The Specification of Geometric Edges by a Plant Rab GTPase Is an Essential Cell-Patterning Principle During Organogenesis in *Arabidopsis*

**Highlights**
- Plant cells possess a discrete class of vesicles that marks their geometric edges.
- These vesicles are defined by a Rab GTPase variant that is unique to plants.
- Inhibiting the activity of this GTPase disrupts growth anisotropy and morphogenesis.
- Edge-directed traffic is a crucial patterning principle, augmenting facial polarity.

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**In Brief**
Organogenesis requires plant cells to adjust their shape through coordinated anisotropic growth of shared walls on individual polyhedral faces. Kirchhelle et. al. now show that the geometric edges of cells represent an additional and important spatial domain, specified by a plant Rab GTPase activity that is essential for morphogenesis.
The Specification of Geometric Edges by a Plant Rab GTPase Is an Essential Cell-Patterning Principle During Organogenesis in *Arabidopsis*

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SUMMARY

Plant organogenesis requires control over division planes and anisotropic cell wall growth, which each require spatial patterning of cells. Polyhedral plant cells can display complex patterning in which individual faces are established as biochemically distinct domains by endomembrane trafficking. We now show that, during organogenesis, the *Arabidopsis* endomembrane system specifies an important additional cellular spatial domain: the geometric edges. Previously unidentified membrane vesicles lying immediately beneath the plasma membrane at cell edges were revealed through localization of RAB-A5c, a plant GTPase of the Rab family of membrane-trafficking regulators. Specific inhibition of RAB-A5c activity grossly perturbed cell geometry in developing lateral organs by interfering independently with growth anisotropy and cytokinesis without disrupting default membrane trafficking. The initial loss of normal cell geometry can be explained by a failure to maintain wall stiffness specifically at geometric edges. RAB-A5c thus meets a requirement to specify this cellular spatial domain during organogenesis.

INTRODUCTION

A central question in morphogenesis is how the behavior of individual cells is coordinated to generate the stereotypical multiscale organization of cells, tissues, and organs during embryogenesis (Blanchard and Adams, 2011). In the case of land plants, apical meristems provide a crucial additional source of undifferentiated dividing cells from which postembryonic lateral organs of diverse morphology may develop. When plant cells divide, daughter cells are separated by a shared cell wall that fixes their relative positions throughout subsequent development. Consequently, plants rely on the coordinated control of both cell division planes and unequal growth of different faces of polyhedral cells to achieve the appropriate morphology (Korn, 1982; Robinson et al., 2013; Smith et al., 1996; Uyttewaal et al., 2012). During morphogenesis, cells also have to accommodate geometric constraints and deviations from mechanical equilibrium that arise at the tissue level, particularly after cytokinesis. In animals this occurs through the regulated reorganization of bonds between cells but in plants, with rigid walls, it requires differential growth of individual walls (Blanchard and Adams, 2011; Cerruti et al., 2013; Korn, 1980). Cell geometry in turn influences the capacity of cells to respond to chemical and mechanical signals (Bassei et al., 2014; Sampathkumar et al., 2014) that act on fields of cells to coordinate their individual polarization with respect to microtubule organization, auxin transport, and wall extensibility (Heisler et al., 2010; Nakayama et al., 2012; Peaucelle et al., 2011, 2015; Ray et al., 2015; Robinson et al., 2013). All these features of regulative development require spatial patterning of cells.

The cell walls and plasma membrane (PM) surrounding individual epidermal cells do exhibit important spatial patterning. For example, the inner periclinal face of epidermal cells in *Arabidopsis* hypocotyls exhibits a distinct pattern of cellulose microfibrils that correlates with organ extensibility (Crowell et al., 2011). Similarly, it has become clear that plant epidermal cells can possess a complex polarity in which the PM at individual cell faces maintains distinct populations of proteins such as nutrient and auxin transporters (Dettmer and Friml, 2011; Langowski et al., 2010). In addition to this facial patterning, the geometric edges of cells have recently been shown to exhibit distinct properties with respect to cell wall stiffness (Routier-Kierzewska et al., 2012) and microtubule organization, which depends on CLASP to stabilize cortical arrays at meristematic cell edges whose high curvature would otherwise cause catastrophe (Ambrose et al., 2011; Ambrose and Wasteneys, 2011; Gunning et al., 1978).

Cell wall deposition and maintenance of membrane polarity are dependent upon the intracellular transport activities of the
endomembrane system (Endler and Persson, 2011; Richter et al., 2009). Some components of the underlying molecular mechanisms have been identified, but the trafficking pathways involved are largely unknown or contentious (Bloch and Yalovsky, 2013; Chan et al., 2010; Dettmer and Friml, 2011). Phylogenomics supported by a number of empirical studies indicate that membrane-trafficking mechanisms diversified independently in multicellular plants, contributing to the distinctive features of facial polarity and cytokinesis (Geldner, 2009; Woollard and Moore, 2008). Here we focus on one important gene family of membrane-trafficking regulators, the Rab guanosine triphosphatases (GTPases), in Arabidopsis.

The Rab family of ras-like small GTPases contains numerous subclasses that regulate diverse aspects of membrane traffic. Individual Rab proteins in their guanosine diphosphate-bound form associate with particular membranes where specific guanine-nucleotide exchange factors (GEFs) convert them to the GTP-bound form to provide transient binding surfaces for assembly of diverse macromolecular complexes before guanosine triphosphate (GTP) is hydrolyzed (Barr, 2013; Olkkonen and Stenmark, 1997; Zhen and Stenmark, 2015). These complexes can contribute to the establishment of membrane identity or the formation, motility, docking, or fusion of transport intermediates (Barr, 2013; Kloepper et al., 2012). Mutations that alter nucleotide affinity, preference, or hydrolysis have been instrumental in revealing the functions of individual Rab proteins (Barr, 2013; Olkkonen and Stenmark, 1997; Zhen and Stenmark, 2015).

Relative to the last common eukaryotic ancestor, it appears that Rab GTPase families underwent independent patterns of loss and diversification in each lineage (Elias, 2008; Kloepper et al., 2012). The most striking radiation in plant genomes is in the Rab-A clade (which includes YPT31/32 of Saccharomyces cerevisiae, and Rab11 and Rab25 of mammals) in which an ancestral gene has radiated into 26 paralogs that form six structural subclasses named Rab-A1 to Rab-A6 (Rutherford and Moore, 2002; Figure S1A). Members of the Rab-A1 to -A5 subclasses have been localized to the plant trans-Golgi network (TGN)/early endosome (Asaoka et al., 2013; Choi et al., 2013; Chow et al., 2008; Feraru et al., 2012; Koh et al., 2009; Lunn et al., 2013; Preuss et al., 2004, 2006; Ueda et al., 1996). The plant TGN is an early site of accumulation of the endocytic dye FM4-64, and in Arabidopsis root tips it is a target of brefeldin A (BFA), which causes its aggregation into BFA bodies (Chow et al., 2008; Dettmer et al., 2006; Richter et al., 2014), caused YFP:RAB-A5c to aggregate with VHA-a1 in these bodies (Figures 1K and S2D). Furthermore, we found that a previously described anti-RAB-A5c monoclonal antibody (Ueda et al., 1996) recognized native RAB-A5c in BFA bodies (Figures 1L and S2E).

RESULTS

Localization of RAB-A5c

The location of Rab GTPases within the endomembrane system is central to their individual function. The Arabidopsis Rab-A5 protein RAB-A5c (At2g43130; ARA4) has been immunolocalized to TGN membranes in pollen grains (Jeda et al., 1996). To investigate the localization of this protein in somatic cells, we generated a functional (see later) fluorescent fusion by inserting the coding sequence of a yellow fluorescent protein (YFP) at the initiation codon of a 6.8-kb genomic DNA fragment encompassing the entire RAB-A5c transcription unit and intergenic regions (5′-A5c-YFP:RAB-A5c-A5c3′). Fluorescence microscopy of 7- to 10-day-old transgenic Arabidopsis seedlings showed that YFP:RAB-A5c localized to the cytosol and numerous punctate structures that were labeled with varying intensity. The more faintly labeled structures frequently colocalized with the TGN as defined by RAB-A2a, VHA-a1, or endocytosed FM4-64 (Chow et al., 2008; Dettmer et al., 2006), but an additional population of brighter structures were distinct from the TGN (Figures 1E–11 and S2A). These structures remained unlabeled by FM4-64, even after 60 min of incubation (Figures 1H and 1I). As FM4-64 is a non-specific lipophilic dye that eventually labels all endosomal and vacuolar membranes in these cells (Viotti et al., 2010), it is unlikely that these structures are endosomal. RAB-A5c compartments were also distinct from Golgi and prevacuolar compartments labeled by ST-GFP and BP80-GFP, respectively (Figures 1J, S2B, and S2C). Thus in the root meristem YFP:RAB-A5c identifies membrane compartments distinct from previously described endomembrane compartments, including those labeled by other Arabidopsis Rab-A subclasses. We refer to these as the RAB-A5c compartments.

Faint labeling of the TGN indicates that YFP:RAB-A5c resides either partially or transiently on this compartment. Consistent with the latter hypothesis, treatment with BFA, which inhibits recycling to the PM and causes TGN aggregation in BFA bodies (Chow et al., 2008; Dettmer et al., 2006; Richter et al., 2014), caused YFP:RAB-A5c to aggregate with VHA-a1 in these bodies (Figures 1K and S2D). Furthermore, we found that a previously described anti-RAB-A5c monoclonal antibody (Jeda et al., 1996) recognized native RAB-A5c in BFA bodies (Figures 1L and S2E). The RAB-A5c cycle was also investigated by introducing mutations into 5′-A5c-YFP:RAB-A5c-A5c3′ that are predicted to affect GTP binding or hydrolysis (Olkkonen and Stenmark, 1997; Zhen and Stenmark, 2015). YFP:RAB-A5c [N125I] carries an Asn–Ile substitution in the nucleotide-binding pocket which is expected to greatly lower the affinity for nucleotides and stabilize the interaction with the exchange factor that catalyzes conversion to the active GTP-bound form after recruitment to the membrane (Batoko et al., 2000; Jones et al., 1995; Olkkonen and Stenmark, 1997; Zhen and Stenmark, 2015). YFP:RAB-A5c [N125I] carries an Asn–Ile substitution in the nucleotide-binding pocket which is expected to greatly lower the affinity for nucleotides and stabilize the interaction with the exchange factor that catalyzes conversion to the active GTP-bound form after recruitment to the membrane (Batoko et al., 2000; Jones et al., 1995; Olkkonen and Stenmark, 1997; Zhen and Stenmark, 2015). YFP:RAB-A5c [Q71L] carrying a substitution that frequently reduces GTP hydrolysis by Rab proteins (Olkkonen and Stenmark, 1997; Zhen and Stenmark, 2015), resulting in less efficient recycling off membranes. This mutant showed no labeling of the TGN, but instead the PM was prominently labeled (Figure 1N; compare with Figure S1B).
Figure 1. RAB-A5c Identifies a Vesicle Population that Is Distinct from the TGN in Young Lateral Organs

(A) Bright-field (left) and fluorescence (right) images of YFP:RAB-A5c in the root showing preferential expression in young lateral roots.

(B) Confocal optical section of YFP:RAB-A5c in a young lateral root.

(C and D) Epifluorescence and (D) confocal optical section showing YFP:RAB-A5c (green) and chlorophyll (blue) in young primary leaves.

(E–N) Confocal optical sections. (E and F) YFP:RAB-A5c (green) predominantly labels a population of structures (arrowheads) that do not colocalize with mCh:RAB-A2a (TGN) (red), but faint TGN labeling is also observed (arrows). (G) YFP:RAB-A5c (red) predominantly labels a population of RAB-A5c compartments (arrowheads) that do not colocalize with VHA-a1:GFP (TGN) (green), but faint TGN labeling is also observed (arrows). (H and I) YFP:RAB-A5c compartments (green) are not labeled by FM4-64 (red), even after 60 min; (I) detail of (H). (J) YFP:RAB-A5c (red) does not colocalize with PVC labeled by BP80:GFP (arrows) but both markers faintly label the TGN (arrowheads). (K) after treatment with brefeldin A (BFA), YFP:RAB-A5c (red) colocalizes with VHA-a1:GFP (green) in BFA bodies. (L) Immunolocalization of endogenous RAB-A5c (red) and KNOLLE (green) in BFA bodies of a mitotic cell stained with DAPI to show the chromosomes (blue). (M) Nucleotide-binding site mutant YFP:RAB-A5c[N125I] (red) colocalizes extensively but incompletely (arrows) with the TGN marker VHA-a1:GFP (green, compare with G). (N) A medial section through epidermal cells showing that the YFP:RAB-A5c[Q71L] (green) mutant shows greatly increased labeling of the PM relative to TGN (both labeled by FM4-64, red) (compare with H and I; also compare with the oblique section in Figure 4A inset).

Scale bars, 10 μm. See also Figures S1 and S2.
and Stenmark, 2015) is that YFP:RAB-A5c is recruited from the cytosol to the TGN from which it traffics on an exocytic pathway to the RAB-A5c compartment but recycles back to the cytosol either before or shortly after reaching the PM (Figure S1C). Consistent with this, in dividing cells where anterograde traffic from the TGN forms the cell plate (Chow et al., 2008; Richter et al., 2014), YFP:RAB-A5c and endogenous RAB-A5c were present in the early and expanding cell plate (Figures 2 and S2G). During expansion, labeling was predominantly at the peripheral region where new membrane vesicles are added, but was lost from the expanded maturing plate (Figures 2E and 2H–2J).

**RAB-A5c Compartments Cluster at the Geometric Edges of Cells in Young Organ Primordia**

RAB-A5c compartments were often located at the extreme periphery of the cell, which is not the case with other cytoplasmic organelles (Figures 1E–1H, 3A, S2A, and S2C). This was confirmed by quantification of RAB-A5c and mitochondrial membranes (Figures 3D, S4A, and S4B; n = 292 cells [control] and n = 136 cells [BFA]). Immunoelectron microscopy confirmed that YFP:RAB-A5c labeled relatively large vesicles (150 ± 35 nm mean sectional diameter; n = 11) close to the PM (mean distance, 93 ± 36 nm; n = 11) (Figures 3E and S4C–S4E). Immunolocalization revealed that the native RAB-A5c protein also localized to cell edges (Figures 3F–3I and S4F–S4K). In fact the pattern of RAB-A5c accumulation at the cell edges allows individual cells of the young lateral roots to be identified in maximum projections of the data (Figure 3L and Movie S2). This was most obvious in the epidermis, where 5’A5c-YFP:RAB-A5c-A5c3’ is most highly expressed (Figures 1B, 3J, and 3K). In the elongation zone of older lateral roots the pattern shifted, with YFP:RAB-A5c accumulating predominantly along the longitudinal edges of the cells (Figure 3M and Movie S4). In still older cells the edge localization was lost entirely and YFP:RAB-A5c abundance waned, but the protein was seen to accumulate strongly at the sites of root hair growth.
initiation in trichoblasts (Figure 3N). Similarly in the shoot, cells in young leaf primordia exhibited YFP:RAB-A5c accumulation at their edges (Figures 3O and 3P), but this pattern was lost in older cells (Figure 3Q).

**Edge Localization Requires Nucleotide Cycling and an Organized Cytoskeleton**

The capacity for edge localization in the meristem was dependent on GTP binding and hydrolysis by YFP:RAB-A5c, as it was lost in the nucleotide-binding N125I mutant and was diminished in the GTPase-deficient Q71L mutant which showed additional PM localization (Figures 4A–4C). The YFP:RAB-A5c compartments were relatively immobile at the cell edges (Figures S5A and S5B), suggesting they may be anchored, and edge localization was sensitive to chemical disruption of actin filaments and microtubules (Figure 4D). In root meristematic cells, the microtubule-stabilizing protein CLASP organizes the cortical microtubule arrays and adopts an edge localization that is reminiscent of the RAB-A5c compartment distribution (Ambrose et al., 2011), suggesting that CLASP could be required for localization of these compartments. The edge localization of RAB-A5c compartments is independent of CLASP and CLASP-mediated microtubule patterning, however, as it was maintained in the *clasp-1* mutant (Ambrose et al., 2007) (Figures 4E, 4F, and S5C). Furthermore, YFP:RAB-A5c showed only limited colocalization with GFP:CLASP (Ambrose et al., 2007) at cell edges, and no colocalization at the cell plate (Figures 4G–4J and S5C). Thus RAB-A5c compartments identify the geometric edges of young meristematic cells as a distinct spatial domain, geometrically distinct from previously described facial polarity and independent of CLASP activity (Figure S1D).

**Inhibition of RAB-A5c Activity Disrupts Cell Geometry**

We next asked whether perturbation of RAB-A5c function influenced cell growth and geometry. As shown above, the N125I mutation efficiently shifted the steady-state location of YFP:RAB-A5c away from the edges towards the TGN (Figures 1M and 4C). Small GTPases carrying this substitution can act as dominant inhibitors by competitively titrating interacting factors (Batoko et al., 2000; Jones et al., 1995; Olikkonen and Stenmark, 1997; Pinheiro et al., 2009; Schmitt et al., 1986). The dominant-inhibitory character of RAB-A5c[N125I] overcomes the difficulties associated with redundancy among gene family members and allows temporal control of mutant phenotypes. For conditional quantitative expression of RAB-A5c[N125I] and wild-type, we used the dexamethasone (Dex)-inducible pOp/LhGR promoter system (Craft et al., 2005). In 16 of 29 independent transgenic lines, induction of RAB-A5c[N125I] on Dex-containing medium resulted in severe inhibition of true leaf development, lateral root formation, and root hair elongation, as well as significant reduction in primary root length (Figures 5A–5D). These mutant phenotypes correlated closely with the expression pattern of 5′A5c–YFP:RAB-A5c–A5c3′ (Figures 1A–1D and 3M–3Q) and were never observed in lines that overexpressed wild-type RAB-A5c (n = 26; p < 10−6, Fisher’s exact test) (Figures 5A and 5E). Notably, when 5-day-old seedlings were transferred to medium containing 20 μM Dex, lateral roots progressively developed grossly perturbed cell shapes (Figure 5F).

The pOp/LhGR expression system is based on the CaMV 35S promoter, which is only weakly active in meristems of primary and lateral roots (Chow et al., 2008) (Figures 5G, 5A, and 5B) where RAB-A5c is most abundant (Figures 1A and 1B). To target Dex-inducible expression of RAB-A5c[N125I] to these cells we replaced CaMV 35S with the AtRPS5A promoter, which is active in lateral root meristems throughout their development (Figures 5G and 5C). This configuration is referred to subsequently as *AtRPS5A>Dex>*[N125I]. Cell geometry in young lateral roots was imaged using the PM marker YFP:NP51 (Wave-131Y; Geldner et al., 2009). *AtRPS5A>Dex>*[N125I] seedlings germinated in the presence of Dex showed severely restricted root growth (Figure 5H) while young lateral roots developed grossly abnormal cell geometries within 48 hr of transfer to dexamethasone (Figures 5I–5M). Lateral roots also showed incomplete and misplaced cytokinesis (Figures 5K–5M, arrows), which is consistent with a role for RAB-A5c in cell plate formation as suggested by localization studies (Figure 2). These cellular phenotypes were induced with Dex concentrations as low as 50 nM (Figure 5D), which is close to the minimal inducing concentration for pOp/LhGR (Craft et al., 2005; Figure S6E). Thus the
Figure 4. Edge Localization Requires GTP Binding but Is Independent of CLASP

(A–F) Maximum projections of series of optical sections from young lateral roots. (A–C) YFP:RAB-A5c wild-type (WT) (A) or the [Q71L] (B) and [N125I] (C) mutant derivatives; insets, single oblique optical sections. Arrowheads and arrows in (B) show, respectively, edge-localized RAB-A5c compartments and PM at medial positions; arrow in (A) shows equivalent medial position on PM. (D) Maximum projections of series of optical sections from young lateral roots showing YFP:RAB-A5c in lateral root tips treated with cytoskeleton disrupting drugs or with DMSO only. (E and F) YFP:RAB-A5c in CLASP (E) and clasp-1 (F).

(G) Quantification of reciprocal colocalization of GFP:CLASP and YFP:RAB-A5c using thresholds of 1 or 2 SD above mean background; error bars denote mean ± SD.

(H–J) YFP:RAB-A5c (magenta) and GFP:CLASP (green). (H) YFP:RAB-A5c partially localizes (arrowheads) with GFP:CLASP. (I) Detail of (H), highlighting structures labeled only by YFP:RAB-A5c (arrows) or GFP:CLASP (arrowheads). (J) Cell plates (arrowheads) labeled by YFP:RAB-A5c only.

Scale bars: 10 μm (A–F, I); 1 μm (G). See also Figure S4.
Figure 5. Inhibition of RAB-A5c Function Perturbs Cell Geometry
Seedlings exhibiting Dex-inducible expression of RAB-A5c (wt) or RAB-A5c[N125I] under control of the CaMV 35S promoter (A–F) or AtRPS5A promoter (H, J–P).
(A) Seedlings grown either in the presence of Dex or the solvent DMSO.
(B and C) Quantification of root system architecture in 14-day-old seedlings on Dex (+) or DMSO (−); error bars denote SD.
(D) Root hair development in seedlings segregating for RAB-A5c[N125I] on Dex; inset shows higher magnification of an [N125I] seedling.
(E) Immunoblot with anti-RAB-A5c antibody showing (arrowhead) relative abundance of RAB-A5c WT and [N125I] in Dex-induced seedlings of independent transgenic lines.

(legend continued on next page)
spatial control of cell growth is highly sensitive to the activity of RAB-A5c[N125I].

**Rescue of Dominant-Negative Mutant Phenotypes by Wild-Type RAB-A5c**

We next asked whether the growth defects induced by RAB-A5c[N125I] were specific to loss of RAB-A5c function. Domi-
ninant-inhibitory GTPase mutants of this sort act by competing with wild-type protein for interactors (Batoko et al., 2000; Jones et al., 1995; Olkkonen and Stenmark, 1997). Such interactors may be specific for RAB-A5c, in which case mutant phenotypes represent loss of RAB-A5c function, but it is possible that they have additional independent interactions, in which case non-specific phenotypes could be induced by titration with RAB-A5c[N125I]. These scenarios can be discriminated experimentally by increasing the dosage of wild-type RAB-A5c, which should quantitatively ameliorate the mutant phenotype if it arises specifically from competition between wild-type and mutant RAB-A5c (Batoko et al., 2000; Jones et al., 1995; Pinheiro et al., 2009). Conversely, if the interactor has additional independent functions that are impaired by competition with RAB-A5c[N125I], increasing wild-type RAB-A5c dosage would exacerbate this competition and any associated phenotype. Figure 6 shows that the growth inhibition and cellular growth defects induced by RAB-A5c[N125I] can be quantitatively suppressed either by introduction of an additional YFP-tagged genomic copy of RAB-A5c (5’A5c-YFP:RAB-A5c-A5c3’; Figure 1) or by inducible expression of wild-type RAB-A5c from the same AtRPS5a promoter as RAB-A5c[N125I], indicating that the mutant phenotype is attributable to loss of RAB-A5c activity and confirming that the YFP:RAB-A5c fusion is functional. In conclusion, RAB-A5c is essential for morphogenesis through maintenance of regular cell geometry in developing lateral roots.

**Inhibition of RAB-A5c Function Perturbs Growth Anisotropy Independently of Cytokinesis**

The severe terminal phenotype of lateral roots expressing RAB-A5c[N125I] is a complex one, most probably arising from loss of control over cell growth anisotropy, cytokinesis, and division plane. These processes can influence each other (Bassel et al., 2014; Besson and Dumais, 2011), so we sought to deconvolve them to determine whether RAB-A5c[N125I] acted primarily through one or the other process. To do this we developed imaging chambers to allow long-term 4D (x,y,z,t) imaging of lateral root development at cellular resolution after the induction of RAB-A5c[N125I] (Figure 7A). Lateral roots in imaging chambers and on agar plates grew at similar rates over 2 days (p = 0.18, n > 22), were morphologically normal in the absence of Dex, and exhibited Dex-induced phenotypes typical of RAB-A5c[N125I] (Figures 7B and 7C). To establish whether misshapen cells can arise independently of cell division defects or only after incomplete or misplaced cell divisions, we captured the development of individual lateral roots by confocal imaging of the PM marker YFP:NPSN12 (Geldner et al., 2009) at 24-hr intervals and analyzed them using MorphoGraphX software (Barbier de Reuille et al., 2014; Barbier de Reuille et al., 2015). In wild-type plants on Dex, cells exhibited the expected anisotropic expansion and stereotypical transverse cell divisions, resulting in files of narrow cells (Figures 7D–7H, 7N–7Q). In plants expressing RAB-A5c[N125I], cells showed greatly reduced longitudinal growth and increased radial expansion (Figures 7I–7M, 7R–7U, S7E, and S7F). This was observed in cells which did not divide throughout the induction period (Figures 7R and 7S) and in cells that completed apparently normal divisions (Figures 7T and 7U).

Abnormal expansion was most pronounced in cells that occupied the elongation zone at 24–48 hr after induction of RAB-A5c[N125I] expression, which reaches its maximum at about 16 hr (Craft et al., 2005). In contrast, cells that had achieved their final length by 24 hr typically showed little abnormality (Figures 7I–7M; marked with “+”). The elongating cells thus appear to be inherently more sensitive to the inhibition of RAB-A5c function. Additionally it became clear that severely radially swollen cells ruptured between 48 hr and 72 hr (Figures 7L, 7M, and 7U; marked with “x”), suggesting that the tensile strength of their wall had become compromised. Finally, we quantified incomplete cytokineses over 72 hr (Figures 7V and 7W) and noted that they could occur within 24 hr of induction in cells that were morphologically normal (Figures 7I and 7J, arrows), indicating that the cytokinesis defect is independent of perturbations to typical cell geometry, and suggesting that RAB-A5c[N125I] acts independently on growth and division. This is consistent with the relocation of RAB-A5c from cell edges to the cell plate during cytokinesis (Figure 2). Thus, RAB-A5c[N125I] can initially perturb the polyhedral geometry of growing cells by independently disrupting either growth anisotropy or cytokinesis, but the terminal phenotype is compounded by misplaced cell divisions and cell rupture.

To investigate how the properties of cell edges may account for the observed perturbation of cell geometry by RAB-A5c[N125I], we used published cell geometries (Dyson et al., 2014) to develop a 2D finite-element linear-elastic model of the root epidermis in which the relative stiffness of the wall at the vertices (equivalent to the 3D edges) could be varied (Figures S7A and S7B). When inflated under turgor, 3- to 10-fold softening at the vertices caused the wall to distend radially, as seen in plants expressing RAB-A5c[N125I] (Figures 5F, 5I–5L, S7E, and S7F), and this effect was exacerbated when the region of softening was increased from 0.5 μm to 1 μm around the vertex (Figure S7C).
Calculation of maximum principal stresses showed that softening at the vertices caused a relocation of load from the vertices to the faces, resulting in delocalized distention of the outer periclinal wall (Figure S7/D). Thus the increased radial expansion that results from inhibition of RAB-A5c activity can be explained by a failure to maintain appropriate wall stiffness specifically at the geometric-edge domain in elongating cells.

**DISCUSSION**

The plant-specific small GTPase RAB-A5c has revealed the existence of a distinct population of membrane vesicles that are aligned along the geometric edges of cells undergoing growth and division in young lateral organs. Thus, plant membrane-trafficking pathways are able to establish a spatial domain that is geometrically distinct from the previously described facial domains (Langowski et al., 2010), adding to our appreciation of the complexity of plant cell patterning.

The extraordinary diversification of the Rab-A clade in the land plant lineage (Woollard and Moore, 2008) has been hypothesized to reflect the advent of membrane-trafficking specializations. This is supported by our observation that RAB-A5c compartments are distinct from the TGN where the Rab-A2 subclass and other Rab-A proteins have been localized (Asaoka et al., 2013; Choi et al., 2013; Chow et al., 2008; Feraru et al., 2012; Preuss et al., 2006; Ueda et al., 1996). Indeed, whereas Rab-A2 proteins cycle between the late Golgi, TGN, and PM (Chow et al., 2008), RAB-A5c apparently cycles between the TGN and the edge-localized RAB-A5c compartments. In addition, although Rab-A5c compartments communicate with the TGN, they fail to accumulate the non-specific membrane dye FM4-64 from this compartment, suggesting that the pathway to the cell edges may be selective rather than a default pathway to the cell surface. This view is supported by our observation that PM and endocytic markers continue to traffic normally to and from the PM even when cell morphology and cytokinesis are grossly perturbed by RAB-A5c[N125I] (Figures S7G–S7N). This implicit diversification of Rab-A functions in plants is analogous to the independent diversification in early metazoans of the ancestral exocytic Rab8 into 13 different Rab sequences with diverse functions (Kloepper et al., 2012). In the case of the plant Rab-A clade, one of these functions is the specification of an edge-associated vesicle population. Indeed the Rab-A5 subclass is conserved across early diverging land plant lineages and so is likely to have been present during the early evolution of multicellular development (Elias et al., 2012; Kloepper et al., 2012; Woollard and Moore, 2008; Figure S1/B). Electron microscopy of the root meristem of the tracheophyte Azolla has revealed that geometric edges are rich in microtubules and putative vesicles that may be homologous to the RAB-A5c-labeled vesicles of Arabidopsis (Gunning et al., 1978).

What is the utility of an edge domain within the endomembrane system? It appears unlikely that the domain acts to define the boundary between adjacent facial domains, because previously described polar membrane markers typically exhibit significant drift into the adjoining faces of the cell, which suggests that the intervening edge is not sharply defined in the PM (Geldner, 2009; Langowski et al., 2010). Furthermore, PIN2:GFP trafficking was found to be insensitive to RAB-A5c[N125I] (Figures S7I).

Edges do, however, have some distinct properties that may require specialized trafficking activity, particularly in epidermal cells that have strong influence on organ growth (Dyson et al., 2014). The cell wall at the periclinal faces of growing cells can increase $10^{10}$-fold in surface area whereas the edges expand only linearly and vertices are fixed, yet a characteristic wall thickness is maintained at all positions, which implies spatially regulated wall assembly at each geometric domain (Roberts, 1994). Furthermore, wall stiffness has been shown to vary between the outer face and edges of turgid epidermal cells, requiring the accumulation of softer material at the edges where anticlinical walls intersect (Routier-Kierzkowska et al., 2012). However, our 2D finite-element model of idealized epidermal root cells revealed that local reduction in cell wall stiffness at these intersections can have a profound effect on cell geometry through redistribution of stresses to cell faces whose stiffness is otherwise unaltered. These considerations suggest a requirement to regulate cell wall stiffness specifically at the edges of growing cells, and RAB-A5c-mediated membrane trafficking may provide the underlying mechanism. Inhibition of RAB-A5c function causes radial swelling, consistent with softening of the wall at the edge, which suggests that RAB-A5c acts to stiffen this domain.
Figure 7. 4D Imaging of Individual Cell Growth in Lateral Roots

(A) A chamber developed for long-term time-lapse imaging of lateral root development. (B and C) Surface-rendered projections of confocal image series of lateral roots after 48 hr in imaging chambers containing medium with DMSO (B) or 20 μM Dex (C). (D–M) A lateral root expressing YFP:NPSN12 (D–H) and one expressing YFP:NPSN12 and Dex-inducible RAB-A5c[N125I] (I–M) imaged successively at 0–72 hr after transfer to imaging chambers containing 20 μM Dex. Individual epidermal cells and their descendants at each time-point are identified by the same color after image segmentation in MorphoGraphX. (H and M) Segmented image of epidermal cells at 72 hr without the overlaid projected image. (I–M) Cells that...
of the cell periphery. The bursting of significant numbers of elongating cells that express RAB-A5c[N125I] also suggests a loss of tensile strength in growing cell walls when RAB-A5c activity is compromised. The implied wall-stiffening activity of RAB-A5c at cell edges may compensate for an inherent weakness in the wall at cell edges owing, for example, to reduced cellulose accumulation (Suslov et al., 2009) or the unusual organization of the cortical microtubule arrays at these positions of high curvature in young cells (Ambrose et al., 2011). Alternatively, RAB-A5c-mediated trafficking may maintain mechanical homeostasis by balancing the activity of some other edge-directed wall-loosening activity in growing cells. Either way, a requirement to specify geometric edges of cells during morphogenesis may explain the presence in land plants of this novel Rab GTPase specificity that provides a membrane-trafficking pathway to this geometric domain. The identification of this pathway also reveals additional complexity in the spatial patterning of plant cells, which are able to specify geometric edges as well as individual faces as distinct spatial domains with respect to membrane trafficking. Together with the associated mutant phenotype and the potential for relocation of stresses between edges and faces, these findings have clear implications for our consideration of mechanisms that maintain and regulate cell geometry during morphogenesis.

**EXPERIMENTAL PROCEDURES**

Detailed methods are described in Supplemental Experimental Procedures.

**Plant Material and Growth Conditions**

The Columbia ecotype was used throughout. Transgenes were introduced into wild-type plants. Lateral roots were imaged from seedlings after 7–12 days in the growth chamber on vertically oriented 0.8% agar (Bacto Agar; Difco BD) plates with half-strength Murashige and Skoog medium (Sigma-Aldrich), and 1% (w/v) sucrose (pH 5.7).

**Plasmids**

Detailed descriptions of plasmid cloning are given in Supplemental Experimental Procedures. All plasmids used for plant transformation were constructed twice independently. Genomic sequence for RAB-A5c was amplified from genomic DNA of *Arabidopsis thaliana* Col-0 and used to generate in-frame fusions to fluorescent proteins. Q71L and N125I point mutations were introduced into the RAB-A5c genomic sequence for making fluorescent fusions and for Dex-inducible expression from the pOp/LhGR expression system (Craft et al., 2005).

**Microscopy and Image Analysis**

Bright-field and fluorescence images of seedlings were collected with a Leica MZFLIII microscope and CoolSNAP camera (Roper Scientific) or Nikon D300 Bright-field and fluorescence images of seedlings were collected with a Leica Microscopy and Image Analysis.

**2D Finite-Element Modeling**

2D finite-element simulations were run in Abaqus 6.14 Standard (SIMULIA, see http://abaqus.software.polimi.it/v6.14/index.html for documentation). Idealized cell geometries and cell wall thicknesses were based on published data (Dyson et al., 2014). Walls were assumed to be quasi-incompressible with a Poisson ratio of 0.45 (a Poisson ratio of 0.5 was avoided to avoid numerical artifacts) and an elastic modulus of 5 × 10^9 Pa. We uniformly pressurized the cell interior with a turgor pressure of 5 bar. The internal periclinal walls were fully constrained. The material was meshed with 70,292 linear quadrilateral elements (C2D4R) and tested for spatial convergence.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedure, seven figures, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2016.01.020.

**AUTHOR CONTRIBUTIONS**

C.K. performed quantitative and 4D imaging and image analysis, phenotypic characterization, mutant rescue, immunolocalization, and finite-element modeling, and wrote and revised the manuscript. C.-M.C. generated plasmid constructs and transgenic plants and performed the YFP/RAB-A5c localization and immunolocalization; C.F., H.N., M.K., N.I., C.W., and I.M. generated constructs and transgenic lines and contributed to microscopy and phenotypic characterization. Y.D.S. performed immunoelectron microscopy; M.F. and R.S.S. provided image analysis software; A.J. contributed to the finite-element modeling; I.M. acquired funding, planned the experiments, and wrote and revised the manuscript.

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