortical division site. AIR9, then moves inward on the young cell plate to form a torus-like structure. When the cell plate is inserted outside the former PPB site no AIR9 torus is formed, suggesting that AIR9 associates with proteins that are retained in the PPB site. For this reason, AIR9 is viewed as a candidate factor involved in the regulation of cell plate maturation (Buschmann et al., 2006). A cell wall hydroxyproline-rich glycoprotein (Hall & Cannon, 2002) may also play a role in cell plate maturation.

Molecules that mark the cortical division zone after the disappearance of PPB MTs		Molecules that appear in the cortical zone at the end of cell plate insertion
Candidates of negative memories	Candidates of positive memories	
<p>Actin (Liu &amp; Palevitz, 1992; Cleary et al., 1992)</p> <p>KCA1 (Kinesin-like protein: Vanstraelen et al., 2006)</p>	<p>TAN (Protein having basic MT-binding domain of vertebrate APC proteins: Walker et al. 2007)</p> <p>RanGAP1 (RanGTPase activating protein: Xu et al., 2008)</p> <p>DCD1/ADD1 (Maize homologous of <i>Arabidopsis</i> FASS/TON2: Wright et al., 2009)</p>	<p>TPLATE (Adaptin-like protein: Van Damme et al., 2006)</p> <p>Clathrin (Van Damme et al., 2011)</p> <p>AIR9 (MT associated protein: Buschmann et al., 2006)</p> <p>RSH (?) (Cell wall hydroxyproline-rich glycoprotein: Hall &amp; Cannon, 2002)</p>

**Table 1.** Candidates molecules for modifiers of the cortical division zone.

### 3. Electron tomography of high-pressure frozen cells

Electron tomography is a powerful method for visualizing and quantitatively analyzing the ultrastructural features of cells in three dimensions (Frank, 1992). In the context of PPBs, electron tomography has enabled us to obtain quantitative information on the organization of cortical and cell plate-associated MTs, and on the types and the distribution of vesicles in large volumes of cytoplasm in defined cellular domains (Austin et al., 2005; Karahara et al., 2009; Seguí-Simarro et al., 2004). This method is particularly effective when employed in conjunction with cryo-fixation, which preserves transient membrane systems much better than chemical fixation. We selected epidermal cells of onion cotyledons for the analysis of membrane structures associated with PPBs, because PPB development in this cell type is well characterized (Mineyuki et al., 1989). Most notably, in the basal region of the cotyledons, the percentage of cells undergoing mitosis is relatively high. Specimen preparation was carried out as described previously (Karahara et al., 2009). In short, a basal part of the cotyledon was cut and immediately frozen using a high pressure freezer. The high pressure-frozen samples were freeze-substituted and then embedded in Spurr's resin (Murata et al., 2002). Our electron micrographs of transverse sections of high-pressure frozen/freeze-substituted onion epidermal cells showed exceptionally-well preserved cells at the ultrastructural level (Fig. 3). Late prophase cells with a narrow PPB can be distinguished from interphase cells based on the staining pattern of the chromosomes. After the staining of 250 nm-thick tangential sections with uranyl acetate and Reynold's lead citrate, colloidal

gold particles were added to both sides of the grid as fiducial markers to align the series of tilted images. These thick tangential sections of outer epidermal cell wall regions were mounted in a tilt-rotate specimen holder and observed using either a high-voltage electron microscope operating at 750 kV, or an intermediate-voltage electron microscope operating at 300 kV. The images were taken from +60° to -60° at 1.5° intervals about two orthogonal axes and collected with a digital camera attached to the electron microscopes. Tomograms were computed for each set of aligned tilts using the R-weighted back-projection algorithm. Tomograms were displayed and analyzed with Imod, the graphics component of the IMOD software package (Kremer et al., 1996).

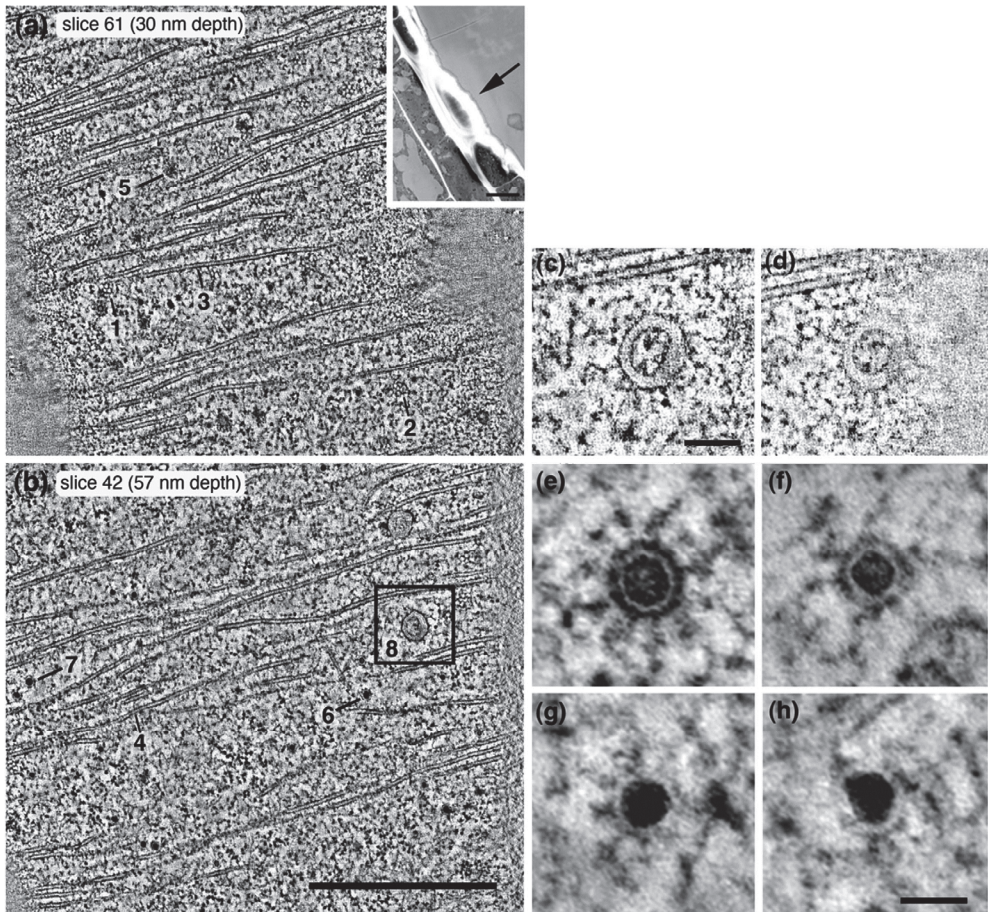
The use of electron tomography has enabled us to identify, map and model the pits, vesicles and MTs of PPB regions in three dimension with a much higher degree of resolution than is possible with conventional ultra-thin sections obtained using an ultramicrotome (Figs. 3a, b & 4). The specimen preparation procedures employed in this study produced characteristic, high-contrast images of the triskelion complexes and lattices associated with the clathrin-coated pits, as well as of the contents of the vesicles. Although most vesicles in the cell cortex examined in the tomographic images were either dark-core, clathrin-coated vesicles (Fig. 3e) or dark-core, non-coated vesicles (Fig. 3h), we did observe some dark-core vesicles with partial coats (Fig. 3f, g). This indicates that the dark-core, non-coated vesicles could be derived from the dark-core, clathrin-coated vesicles.

To test this postulated relationship, we have also quantitatively analyzed the distance between the center of the darkly stained clathrin-coated and non-coated vesicles and the plasma membrane. If the darkly stained, non-clathrin-coated vesicles were derived from clathrin-coated, endocytic vesicles, then, on average, they should be found at a greater distance from the plasma membrane than the clathrin-coated ones. As illustrated in Fig. 5, in the cytoplasm underlying PPBs, the non-coated, darkly stained vesicles were found to be further away from the plasma membrane ( $74.4 \pm 2.6$  nm, mean  $\pm$  SEM,  $n=168$ ) than the clathrin-coated vesicles ( $52.8 \pm 6.4$  nm, mean  $\pm$  SEM,  $n=29$ ). This supports the idea that the non-coated, darkly stained vesicles were the uncoated form of the clathrin-coated vesicles on the way to endosomal compartments.

To confirm that clathrin molecules are present in the PPB, we examined the localization of clathrin in interphase and prophase cells of onion epidermal cells by immunofluorescent microscopy. The anti-clathrin heavy-chain antibodies cross-reacted with two types of intracellular structures in the onion epidermal cells, large, brightly stained objects and small, dim structures. The small, dim fluorescent structures seen in the confocal images correspond to the clathrin-coated pits and vesicles seen in the cell cortex of thin-sectioned cells (see Fig. 6 in Karahara et al., 2009). We have roughly quantified the frequency of the anti-clathrin stained, small, dim fluorescent dots observed in the PPBs of cells visualized by immunofluorescence microscopy (see Table S1 in Karahara et al., 2009). However, because the number of clathrin-containing dim fluorescent dots per square micron was smaller than the number of clathrin-coated pits and vesicles determined by electron tomography, one

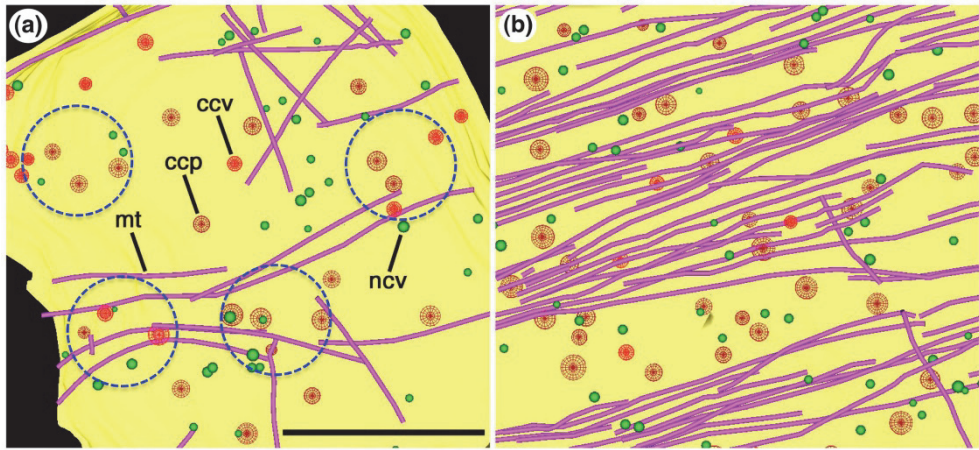


fluorescent dot may in some instances correspond to a cluster of several clathrin-coated pits and vesicles (Fig. 4a, circles of dashed blue lines).

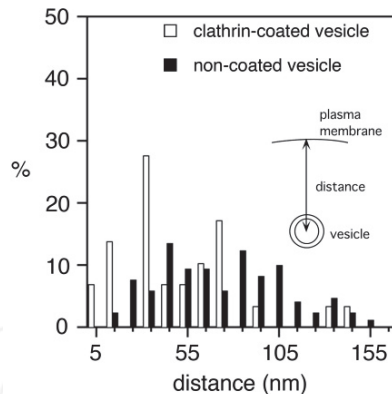


**Figure 3.** Tomographic images of a tangentially-sectioned PPB in a late prophase onion epidermal cell. The tomogram contained a total of 110 slices, with the higher slice numbers showing areas closer to the plasma membrane. Structures 1 and 2: Clathrin lattices associated with shallow pits. Structures 3 and 4: Two cortical MTs. Structure 5: A detached clathrin-coated vesicle. Structures 6 and 7: Partially uncoated and non-coated dark vesicles. Structure 8: Horseshoe-shaped plasma membrane infoldings. (a, b) Two images of 1.42-nm thick tomographic slices. Inset: overview electron micrograph of the 250 nm section used to make the tomogram. (c, d) Higher magnification tomographic slices images of a horseshoe-shaped plasma membrane infoldings shown in the black rectangular in (b). (c) (slice 54) and (d) (slice 60) are different sections through the same horseshoe structure framed in (b). (e-h) Gallery of 21.3-nm thick, composite tomographic slice images illustrating the morphological similarities between clathrin-coated (a), partially-coated (b, c) and non-coated (d) dense-core vesicles. Bars=(a, b) 1  $\mu$ m, (c, d) 1  $\mu$ m, (e-h) 50 nm and (inset Figure in (a)) 10  $\mu$ m. Figure adapted from Karahara et al. (2009).





**Figure 4.** Tomography-based reconstructions of the cortical region at the nuclear level (i.e. PPB region) of an interphase cell (a), at the PPB region of a late prophase cell (b). ccv, clathrin coated vesicle (bright red); ccp, clathrin-coated pit (deep red); ncv, non-coated vesicle (green); mt, MT (purple); pm, plasma membrane (yellow). Clusters of several clathrin-coated pits and vesicles shown in circles of blue broken line, which may correspond to fluorescent dots seen in immunofluorescence photographs. Bar = 1  $\mu$ m. Figure modified from Karahara et al. (2009).



**Figure 5.** Histograms illustrating the distances between the center of the dark-core vesicles (clathrin-coated and non-coated) and the plasma membrane as measured in tomograms of late prophase cells. Open column, clathrin-coated vesicle; closed column, non-coated vesicle.

#### 4. Endocytosis at the future site of cell division

Clathrin-mediated endocytosis is an attractive mechanism for locally changing the composition of the plasma membrane at the PPB site, because clathrin-coated pits are known to concentrate specific types of membrane molecules prior to budding from the plasma membrane (Bonifacino & Traub, 2003; Chen et al., 2011; Kirchhausen, 2000). The original term for endocytosis in plant cell was pinocytosis (Conner & Schmid, 2003), which

included both clathrin-mediated and clathrin-independent endocytosis. The former one is considered to be the major pathway while the importance or even existence of the latter one is still being debated (Chen et al., 2011). Clathrin-mediated endocytosis in plants has been shown to involve molecules, such as adaptor proteins (Holstein, 2002; Takano et al., 2010; Van Damme et al., 2011), accessory adaptor proteins (Bar & Avni, 2009), dynamins (Bednarek & Backues, 2010), small GTPases (Naramoto et al., 2010), actin filaments (Bar et al., 2009; Lam et al., 2001), as well as post-translational protein modifications such as phosphorylation and ubiquitination (Chen et al., 2011). TPLATE, an adaptor-like protein, also appears to participate in endocytic activities associated with cell plate formation during cytokinesis (Van Damme et al., 2004, 2006, 2011).

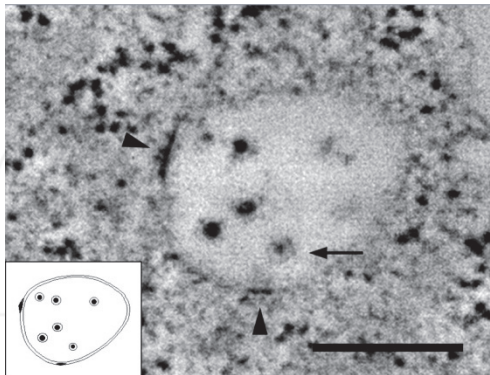
#### 4.1. Endocytic membrane structures in the PPB region

Quantitative analysis of the distribution of the clathrin-coated pits in the cortical region closest to the nucleus of late prophase and of interphase cells showed that the average frequency of clathrin-coated pits between the PPB (nuclear) and the non-PPB (extra nuclear) regions of the plasma membrane was reduced in the non-PPB domains (see Figure 4 in Karahara et al., 2009). Furthermore, the average frequencies of dark-core, clathrin-coated and non-coated vesicles in the cytoplasm underlying the external wall at the nuclear (PPB) and extra nuclear (non-PPB) levels of late prophase cells, and those at the nuclear level of interphase cells, demonstrated that in late prophase cells the frequency of the clathrin-coated vesicles underlying the PPB region was 3.7 fold higher than in the region outside the PPB. On the other hand, the frequency of the dark-core, clathrin-coated vesicles in the PPB region of late prophase cells was two-fold higher than in the interphase cells, and the frequency of dense-core, non-coated vesicles in late prophase cells was over three-fold higher compared to interphase cells (see Table 1 in Karahara et al., 2009). These data demonstrate that the PPB regions are sites of endocytic activity mediated by clathrin-coated vesicles.

To determine whether some of the dense core vesicles underlying the thicker, cuticle-covered outer cell wall of the epidermal cells could be secretory vesicles, we counted all of the vesicles with dark cores in the cortical cytoplasm underlying the inner and outer cell wall regions in serial thin sections of cells sectioned in the plane of their PPBs. No significant differences in the frequency of dark-core vesicles underlying inner and outer cell walls in the PPB region and in the non-PPB regions was observed. However, we did confirm the noted increase in vesicle frequency in the cytoplasm underlying the PPB, both adjacent to the thick outer and the thinner inner cell walls of the epidermal cells (see Table 2 in Karahara et al., 2009).

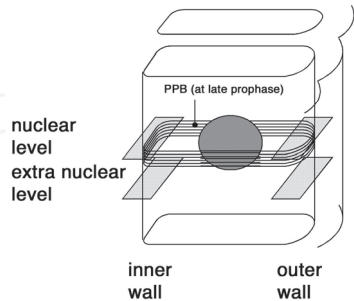
In plants, endocytosed vesicles have been shown to be transported via the trans Golgi network (TGN) to multivesicular bodies (MVBs), where both membrane and cargo molecules are sorted for recycling or for degradation in vacuoles (Haas et al., 2007; Kang et al., 2011; Reyes et al., 2011; Viotti et al., 2010). The onset of clathrin vesicle-mediated endocytosis from cell plates leads to a temporary increase in MVBs in apical meristem cells of *Arabidopsis thaliana* (Seguí-Simarro & Staehelin, 2006). However, in our study of onion epidermal cells we have observed few MVBs in the cortical cytoplasm (Fig. 6) and have been

unable to detect any significant increase in MVBs in the PPB (Table 2). This suggests that the plasma membrane molecules endocytosed from the PPB membrane domains could be recycled back to the plasma membrane via the TGN and not transferred to the MVBs and vacuoles for degradation.



**Figure 6.** Electron micrograph of a thin sectioned multivesicular body (MVB) in a late prophase onion epidermal cell. The MVB contains intraluminal vesicles (arrow). Characteristic electron dense patches are seen on the surface of the MVB membrane (arrowheads). Schematic illustration depicting the MVB is shown (inset). Bar = 50 nm.

	interphase	late prophase	<i>P</i> (cell stage comparison)
nuclear level	0.16 ± 0.07	0.20 ± 0.10	0.59 ( <i>z</i> =-0.53)
extra nuclear level	0.23 ± 0.06	0.11 ± 0.06	0.22 ( <i>z</i> =-1.20)
<i>P</i> (level comparison)	1.00 ( <i>z</i> =0.00)	0.77 ( <i>z</i> =0.29)	



**Table 2.** Average frequency of MVBs observed in PPB (inner and outer cortical regions at nuclear level in late prophase cell) and in non-PPB cortical region (inner and outer cortical regions at extra nuclear level in late prophase cell and inner and outer cortical regions in interphase cell) determined from serially thin cross-sections of onion epidermal cells. The frequency of MVBs was expressed as numbers of MVBs per  $\mu\text{m}^3$  (mean  $\pm$  SEM, *n*=6). Thickness of each section was 70-90 nm. The Mann-Whitney *U*-test (two-tailed) was performed at each level. Schematic illustration depicting the plane of the sections is shown, which is adapted from Karahara et al. (2009).

Enhanced rates of endocytosis confined to PPB regions has also been observed in FM4-64 uptake studies in tobacco BY-2 cells (Dhonukshe et al., 2005). However, in our study, both the tomographic data and immunofluorescent microscopy with anti-clathrin antibodies clearly showed that the frequency of clathrin-bearing structures (clathrin-coated pits and vesicles) does not decrease abruptly at the edge of the PPB region but decreases gradually. Thus, our tomographic models demonstrate that a significant amount of the clathrin-bearing structures are also formed in the region adjacent to the PPB MTs (see Fig. 4 in Karahara et al. 2009). Based on this observation we have postulated that the formation of clathrin-coated pits is not tightly coupled to PPB MTs. Instead, the observed distribution of the MTs and of the endocytic vesicles in the PPB region can be better explained by the hypothesis that the local removal of selected molecules from the plasma membrane via endocytosis creates a membrane gradient in the PPB region that stimulates the assembly of MTs in that region. In this context, the function of the PPB MT array might be both to create a planar reference structure and an associated membrane domain in which the molecules involved in defining the division site can become organized. Therefore, the PPB region can be defined not only as a localized array of MTs but also as a localized region of clathrin-mediated endocytic activity. The fact that the PPB-associated p34<sup>cdc2</sup> kinase homolog (A-type cyclin-dependent kinase CDKA;1) forms a band that is narrower than the PPB (Mineyuki, 1999; Mineyuki et al., 1991b) is consistent with this idea.

## 4.2. Exocytic membrane structures in the PPB region

The outer tangential walls of epidermal cells are considerably thicker than the inner walls. In addition, the outer walls are covered by a cuticle. Since it is possible that there is a difference in secretory activity between the outer and the inner walls in epidermal cells, secretory activities were assessed inside and outside of the PPB region. When a secretory vesicle fuses with the plasma membrane of a plant cell, the vesicle collapses and forms a characteristic, horseshoe-shaped infolding (Staehelin & Chapman, 1987). These horseshoe-shaped membrane structures can be identified in cryofixed and freeze-substituted cells and used as a diagnostic tool for assessing secretory activities (Fig. 3c and d). We have analyzed the distribution of horseshoe-shaped structures in serial thin-sectioned onion epidermal cells and have demonstrated that there was no significant difference between the frequency of horseshoe-shaped structures in the cell cortex at the nuclear level as well as at the extra-nuclear level in the late prophase cells (i.e. PPB region) and in the interphase cells (see Table 3 in Karahara et al., 2009). It has been reported that in 10% of tobacco BY-2 cells there is an increase in Golgi stacks underlying the PPB (Dixit & Cyr, 2002). To determine if onion epidermal cells also accumulate Golgi stacks in the cortical cytoplasm underlying the PPB, we have analyzed the distribution of Golgi stacks in our serial thin-sectioned cells and found that there was no significant difference between the frequency of Golgi stacks in the cell cortex at the nuclear and the extra-nuclear level in late prophase cells and interphase cells (see Table 4 in Karahara et al., 2009).

In a recent study of tobacco BY-2 cells, Toyooka et al. (2009) have described what they claimed was a new exocytic structure, and which they called secretory vesicle cluster.

However, as demonstrated in a recent electron tomography study, the so-called secretory vesicle clusters are free TGN cisternae that release their vesicles by means of cisternal fragmentation prior to the fusion of the individual secretory vesicles with the plasma membrane (Kang et al., 2011).

By quantifying the frequency of secretory structures we have demonstrated that the number of secretory events inside and outside of the PPB is essentially the same and that at this stage of the cell cycle the number of secretory events is low. The paucity of secretory structures observed in the PPB region is also consistent with the conclusion of Dixit and Cyr (2002) that Golgi secretion is not required for marking the PPB site.

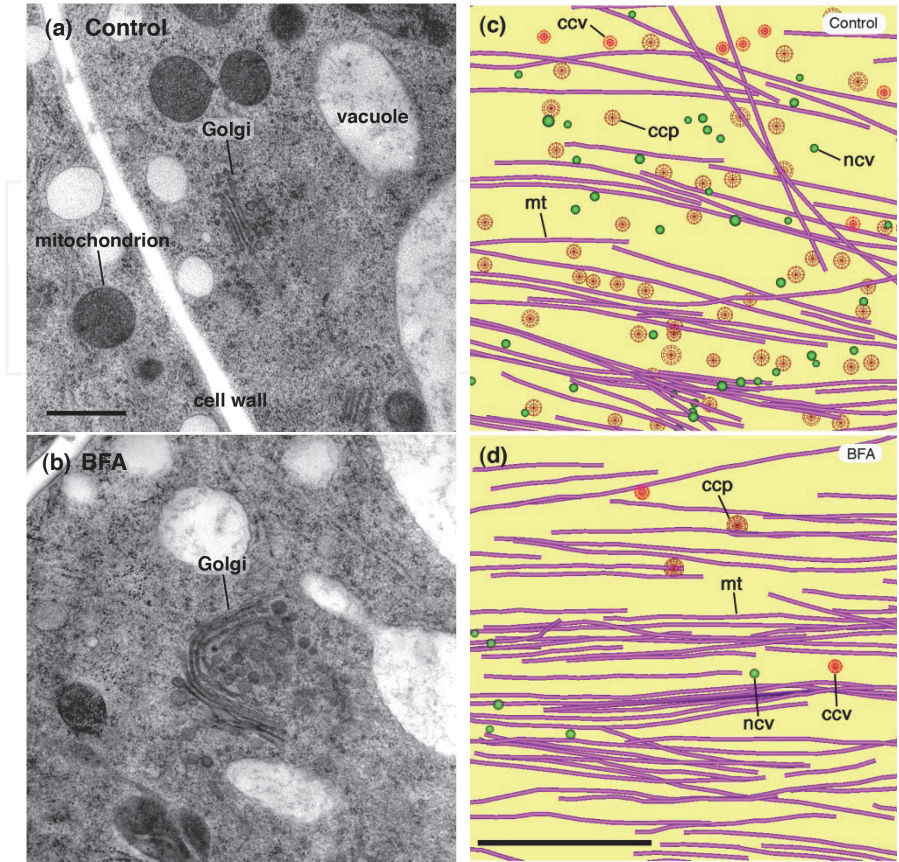
### **4.3. Role of endocytosis in the establishment of the cortical division zone**

The discovery that PPB formation involves increased rates of endocytosis at the PPB zone leads to the question as to what types of plasma membrane molecules could be selectively retrieved from this zone by means of the clathrin-coated vesicles. If molecules, that are necessary for the attachment of actin filaments or KCA1 molecules to the plasma membrane were selectively removed by endocytosis, then this could lead to the formation of actin or KCA1 depleted zones. One class of candidate proteins might be the plasma membrane-associated, actin filament-nucleating proteins called formin homology (FH) proteins (Banno & Chua, 2000; Favery et al., 2004). Several plant formins have been shown to have the ability to nucleate actin filaments, and overexpression of AtFH1 induces the formation of arrays of actin cables that project into the cytoplasm from the plasma membrane (Cheung & Wu, 2004). Thus, one possible function of the enhanced endocytic activity at forming PPBs might be the retrieval of actin-nucleating/binding proteins from these plasma membrane domains to create an actin-free zone to which the expanding cell plate is guided and where it can fuse. A similar function for the removal of KCA1 can also be envisaged. Together, our data suggest a mechanism for how endocytosis could help create a “negative memory” structure in the PPB region of preprophase cells.

## **5. Effects of brefeldin A on the formation of clathrin-coated membrane vesicles at the future division site**

It is known that brefeldin A (BFA) interferes with the functioning of Arf proteins, which are important both for the assembly of COPI as well as for clathrin-coated vesicles that are formed both on TGN cisternae and at the plasma membrane (Nebenführ et al., 2002). To determine if BFA can also inhibit the endocytosis events associated with PPB formation, we examined the effects of BFA on the formation of clathrin-coated pits and vesicles as well as dark core vesicles in the PPB regions of epidermal cells. For the BFA treatment, we made a stock solution in methanol and diluted it in an aqueous solution of 0.1 M sucrose to achieve an effective working concentration of 100  $\mu$ M BFA. The control solution contained 0.2% (v/v) methanol and 0.1 M sucrose. Onion seedlings were treated with the solution for 20 minutes before high-pressure freezing. To evaluate the responses of Golgi stacks to BFA

treatment, three separate tissue regions (each including 5-6 cells) were selected, and the numbers of normal and BFA-perturbed Golgi stacks were determined.

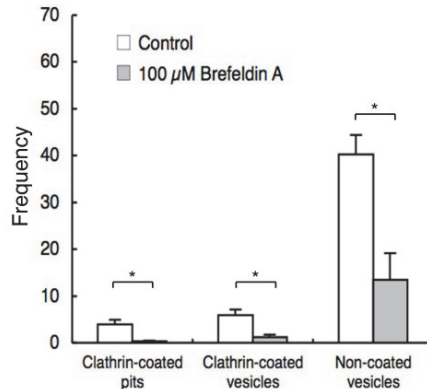


**Figure 7.** Effects of BFA on the Golgi architecture and on the density of clathrin-coated pits and of clathrin-coated and non-coated, dark vesicles in the PPB zone of the cytoplasm of onion epidermal cells. (a, b) Electron micrographs of thin sections showing Golgi architecture observed in a control (a) and a BFA-treated (100  $\mu\text{M}$  for 20 min) (b) onion epidermal cell at the nuclear level. (c, d) Tomography-based reconstructions of the cortical region of a control (c) and a BFA-treated (d) late prophase cell. ccv, clathrin coated vesicle (bright red); ccp, clathrin-coated pit (deep red); ncv, non-coated vesicle (green); mt, MT (purple); pm, plasma membrane (yellow). Bar = (a, b) 0.7  $\mu\text{m}$  and (c, d) 1  $\mu\text{m}$ .

After treatment with BFA for 20 min, the onion epidermal cells contained a mixture of both normal looking and BFA-perturbed Golgi (Fig. 7a, b). In particular, the normal looking Golgi, which made up  $31 \pm 3\%$  (mean  $\pm$  SEM) of the total Golgi population, displayed polar stack architecture together with one or several TGN cisternae, and resembled control Golgi (Fig. 7a). In contrast, the BFA-perturbed Golgi ( $69 \pm 3\%$ ) consisted of stacks that lacked a polar architecture, and whose cisternae resembled wider than normal and curved trans



cisternae (Fig. 7b). Many secretory-type vesicles ( $84.3 \pm 3.5$  nm (diameter), mean  $\pm$  SEM,  $n=73$ ) were also seen in the vicinity of the altered stacks. In contrast to the variable appearance of the Golgi/TGN units in the BFA-treated cells, the responses of the endocytic membrane compartments to BFA were both pronounced and consistent.



**Figure 8.** Average densities of clathrin-coated pits and vesicles at the nuclear level of late prophase (PPB region) of BFA-treated (100  $\mu$ M for 20 min) cells. The results are based on measurements made on tomographic data sets. The frequency is expressed as number of pits per  $\mu\text{m}^2$  in the case of clathrin coated pits, and numbers of vesicles per  $\mu\text{m}^3$  in the case of vesicles (mean  $\pm$  SEM;  $n=3$ ). The Mann-Whitney *U*-test (two-tailed) was used to determine whether the difference were statistically significant compared with the control. \*;  $P=0.0495$ ,  $z=-1.964$ .

The number of clathrin-coated pits was decreased by ~90%, the number of clathrin-coated vesicles by ~80%, and the number of non-coated, dark-core vesicles by 67% (Fig. 8), consistent with the hypothesis that these three structures are causally related and involved in the endocytic pathway. By limiting the exposure time of the seedlings to BFA to 20 min, we have been able to differentially perturb the secretory and endocytic pathways, and thereby obtain data that are consistent with the hypothesis that the dense-core, non-coated vesicles underlying the plasma membrane are derived from clathrin-coated pits and vesicles that originate at the plasma membrane, and that they are not Golgi-derived secretory vesicles.

## 6. Conclusion

How the PPB marks the future site of cell division has been the subject of many studies and discussions since its discovery in 1966 (Pickett-Heaps & Northcote, 1966a, b). In a recent paper, we have quantified the distribution of clathrin-coated pits and vesicles as well as of secretory structures during PPB formation in onion epidermal cells using a combination of high-pressure freezing and electron tomography techniques. This quantitative characterization demonstrated that the rate of endocytosis is enhanced in PPB regions and suggests that the reported changes in composition of the plasma membrane of PPB regions could be brought about by the selective removal of specific plasma membrane molecules via

BFA-sensitive, clathrin-coated pits and vesicles. One possible function of the enhanced endocytic activity at forming PPBs might be the retrieval of actin-nucleating/binding proteins or KCA1 from these plasma membrane domains to create membrane zones that are depleted of such molecules. In turn, these modified plasma membrane regions could help guide the expanding cell plate to the division site and facilitate fusion of the cell plate margins to that site (Karahara et al., 2010). Thus, endocytosis appears to play an essential role in the creation of PPB "memory" structures in plants.

## Author details

Ichirou Karahara  
University of Toyama, Japan

L. Andrew Staehelin  
University of Colorado, USA

Yoshinobu Mineyuki\*  
University of Hyogo, Japan

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\* Corresponding Author

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