

Cell Cycle-Dependent Targeting of a Kinesin at the Plasma Membrane Demarcates the Division Site in Plant Cells

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Summary

Eukaryotic cells have developed different mechanisms to establish the division plane [1]. In plants, the position is determined before the onset of mitosis by the preprophase band (PPB) [2, 3]. This ring of microtubules surrounds the nucleus and disappears completely by prometaphase. An unknown marker is left behind by the PPB, providing the necessary spatial cues during cytokinesis. At the position of the PPB, cortical actin is removed or modified to generate an actin-depleted zone that was proposed to provide the structural means for phragmoplast guidance [4–6]. Here, we identify a plasma membrane domain that emerges at the onset of mitosis and persists until the end of cytokinesis. The narrow band in the plasma membrane corresponds to the position of the PPB and is prevented from accumulation of a GFP-tagged kinesin GFP-KCA1; hence, it is called the KCA-depleted zone (KDZ). The KDZ demarcates the cortical division site independent from the mitotic cytoskeleton. Cell divisions in the absence of a KDZ resulted in misplaced cell plates, suggesting that the PPB transmits a signal to the plasma membrane required for correct cell plate guidance and vesicular targeting to the cortical division site.

Results and Discussion

A KCA-Depleted Zone in the Plasma Membrane Demarcates the Division Site

Previously, we showed that GFP-tagged kinesin KCA concentrates at expanding cell plates in BY-2 cells [7]. To investigate the localization of endogenous KCA, an antibody was raised against the KCA stalk domain (α KCA;stalk) that recognizes KCA on Western blot (see Figure S1 in the Supplemental Data available with this article online). Immunolocalization analysis of *Arabidopsis thaliana* root tips supports the association of KCA with the cell plate and in addition suggests that it is also targeted to the plasma membrane (Figure S1).

Confocal microscopy of GFP-tagged KCA1 in BY-2 cells was consistent with accumulation at the cell plate and the plasma membrane (Figure 1A and Figure S2). The fluorescence intensity at the plasma membrane was strongest in dividing cells and showed a sharp increase in cells that entered mitosis (Figure 1B). Once the cell plate reached the mother cell wall, plasma membrane-associated fluorescence quickly decreased to the levels from before mitosis (Figure 1B).

The predicted structure of KCA1 does not contain membrane-targeting signals. It consists of a tripartite domain organization typical for kinesins, with a head, stalk, and tail (Figure 2A). To investigate the targeting properties of the KCA1 domains, we examined the localization patterns of GFP-KCA1 deletion fragments (Figures 2B–2E). A KCA1 fragment, lacking the N-terminal sequence, the motor domain, and the stalk, concentrated at the midline of the phragmoplast, indicating that accumulation at the cell plate relies on targeting signals present in the tail and does not depend on the motor domain (Figures 2B–2D) [7]. A different behavior was noticed with regard to plasma membrane targeting. The concentration of the GFP-tagged deletion fragments at the plasma membrane was analyzed by fluorescence intensity measurements of diving cells stained with the membrane dye FM4-64 (Figures 2F–2J). The fluorescence plot at the plasma membrane intersection produced a sharp FM4-64 peak that coincided with GFP fluorescence from GFP-KCA1-del-Ncoil or GFP-KCA1-del-Nmotor. Deletion of the central stalk (GFP-KCA1-del-Nstalk) caused the loss of plasma membrane association (Figures 2I and 2J). Thus, plasma membrane targeting requires the stalk domain whereas cell plate targeting does not. Brefeldin A (BFA) is an inhibitor of ARF-GTPase-dependent Golgi-ER traffic in BY-2 cells [8], preventing the targeting of Golgi-derived vesicles to the cell plate. GFP-KCA1-expressing cells challenged with BFA produced nonfluorescent cell plates; however, GFP fluorescence still accumulated at the plasma membrane (Figure S3). Therefore, plasma membrane targeting of GFP-KCA does not depend on BFA-sensitive ER-Golgi vesicular trafficking, whereas targeting to the cell plate does.

Previously, it was shown that the stalk domain binds to the cyclin-dependent kinase CDKA;1 [7, 9]. Next to the stalk, the tail domain contains a cluster of putative serine/threonine phosphorylation sites (Figure S4). The functional significance of S⁸⁴¹ and S⁸⁴⁵, two adjacent, putative CDKA;1 phosphorylation sites, was assessed by site-directed mutagenesis and analysis of the localization of GFP-tagged recombinant protein. KCA with serines (S⁸⁴¹ and S⁸⁴⁵) replaced by alanine was targeted to the cell plate and the plasma membrane in mitotic cells, similar to the localization of unmodified GFP-tagged KCA protein (Figure S4). When the same serines were changed to glutamate residues, the resulting recombinant protein no longer associated with the cell plate or plasma membrane (Figure S4). These data point

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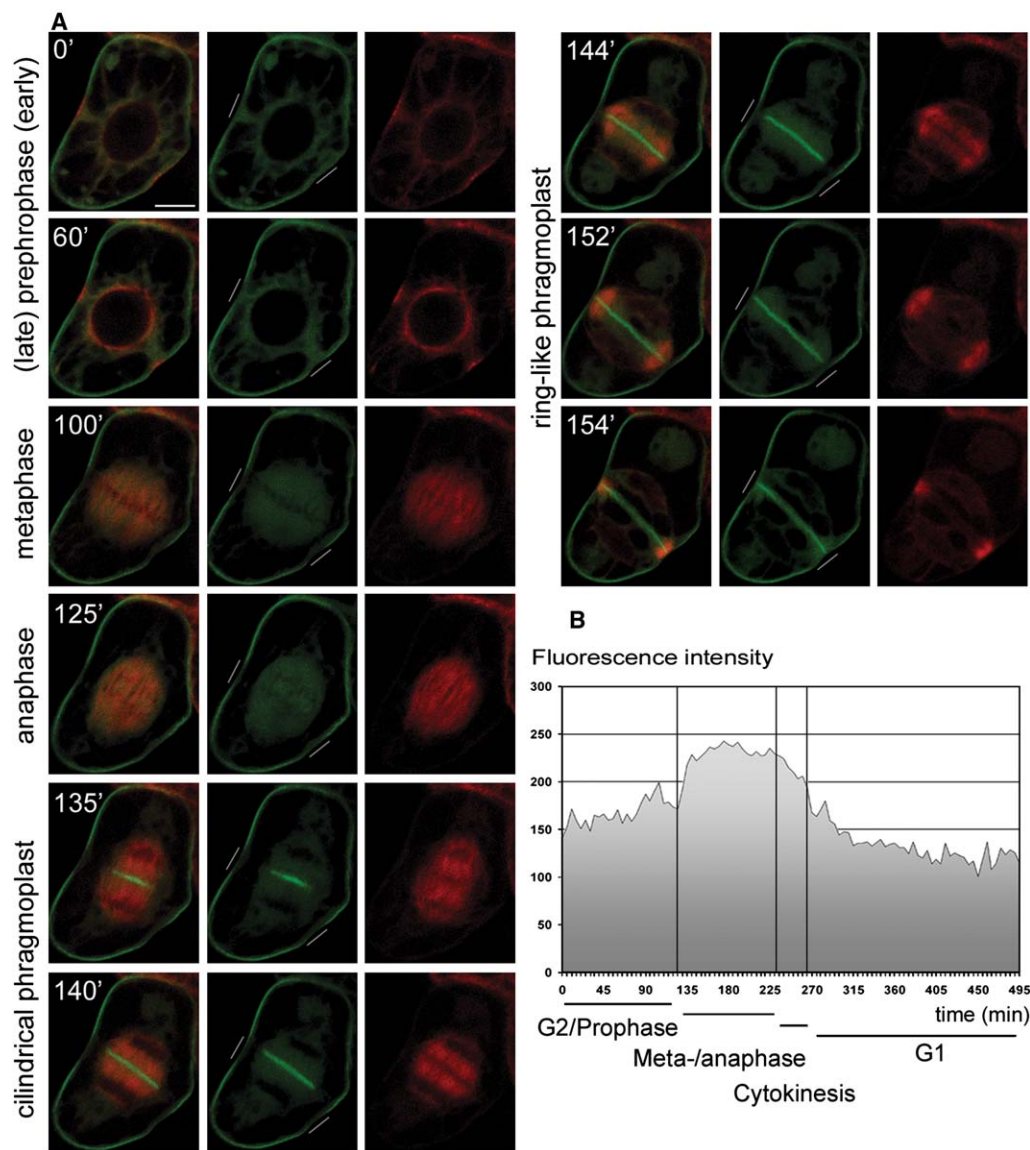


Figure 1. GFP-KCA1 Accumulates at the Cell Plate and the Plasma Membrane during Cell Division

(A) Time-lapse photography of a BY-2 cell expressing GFP-KCA1 and TUA2-RFP throughout mitosis. The different mitotic stages are indicated at the left. The left column shows the merged fluorescence, the middle GFP-KCA1 in green, and the right TUA2-RFP in red. Time points are indicated in the top left corner in minutes. The brackets in the middle panel indicate the position of the final division site. Scale bar equals 10 μm . (B) Quantification of fluorescence intensity at the plasma membrane throughout the cell cycle. The absolute fluorescence intensity (y axis) indicated as arbitrary units is plotted as a function of time (x axis). The corresponding mitotic phase is indicated below.

to a role for S⁸⁴¹ and S⁸⁴⁵ in cell plate as well as plasma membrane targeting. As the affected serines locate at the junction of the stalk and the tail where also flexible hinge regions occur, conformational changes may be inflicted by their (de-)phosphorylation. The failure of the serine to glutamate mutant to localize suggests that dephosphorylation of S⁸⁴¹ and S⁸⁴⁵ is required for cell plate and plasma membrane targeting.

The GFP-KCA1 label in the plasma membrane was not homogenous and showed a zone at the equatorial plane where it was reduced by 3- to 4-fold (Figures 1 and 2). We therefore compared the distribution of GFP-KCA1 fluorescence to that of the styryl membrane dye FM4-64 and the plasma membrane marker AtFH6-GFP [10, 11]

(Figure 2F, Figure S5). In contrast to GFP-KCA1, FM4-64 and AtFH6-GFP labeled the plasma membrane without showing regions of reduced fluorescence. Green and red fluorescence were quantified along a short plasma membrane stretch in a dividing cell (Figure 2F). The yellow/orange color at the plasma membrane in Figure 2 indicates colocalization of GFP-KCA1 and FM4-64 during metaphase, except for a narrow zone surrounding the spindle. The size of the depleted zone was on average 9.7 μm ($\geq 50\%$ reduction in fluorescence, $n = 35$) and occurred at a position opposite to the leading edges of the developing cell plate (Figures 2B and 2C). The region with reduced GFP-KCA1 fluorescence was called the KCA-depleted zone or KDZ.

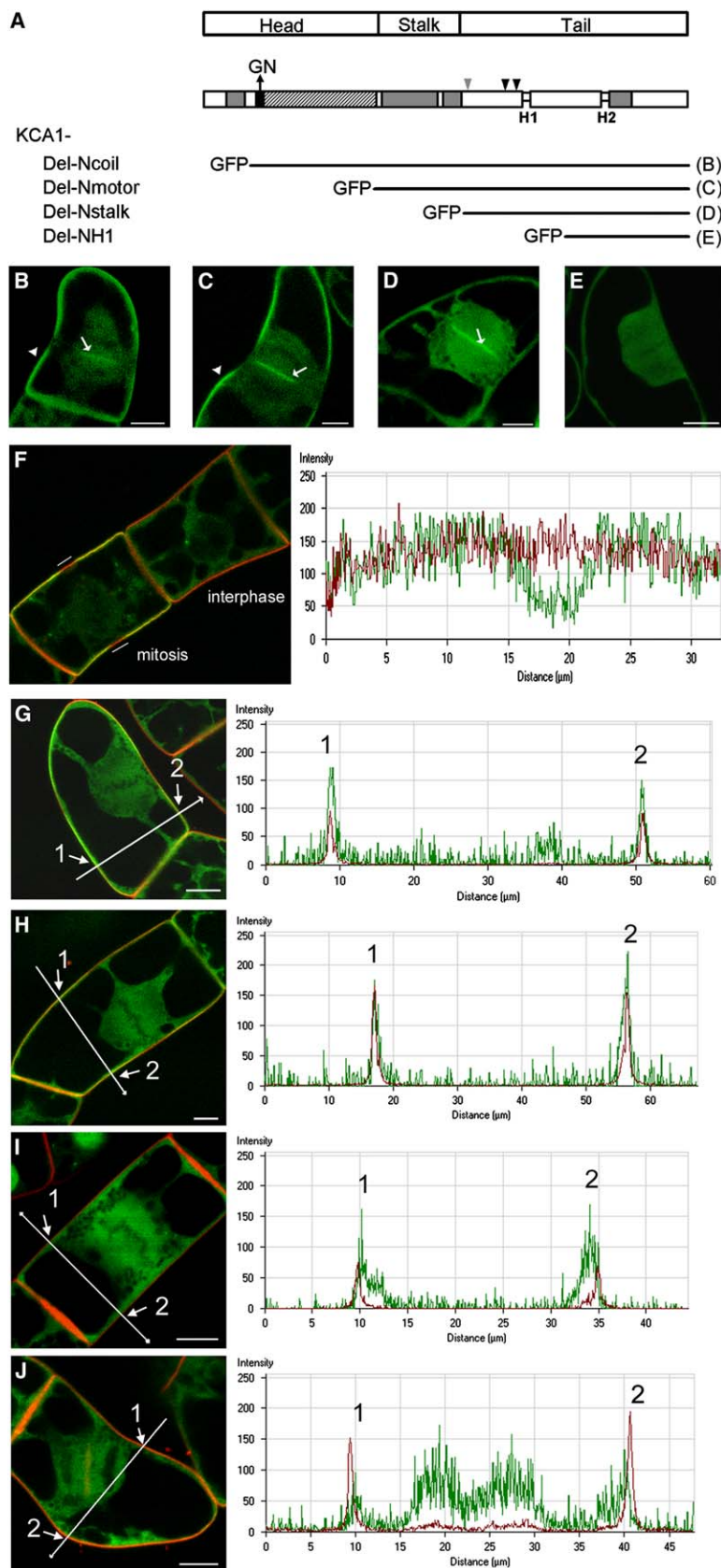


Figure 2. GFP-KCA1 Is Excluded from a Cortical Zone at the Equator during Division

(A) Secondary structure and domain organization of KCA1. Neck with conserved GN motif for minus end-directed motility; black box, motor domain; dashed box, coiled coils; gray boxes, CDKA1 phosphorylation sites; arrowheads (black: sites present both in KCA1 and its homolog) and hinge regions, H1 and H2. Nested KCA1 N-terminal deletion fragments were constructed with GFP fused N-terminally.

(B–E) Localization of the KCA1 fragments in dividing BY-2 cells: GFP-KCA1-del-Ncoil (B), GFP-KCA1-del-Nmotor (C), GFP-KCA1-del-Nstalk (D), GFP-KCA1-del-NH1 (E). GFP-KCA1 signal at the cell plate (arrow) and at the plasma membrane (arrowhead) are indicated.

(F) GFP-KCA1 accumulates at the plasma membrane during mitosis, except at region near the equator (GFP-KCA1 in green, FM4-64 in red). The image shows a cell file with an interphase cell (little GFP-KCA1 fluorescence at the plasma membrane) and an adjacent mitotic cell with strong colocalization of GFP-KCA1 and FM4-64 at the plasma membrane, except at the equator (bar). Fluorescence intensity was measured along the plasma membrane. A 3-fold reduction of GFP-KCA1 fluorescence was observed at the equatorial region, whereas FM4-64 fluorescence remained constant along the plasma membrane.

(G–J) FM4-64-stained cells expressing the nested KCA1 deletion fragments (KCA1-del-Ncoil [G], KCA1-del-Nmotor [H], del-Nstalk [I], and del-NH1 [J]). Red and green fluorescence intensity plots across the white line shown in the pictures are indicated on the right. 1 and 2 point to the plasma membrane intersection. GFP-KCA1-del-Ncoil and GFP-KCA1-del-Nmotor show a fluorescence peak at the plasma membrane, whereas GFP-del-Nstalk and GFP-del-NH1 do not. Scale bar equals 10 μm.

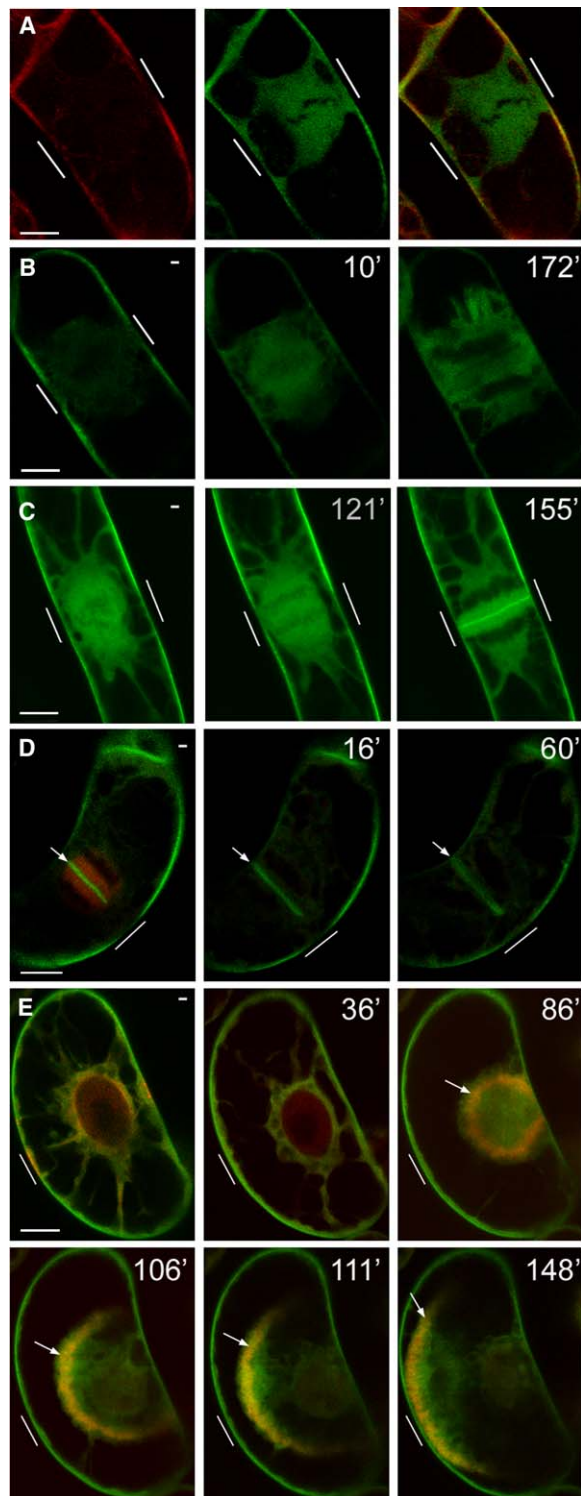


Figure 3. The KCA-Depleted Zone Depends on the PPB
(A) Confocal section of a metaphase cell expressing ABD2-RFP (red), GFP-KCA1 (green). Merged image is at the right. The KDZ colocalizes with the actin-depleted zone (bar).
(B) Confocal section of a metaphase cell producing ABD2-GFP (green) treated with LatB. Actin filaments and the actin-depleted zone are destroyed in 10 min.
(C) Time-lapse recording of a GFP-KCA1-producing cell treated with LatB. The drug treatment does not affect the localization of GFP-KCA1.
(D) Time-lapse recording of a GFP-KCA1/TUA2-RFP-producing cell treated with microtubule-destabilizing agent amiprophos. Microtubules are lost in 16 min whereas cell plate GFP-KCA1 targeting (arrow) and KDZ formation (bar) are unaffected. After 60 min, the overall fluorescence intensity at the plasma membrane decreased.
(E) Cell plate positioning in the absence of a KDZ. GFP-KCA1/TUA2-RFP cells with a PPB (red band at either side of the cell) were treated with propyzamide in early preprophase. The drug was washed out after TUA2-RFP-labeled MTs had depolymerized (36 min) and cells were followed throughout cell division (86–148 min). The PPB was not reconstructed and no corresponding KDZ was established. At cytokinesis, the division plane had tilted 90°. The phragmoplast was formed in the same plane as the confocal section and expanded as a ring-like structure (arrow, 86–148 min). Bars indicate the former position of the PPB. The cell plate (green area within the red ring-like phragmoplast) was not inserted at the original position of the PPB. Scale bar equals 10 μ m.

At the end of G2, the cortical MTs disassemble and are replaced by PPB MTs. Actin remains at the cell cortex throughout cell division and disassembles only at the equatorial plane, resulting in the actin-depleted zone (ADZ) [4–6]. To visualize actin together with the KCA fusion protein, the actin binding domain 2 of fimbrin was fused N-terminally to RFP (ABD2-RFP) and stably transformed in GFP-KCA1 BY-2 cells. ABD2-RFP associated with the fine meshwork of actin filaments at the cell cortex except at the division site. Dual emission imaging showed that the ADZ and the KDZ colocalize (Figure 3A). Cells challenged with actin-destabilizing latrunculin B (Lat B), or swinolidide, lost their actin filament network within 10 min and consequently also the ADZ disappeared (Figure 3B). Therefore, cells were imaged after 20 min of treatment. A noticeable difference was that the progression from metaphase till the end of cytokinesis took much longer (3 hr 26 min for the cell shown in Figure 3B) than typically recorded for untreated cells (about 60 min). Nevertheless, cells progressed through mitosis and cytokinesis ($n = 41$). In these cells, we observed the KDZ similar to untreated cells (Figure 3C).

The KDZ Depends on PPB Formation

In several time-lapse recordings, we observed that at first the axis of the growing cell plate did not align with the position of the KDZ. Remarkably, in each of these cases, the position of the cell plate was readjusted to fit with that of the KDZ so that the plate eventually fused with the mother cell wall at the site that was marked by the KDZ. An example of such an event is shown in Figure 1A. At time point 135 min into mitosis, the cell plate matched with the KDZ at one side of the cell but not with the opposite side. In the following recordings, the entire phragmoplast and cell plate had moved slightly so that now both of the leading edges pointed toward the KDZ at either side of the cell (Figure 1A). Thus, the KDZ marked a zone to which the cell plate was guided. It has been proposed that the PPB establishes the division site by steering the localized deposition of factors necessary for cell plate guidance and insertion prior to mitosis [12]. This feature of the PPB prompted us to ask two questions: does the KDZ match with the former position of the PPB, and is KDZ formation coincident/dependent on PPB formation? Time-lapse recordings of BY-2 cells expressing the microtubule marker TUA2-RFP and GFP-KCA showed that the KDZ was established during

(D) Time-lapse recording of a GFP-KCA1/TUA2-RFP-producing cell treated with microtubule-destabilizing agent amiprophos. Microtubules are lost in 16 min whereas cell plate GFP-KCA1 targeting (arrow) and KDZ formation (bar) are unaffected. After 60 min, the overall fluorescence intensity at the plasma membrane decreased.

(E) Cell plate positioning in the absence of a KDZ. GFP-KCA1/TUA2-RFP cells with a PPB (red band at either side of the cell) were treated with propyzamide in early preprophase. The drug was washed out after TUA2-RFP-labeled MTs had depolymerized (36 min) and cells were followed throughout cell division (86–148 min). The PPB was not reconstructed and no corresponding KDZ was established. At cytokinesis, the division plane had tilted 90°. The phragmoplast was formed in the same plane as the confocal section and expanded as a ring-like structure (arrow, 86–148 min). Bars indicate the former position of the PPB. The cell plate (green area within the red ring-like phragmoplast) was not inserted at the original position of the PPB. Scale bar equals 10 μ m.

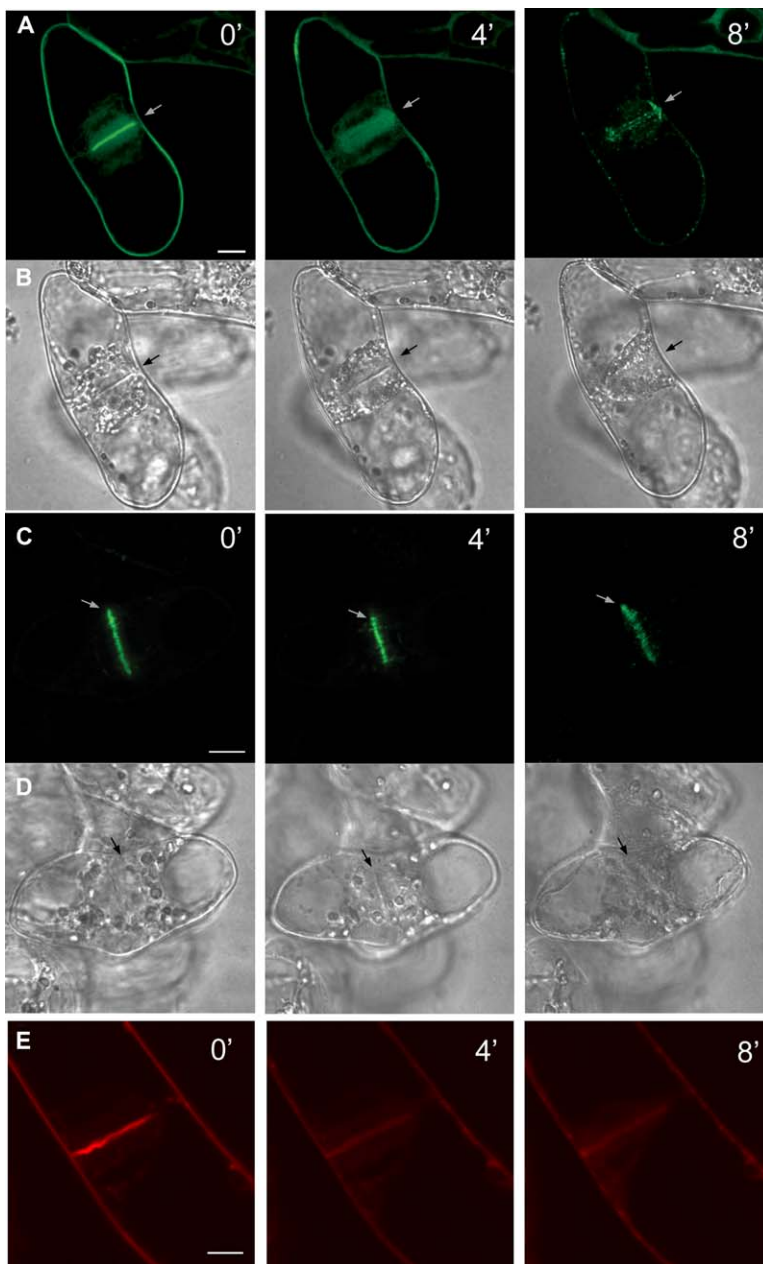


Figure 4. GFP-KCA1 Associates with Microdomains

(A and B) GFP-KCA1 fluorescence pattern (A) and differential interference contrast (DIC) (B) image of a BY-2 cell treated with 0.1% Triton X-100. GFP-KCA1 fluorescence reduced at the cell plate and plasma membrane in 4 min without morphological changes. Longer incubation resulted in a dotted pattern at the plasma membrane and the cell plate. (C and D) The microtubule binding protein MAP65-3-GFP does not dissociate from the cell plate in the presence of Triton X-100. (E) FM4-64-stained, wild-type BY-2 cell treated with Triton X-100. Notice that the treatment does not result in the formation of aggregates. The black and white arrows indicate the position of the cell plate. Times after Triton X-100 addition are indicated at the top right. Scale bar equals 10 μm .

preprophase and, unlike the PPB, that it persisted until the cell plate inserted into the sidewall (Figure 1). The PPB may therefore be necessary for the formation of a KDZ, but it is clearly not engaged in its preservation. The KDZ was unambiguously present when the PPB started to narrow down and thus its formation goes hand in hand with PPB formation (compare time points 0 min and 60 min in Figure 1). We observed that in all other independent observations, the position of the KDZ invariably corresponded to that of the PPB. This reinforced a possible interdependence of the two structures. To investigate the relationship between the PPB and the KDZ, MT-destabilizing drugs were added to dividing GFP-KCA1/TUA2-RFP cells. Disappearance of TUA2-RFP red fluorescence was indicative of the depolymerization of the microtubules. Amiprophos-methyl (APM) added to a cell in early cytokinesis cleared phragmoplast

microtubules within 16 min (Figure 3D). The KDZ was unaffected by APM, and even after 60 min of treatment, only a small reduction in GFP-KCA1 concentration at the cell plate and the plasma membrane was detected. Microtubules were not required to keep GFP-KCA1 at the cell plate or to maintain a KDZ in the plasma membrane. Application of propyzamide in the early stages of PPB formation allowed the reversible destruction of MTs (Figure 3E). After wash out, most of the cells were able to reconstitute a PPB as well as a KDZ. As the cells continued mitosis, they formed a cell plate that was correctly positioned ($n = 43$). Cells that did not produce a new PPB did not form a KDZ ($n = 5$). Despite the absence of a PPB or a KDZ, mitosis was completed, producing a cell plate that expanded and finally fused with the cell wall. However, the cell plate in these cells was not positioned in accordance with the expected division

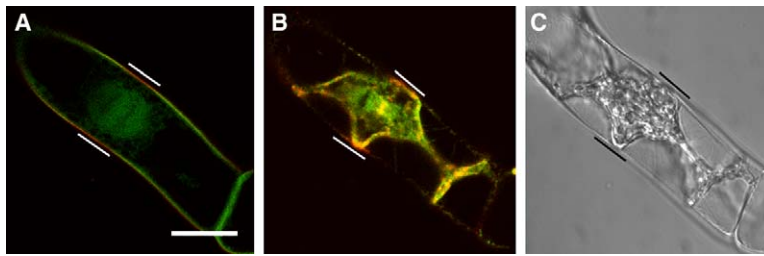


Figure 5. The KDZ Is Anchored to the Cell Wall by Membrane Linkers

Dividing GFP-KCA1 BY-2 cells were stained with FM4-64 and placed in hypertonic solution (0.3 M sucrose). The cell in (A) is imaged just prior the osmotic treatment at early anaphase. The KDZ is evident from red FM-4-64 fluorescence and is marked by white bars. (B) Upon plasmolysis, a plasma membrane-cell wall attachment zone at the same position as the KDZ emerges. The retracted plasma membrane is evident from the DIC image in (C). Scale bar equals 10 μ m.

plane. An example is given in Figure 3E where the cell plate had turned 90° to the position of the original PPB so that it cleaved the sister cells over the longest axis, a division that is very rare in BY-2 cultures. Previously, it had been reported that BY-2 cells occasionally produce aberrant PPB structures [13]. We observed double and bifurcated KDZ structures in BY-2 cells transformed with GFP-KCA1 (Figure S6). In these instances, a single KDZ position was chosen as the division plane. A similar selection of the division site takes place in cells with a doubled or bifurcated PPB, further supporting the intricate relationship of the KDZ and the PPB.

A Role for the Plasma Membrane in Determining the Cortical Division Site in Plant Cells

At later stages after the PPB is removed, the KDZ persists independent from the cytoskeleton. If neither actin nor microtubules are required for excluding GFP-KCA1 from the KDZ, what is? In fission yeast, a sterol-rich membrane domain forms at the cell division sites [14, 15]. Sterol-containing membrane domains can be disturbed by mild detergent treatments [16–18]. Addition of 0.1% Triton X-100 removed GFP-KCA1 from the plasma membrane and cell plate in 4 min without changing the cellular morphology (Figures 4A and 4B). Similar treatments did not dissolve the microtubule-associated protein AtMAP65-3-GFP that marks the phragmoplast midline [11] (Figures 4C and 4D). Upon extended incubation, GFP-KCA1 concentrated into fluorescent dots that associated with the plasma membrane and the phragmoplast (Figure 4A, 8 min). These aggregates are reminiscent to microdomains or detergent-insoluble lipid rafts known to occur in yeast and animal organisms [16]. Triton X-100 treatments of FM4-64-stained BY-2 cells showed a gradual loss of red fluorescence without the formation of aggregates (Figure 4E). The aggregation of GFP-KCA1 holds the premise that GFP-KCA is associated to triton-insoluble lipid rafts that may be contributing to a differential distribution at the PM. When placed in a hypertonic solution, plant cells plasmolyse, whereby the plasma membrane detaches from the external wall with the exception of cell wall plasma membrane connections known as Hechtian strands. Plasmolysed BY-2 cells showed in addition to Hechtian strands also a broad area where plasma membrane and cell wall are connected that corresponded to the position of the KDZ (Figure 5). The data suggest the presence of a plasma membrane cell wall linker protein at the position of the KDZ and support a role for membrane and cell wall depositions overlaying the PPB [19–21]. Plasma membrane cell wall linker proteins are then possible candidates

for maintaining the KDZ and keeping the division site at its predefined position.

Supplemental Data

Supplemental Data include six figures and Supplemental Experimental Procedures and can be found with this article online at <http://www.current-biology.com/cgi/content/full/16/3/308/DC1>.

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