Cell polarity is a fundamental feature underlying cell morphogenesis and organismal development. In the Arabidopsis stomatal lineage, the polarity protein BASL controls stomatal asymmetric cell division. However, the cellular machinery by which this intrinsic polarity site is established remains unknown. Here, we identify the PRAF/RLD proteins as BASL physical partners and mutating four PRAF members leads to defects in BASL polarization. Members of PRAF proteins are polarized in stomatal lineage cells in a BASL-dependent manner. Developmental defects of the praf mutants phenocopy those of the gnom mutants. GNOM is an activator of the conserved Arf GTPases and plays important roles in membrane trafficking. We further find PRAF physically interacts with GNOM in vitro and in vivo. Thus, we propose that the positive feedback of BASL and PRAF at the plasma membrane and the connected function of PRAF and GNOM in endosomal trafficking establish intrinsic cell polarity in the Arabidopsis stomatal lineage.
Asymmetries in morphology and molecular distribution are fundamental features of the cells. Unevenly distributed, or “polarized”, proteins are particularly critical for maintaining cellular structure and function in living organisms. Asymmetric cell division (ACD) is a hallmark of stem cells that divide to self-renew while generating new cell types in the development of multicellular organisms. Stem cell ACD requires polarly localized protein complexes to regulate the asymmetries of division-plane placement and daughter-cell-fate determination. In Arabidopsis, stomatal lineage cells are dispersed stem cells that undergo ACD to produce stomatal guard cells and pavement cells in the epidermis (Fig. 1a and Supplementary Fig. 1a). During stomatal ACD, the plant-specific protein BASL defines an intrinsic polarity pole by asymmetrically distributing to the cell cortex (Fig. 1a), where BASL assembles a polarity complex comprised of scaffold proteins (POLAR and BRX) and signaling regulators (YODA kinase, BIN2 GSK3-like kinases, and BSL phosphatases)11–13. The loss-of-function of BASL results in

![Diagram](image_url)

**Fig. 1 Identification of PRAF/RLD proteins as BASL physical partners.** a Localization of BASL (green) in Arabidopsis stomatal asymmetric cell division (ACD). PrC protodermal cell, MMC meristemoid mother cell, M meristemoid, SLGC stomatal lineage ground cell. Bottom schematic shows BASL polarization requires MAPKmediate protein phosphorylation and the plasma membrane-associated BRX proteins. Unknown regulators (orange) are anticipated to mediate BASL polarization. PM, plasma membrane; NE, nuclear envelope. b Results of GFP-BASL co-immunoprecipitation (IP) coupled with fluorescence complementation (BiFC) indicate Gal4 activation domain (AD) and interacting domain/motif. c Phylogenetic tree for the PRAF/RLD family, generated by Clustal W and based on the full-length proteins. Bold indicates proteins further characterized in this study. The AGI numbers can be found in “Methods”. e-g Pairwise yeast two-hybrid assays. Bait, designated proteins fused with Gal4 DNA-binding domain (BD). Prey: “-” indicates Gal4 activation domain (AD) only, and the others are AD fused with designated protein or protein domain. 3F > 3K (BASL FxxFxX mutated to KxxKxK). “Test” indicates interaction assays on synthetic dropout media (-LeuTrpHis, -LTH); “Control” indicates yeast growth in rich media (-LeuTrp, -LT). When needed, specific concentrations of 3-Amino-1,2,4-triazole (3-AT) were supplied to suppress bait auto-activation. h Bimolecular fluorescence complementation (BiFC) assays in N. benthamiana leaf epidermal cells. Positive YFP signals indicate positive protein-protein interactions (BASL with PRAF4). Deletion of the BRX domain (PRAF4ΔBRX) or mutating the FxxFxX motif (BASL_3F > 3K) abolished YFP signals. White arrows show the interaction occurs in a polarized manner. Data represent the results of three independent experiments. Scale bars = 25 μm. (z), z-stacked confocal image.

| Protein ID | #Unique | %Coverage | #Unique | %Coverage |
|------------|---------|-----------|---------|-----------|
| BASL       | 561     | 100+      | 0       | 0         |
| BRX        | 11      | 71        | 0       | 0         |
| BRX1       | 3       | 39        | 0       | 0         |
| BRX2       | 24      | 71        | 0       | 0         |
| BRX3       | 4       | 39        | 0       | 0         |
| BRX4       | 14      | 90        | 0       | 0         |
| PRAF4      | 2       | 36        | 0       | 0         |
| PRAF8      | 30      | 68        | 0       | 0         |
| PRAF9      | 10      | 31        | 0       | 0         |
| **PRAF/RLD family members** | |
| PRAF1      | 3       | 39        | 0       | 0         |
| PRAF2      | 24      | 71        | 0       | 0         |
| PRAF3      | 14      | 90        | 0       | 0         |
| PRAF4      | 2       | 36        | 0       | 0         |
| PRAF5      | 3       | 39        | 0       | 0         |
| PRAF6      | 11      | 71        | 0       | 0         |
| PRAF7      | 561     | 100+      | 0       | 0         |
| PRAF8      | 30      | 68        | 0       | 0         |
| PRAF9      | 10      | 31        | 0       | 0         |

Methods: Plasmid DNA was used to generate the interaction constructs. Yeast two-hybrid assay was performed as previously described. cYFP-PRAF4ΔBRX (1 mM 3-AT) and cYFP-PRAF4 were used as bait and prey controls.
compromised stomatal division and abnormal patterning of epidermal cells in Arabidopsis® (Supplementary Fig. 1a).

BASL is a peripheral membrane protein required for polarization of the other components in the polarity complex. However, how BASL polarization is initiated and maintained in the stomatal lineage cells remains largely unknown. Previous studies showed that protein phosphorylation mediated by mitogen-activated protein kinases (MAPKs) and the GSK3-like kinases regulates BASL polarization and turnover. Fluorescence Recovery After Photobleaching (FRAP) analyses that monitor protein intracellular dynamics revealed slow recovery of the BASL protein at the cell cortex, resembling that of an integral membrane protein, the PIN3 auxin efflux transporter in stomatal lineage cells. It was thus hypothesized that the polarization of BASL protein is likely regulated by endomembrane trafficking and activities.

Directional auxin flows underlying developmental patterning and growth in plants is mediated by a network of auxin transporters, particularly the type I PIN effluxers, some of which are polarized to one side of the cell. The PIN polarity maintenance heavily relies on the endomembrane trafficking system. In these processes, an ADP-ribosylation factor guanine-nucleotide exchange factor (Arf GEF) GNOM switches on the activities of Arf small GTPases to promote endosomal recycling, thus the polar distribution of PIN1 at the plasma membrane.

Here, we identify a group of plant-specific PRAF/RLD proteins as physical partners of BASL and determine the essential role of PRAF/RLD for BASL polarization and stomatal ACD. We further find a direct functional connection between PRAF/RLD and the Arf GEF GNOM. We thus propose that the connected functions of PRAF/RLD with BASL and PRAF/RLD with GNOM underlie the intrinsic polarization of the peripheral membrane protein BASL in Arabidopsis stomatal lineage cells.

Results
PRAF/RLD proteins are physical partners of BASL. To identify molecular components required for BASL polarization, we performed a genome-wide in vitro yeast two-hybrid screen and an in vivo co-immunoprecipitation (co-IP) assay coupled with mass spectrometry (MS). In the yeast two-hybrid, by using the full-length BASL protein as bait, 14 out of 17 positive interactions corresponded to a Brevis radix (BRX) domain that belongs to two plant-specific protein families, BRX and PRAF. We propose that the connected functions of PRAF/RLD with BASL and PRAF/RLD with GNOM underlie the intrinsic polarization of the peripheral membrane protein BASL in Arabidopsis stomatal lineage cells.

In the pairwise yeast two-hybrid assays, we confirmed the physical interaction between BASL and the BRX domain of the four PRAFs (Fig. 1e). However, neither the full-length PRAFs nor the other individual subdomains of PRAF outside of the BRX domain showed detectable interaction with BASL in yeast (Fig. 1f and Supplementary Fig. 1b). We suspected that intramolecular folding of the full-length PRAF protein might interfere with BASL interaction, or that lipid-binding motifs would make PRAF translocation that makes the assays in the nucleus difficult in yeast. In vitro pull-down assays using E. coli-made recombinant proteins showed that BASL interacts with the carboxyl side of PRAF4, PRAF5, PRAF8, and PRAF9, while the PRAF protein might interfere with BASL interaction (Supplementary Fig. 1b), indicating that the MCC domain might negatively influence the full-length PRAF to interact with BASL. On the other hand, when BASL subdomains (N, I, and C) were tested, the internal BASL_I domain was the only one exhibiting a positive interaction with PRAF4_BRX in yeast (Fig. 1g). Further assays showed us to identify a small fragment containing a hydrophobic motif FxxxFx that once mutated to hydrophilic KxxKxK (BASL_3F > 3K) the interaction with PRAF4_BRX was disrupted in this yeast system. Therefore, our data suggest that the physical contact between BASL and PRAF proteins occurs through the FxxxFx motif of BASL and the BRX domain of PRAF (Fig. 1c).

To test the BASL-PRAF interaction in plant cells, we performed the bimolecular fluorescence complementation (BiFC) assay in Nicotiana benthamiana epidermal cells. The BiFC assay is based on the reconstitution of an intact fluorescent protein (YFP) when two complementary non-fluorescent fragments are brought together by a pair of interacting proteins. In the BiFC, we detected positive interactions between BASL and all four PRAF proteins, whilst the negative controls (nYFP or cYFP alone coupled with corresponding protein fusions) did not give observable signals (Fig. 1h). Interestingly, compared with the subcellular localization of individual proteins (YFP-BASL, cytoplasmic/PM and nuclear; YFP-PRAF, PM and cytoplasmic fine punctate. Supplementary Fig. 1d), the interaction of BASL with all four PRAFs commonly occur at the PM and in a highly polarized manner (Fig. 1h and Supplementary Fig. 1e). Such polarization events were also detected when BASL was co-expressed with other established components of the polarity complex. PRAF4 and PRAF5, PRAF8 and PRAF9 are close homologous pairs (the amino acid sequences of PRAF4 and 5 are 70% identical, and PRAF8 and 9 are 85% identical). Thus, we often used PRAF4 and/or PRAF8 as representative members for various assays in this study. Deleting or mutating the interacting domain/motif of either side (PRAF4aBRX or BASL_3F > 3K) abolished the BASL-PRAF interactions in the BiFC assay, again indicating their interaction requires the BRX domain of PRAF and the FxxxFx motif of BASL, respectively. Taken together, we provided in vitro and in vivo data demonstrating that the PRAF proteins have the characters of being BASL physical partners.
PRAFs are required for stomatal ACD. To genetically characterize the biological function of PRAFs in vivo, we deployed the CRISPR/Cas9-mediated mutagenesis32 to knock out PRAF4, 5, 8, and 9 simultaneously in Arabidopsis. In our experiments, each of the four PRAF genes was targeted by one guide RNA on one of the exons (Supplementary Fig. 2a). Among the second generation of transgenic plants (T2), we found dwarfed seedlings with dark green cotyledons segregating in some of the populations (Supplementary Fig. 2b). Genotyping results showed that these mutant plants are somatically chimeric (Supplementary Fig. 2c). For example, each of such two individual mutants (C6 and C8, Supplementary Fig. 2b) harbors homozygous premature termination mutations in PRAF4 and PRAF5, also contains more than two different alleles of praf8 and praf9 mixed with the wild-type copies (detailed information in Supplementary Fig. 2c). These somatically mutated plants were named praf4c;5c;8c;9c that can rarely survive through the seedling stage. To create a null quadruple mutant, we first isolated a Cas9-free triple mutant praf5c;8c;9c that carries three homozygous premature termination mutations (sequences in Supplementary Fig. 2c). praf5c;8c;9c was then crossed with a T-DNA insertion null allele praf4t (SALK_067605, Supplementary Fig. 2a). The homozygous quadruple praf4t;5c;8c;9c mutants are phenotypically more severe than praf5c;8c;9c (Supplementary Fig. 2b) but can be maintained as heterozygous praf4t/+;5c;8c;9c.

Close examination of the cotyledon epidermis showed that both quadruple mutants, praf4c;5c;8c;9c and praf4t;5c;8c;9c, produced extra numbers of stomatal lineage cells (Fig. 2a, b), the identity of which was verified by the expression of the stomatal lineage-specific receptor protein Too Many Mouths, TMM-GFP33 (Supplementary Fig. 2d). Furthermore, the typical divisional asymmetry of the stomatal lineage cells (calculated as ratios of the smaller size A relative to the large size B, Fig. 2c) was disturbed by the praf quadruple mutations, to some extent mirroring what was observed in basl mutants (Fig. 2a, c)8. The disruptions of both physical asymmetry and cell-fate asymmetry were found in basl mutants. By using the expression of the late meristemoid marker MUTE as readout34, which is usually only found in the small daughter in the wild type, was indeed identified in both daughter cells in praf4t;5c;8c;9c mutants, as in basl-2 (Supplementary Fig. 2e). However, none of the single T-DNA insertional praf null mutants showed obvious stomatal defects (Supplementary Fig. 3a, b), suggesting the four PRAF genes are redundantly needed in the regulation of stomatal development. When the four praf mutations combined with the basl null, the quintuple mutants phenocopied the praf quadruple mutants in both plant growth and stomatal development (Fig. 2a–c), suggesting that BASL and its function in stomatal development might be one of the pathways that PRAFs regulate. Then, we introduced GFP-BASL in the praf quadruple mutants and found that the BASL polarization was clearly affected in both praf4c;5c;8c;9c and praf4t;5c;8c;9c mutants (Fig. 2d, e and Supplementary Fig. 3c, d), suggesting the presence of the four PRAF proteins are required for BASL to polarize at the cell cortex. Furthermore, after mutating the PRAF-interacting FxxFxG motif of BASL, GFP-BASL_3F > 3K failed to polarize (Fig. 2f and Supplementary Fig. 3d) or to rescue basl-2 mutant stomatal defects (brackets in Fig. 2f). Thus, we propose that the four PRAF proteins are required for stomatal ACD, at least partially through promoting BASL polarization in the stomatal lineage cells.

PRAF proteins are localized to the plasma membrane, Golgi, TGN/EE, and endosomes. To elucidate the in vivo subcellular localization of the PRAF proteins, we generated fluorescent-protein tagged PRAF proteins in Arabidopsis. In general, the PRAF genomic region containing the promoter was fused with YFP and introduced into the loss-of-function mutants for functional tests. We found that both native-promoter-driven PRAF4-YFP and PRAF8-YFP can rescue the growth phenotypes of praf4t;8t-1;9t-1 and praf5c;8c;9c mutants, respectively (Fig. 3a and Supplementary Fig. 4a). The differential orientation of the fluorescent tag was also tested for function by comparing PRAF4-YFP with GFP-PRAF4 in praf4t;8t-1;9t-1 mutants. Results show that the two orientations display similar subcellular distribution and are equally efficient in complementation (Supplementary Fig. 4a, b). Thus, the N-terminal and C-terminal PRAF protein fusions were interchangeably used in this study.

In Arabidopsis leaves, the overall distribution patterns of YFP-tagged PRAF proteins were similar, all of which showed predominant association with the plasma membrane and formed endosome-like accumulations in the cytoplasm (Fig. 3b and Supplementary Fig. 4a–d). The plasma membrane association of PRAF4/8 was verified by the plasmolysis experiments, in which signals of PRAF4/8-YFP at the cell periphery retracted with the plasma membrane when detached from the cell wall (Supplementary Fig. 4e). The endosomal localization of PRAF became more evident when protein levels were expressed highly, such as native promoter-driven PRAF8 in mature guard cells (arrows in Fig. 3b) or overexpressed in the stomatal lineage cells (driven by the TMM promoter) (Fig. 3c and Supplementary Fig. 4d).

Interestingly, when highly expressed in the stomatal lineage cells, PRAF8 became obviously polarized (arrows in Fig. 3d), whereas this polarization was lost in the absence of BASL (Fig. 3e and quantification in 3f). In addition, when co-expressed in Arabidopsis, GFP-BASL and mCherry-PRAF8 became highly overlapping. More specifically, the nuclear pool of BASL was diminished but enriched to the PRAF8-positive endosomes, whilst PRAF8 became highly polarized together with BASL at the plasma membrane (Fig. 3g). These data further supported the in vivo physical interaction of BASL and PRAF.

Because the PRAF proteins are localized to the plasma membrane and the endosome-like structures, to test the functional contribution of the plasma membrane pool, we expressed an N-terminally myristoylated PRAF8 (modification sequence in ref. 35) in the loss-of-function mutants. Indeed, myr-PRAF8, compared to the wild-type PRAF8, was predominantly accumulated at the plasma membrane, and hardly formed endosomal puncta (Fig. 3h vs. 3b). Results showed that myr-PRAF8 partially complemented the dwarf phenotype of praf5c;8c;9c mutants (Fig. 3i), indicating that the plasma membrane pool of PRAF8 contributes significantly to its function.

To verify the endosomal-like localization of PRAF proteins, we used the styryl dye FM4-64 that intercalates into the plasma membrane, is then taken into the cells by endocytosis36. The Arabidopsis seedlings (3-day old) expressing YFP-tagged PRAF4/5/8/9 (driven by the native promoter) were incubated with 8 μM FM4-64 for 40-min. We found PRAF proteins colocalized with FM4-64 at the PM and partially overlapped with the FM4-64-positive puncta in the cytoplasm (Fig. 4a and Supplementary Fig. 5a), supporting the physical association of PRAF proteins with the endosomes. To test whether the PRAF-associated endosomes participate in the endocytic recycling pathway, we further treated the seedlings with Brefeldin A (BFA), an Arf GEF inhibitor that disturbs endomembrane trafficking, leading to the formation of the so-called “BFA-body” compartments that contain aggregated Golgi and trans-Golgi network (TGN)/early endosome (EE) membranes37. Recent study by Qi et al.37 demonstrated that 30–90 μM BFA effectively induces the formation of similarly structured BFA bodies in Arabidopsis stomatal lineage cells. Our results showed that 60-min 70 μM BFA treatment, in the presence of a protein synthesis inhibitor, cycloheximide (CHX, 50 μM),
triggered the aggregation of existing PRAF8 proteins around the basl-2 mutant (Fig. 4a). Additional 2-h wash-out released the aggregation of PRAF8-YFP and recovered its original localization pattern (Fig. 4a). Similar responses were consistently observed for the other PRAF proteins (Supplementary Fig. 5a). Thus, the results suggested that cytoplasmic PRAF proteins associate with the endomembrane compartments that are sensitive to BFA in plant cells. On the other hand, we applied a selective phosphoinositide 3-kinases (PI3Ks) inhibitor, Wortmannin (WM), which disturbs EE markers, VHAa1, and VAMP72, and the post-Golgi endosomal markers (defined by RabC1, RabD1, RabD2a, and RabE1d; the endosomal/recycling endosomal (E/RE) markers, RabA1e and RabA5d; and the late endosomal/prevacuolar compartment (LE/PVC) markers, RabF2a and RabF2b). The results show the most robust co-localization with PRAF8, based on the Pearson correlation coefficient (PCC) values, were RabC1- and RabE1d-decorated membrane compartments (Fig. 4b and Supplementary Fig. 6a, b).
The RabC1 structures have not been well characterized yet \(^4\), whilst RabE1d was reported to localized to the plasma membrane and Golgi \(^49,50\). Partial co-localization of PRAF8 was also detected with the Golgi marker ST, the TGN/EE marker VAMP721, and LE/PVC RabF2b (Fig. 4b–d and Supplementary Fig. 6a, b). The association of PRAF8 with ST (Golgi) and VAMP721 (TGN/EE) appeared to be less stable or occur under certain conditions because only a portion of expressing cells showed positive co-localization (population a vs. b, Fig. 4c). In summary, results of protein co-expression in \(N.\) benthamiana epidermal cells suggested that cytoplasmic PRAF8 partly associates with the Golgi, TGN/EE, and a subset of endosomal populations (Supplementary Fig. 6c).

\textbf{praf} mutants highly resemble \textit{gnom} mutants. In the process of characterization of the \textit{praf} mutant phenotypes, we noted that, strikingly, the developmental and growth defects of the \textit{praf}
**Fig. 4 Intracellular PRAF8 partially associates with the Golgi, TGN/EE, and endosomes.**

a) **PRAF8-YFP associates with the endosomes.** Top panel: confocal images of PRAF8-YFP (green) in cotyledon epidermal cells stained with the endocytic tracer marker, 8 μM FM4-64 (red) for 40-min (step 1). White circles: overlapping endosomal signals. Middle, step 2, following FM4-64 staining, seedlings were treated with 50 μM cycloheximide (CHX, a protein synthesis inhibitor) for 90-min, followed by the addition of the Arf GEF inhibitor, 70 μM Brefeldin A (BFA), for 60-min. Step 3 (bottom), wash-out with water for 2 h. Yellow arrowheads mark the formation of "BFA-bodies" that overlap with PRAF8-YFP-positive aggregates. Data represent the results of three independent experiments.

b–c) Co-localization of YFP/mRFP-PRAF8 (green) with mCherry/YFP-tagged endomembrane markers (magenta) in *Nicotiana benthamiana* leaf epidermal cells. Data represent results of three independent experiments. The co-localization rates (PCC, Pearson correlation coefficient values) are specified at the upper-right corner (cyan). G Golgi, E endosome, TGN/EE trans-Golgi network/early endosome, LE/PVC late endosome/pervacuolar compartment.

d) Quantification of protein co-localization based on PCC values in (b) and (c). For each pair of co-expression, n > 100 regions-of-interest (ROIs, each 228.3 μm²) were selected to obtain PCC values. ST showed 61/100 ROIs with PCC > 0 (population a), and VAMP721 showed 18/100 ROIs with PCC > 0 (population a). Box plots show the first and third quartile (box), median (line), and mean (cross). Data represent the results of three independent experiments. (z), z-stacked confocal image. Scale bars are 10 μm (a) and 20 μm (b and c).
Fig. 5 gnom mutants highly resemble prof mutants. a 4-day-old seedlings of wild type, prof4t;5c;8c;9c, and loss-of-function gnom (segregated from gnomB/E) mutants. b 4-week-old plants of wild type, prof triple mutants (prof4t;8c;9c and prof5c;8c;9c), and gnomB/E (trans-heterozygously complementing B4049 and emb30-1 alleles). c Confocal images show stomatal phenotypes of 5-day-old adaxial cotyledon epidermis of the designated genotypes. Stomatal lineage and guard cells are manually traced and highlighted. Three independent experiments were performed. Scale bar = 10 μm. d Box plots show quantification of stomata lineage index for (c). Box plots show first and third quartile (box), median (line) and mean (cross). n.s., not significant; *P < 0.05. e Box plots show quantification of divisional asymmetry in the stomatal lineage for (c). Box plots show first and third quartile (box), median (line) and mean (cross). n, # cotyledons counted. Student’s unpaired t tests were used for comparison. Two-sided P values are 0.4439 (for gnom vs. prof4t;5c;8c;9c), 0.0258 (for gnom vs. gnomprof4t;5c;8c;9c), and 0.2922 (for basl-2;gnom vs. gnom). n.s., not significant; f GFP-BASL (green) lost polarization in gnom. Magenta, cell outlines stained by PI. Data represent the results of three independent experiments. g Confocal images show GFP-BASL (green) merged with 8 μM FM4-64 staining (red) (top) or GFP-channel only (bottom). Three-day old seedlings were pre-incubated with FM4-64 for 40-min (left), then treated and functions in endomembrane trafficking to promote the recycling of internalized proteins, including the auxin-efflux carrier PIN1, to the basal plasma membrane. The loss-of-function gnom mutants are defective in the directional transport of auxin, resulting in seedling-lethal phenotypes. The loss-of-function prof4t;5c;8c;9c mutants replicated the strong gnom mutants (homozygous B4049 or emb30-1 alleles segregated from a trans-heterozygotes gnomB/Eemb30-1 plant) (Fig. 5a). Both mutants at 4-day old are dwarfed, produce fused cotyledons, and barely make any roots (Fig. 5a). Furthermore, the prof triple mutants, i.e. prof4t;8c;9c and prof5c;8c;9c, resembled the weak allele of gnomB/E mutants (carrying trans-heterozygously complementing B4049 and emb30-1 alleles) and both prof triple and gnomB/E mutants grow smaller and produce narrower rosette leaves (Fig. 5b). These highly resembling phenotypes hinted the possible functional connection between PRAF and GNOM in plant growth and development. In stomatal development, prof4t;5c;8c;9c and gnom null mutants are also highly mirroring each other; both mutants produce elevated numbers of abnormal cell divisions (Fig. 5c and quantification in Fig. 5d, e) and both prof and gnom mutants produce strikingly similar, peanut-shaped guard cells. Among the lower-order mutants, i.e. prof4t;8t-1;9t-1 and prof5c;8c;9c, resembled the weak gnomB/E mutants in producing mildly clustered stomatal lineage cells (Supplementary Fig. 7a). To assay the genetic interaction between PRAF and GNOM, we generated the quintuple null mutant prof4t;5c;8c;9c;gnomT, in which a T-DNA insertional null allele of gnom was used. We found that the stomatal phenotypes of a quintuple prof4t;5c;8c;9c;gnomT mutant phenocopied that of the quadruple prof4t;5c;8c;9c or gnom mutants (Fig. 5c–e). Furthermore, basl-2;gnom was also indistinguishable from basl-2;prof4t;5c;8c;9c (Figs. 2a and 5c). Taken together, our phenotypic analyses revealed highly resembling phenotypes of the prof and gnom mutants in overall plant growth and stomatal development.

GNOM is required for BASL polarization at the plasma membrane. Because similar defects were observed in prof and gnom mutants, we suspected BASL polarity is also regulated by GNOM. Thus, we introduced the native promoter-driven GFP-
GNOM-Myc was identified in plants expressing PRAF9-YFP. The co-IP results show that when the two proteins co-expressed in 35S PRAF-GNOM, we performed co-IP experiment by co-expressing recombinant proteins produced by E. coli. Further investigation whether the PRAF and GNOM proteins directly interact. In the yeast two-hybrid assays, because the expression of full-length GNOM kills the yeast cells, we split GNOM into two halves (GNOM_N and GNOM_C, Fig. 6a), and no interactions were detected with the four full-length PRAF proteins (Supplementary Fig. 8a). We then tested whether GNOM_N or GNOM_C may interact with the subdomain of PRAFs. By using PRAF4 as a representing member, we tested PRAF4_PH-RCC1 (the fragment containing PH and RCC1) and PRAF4_FYVE-CC (containing FYVE and CC). Positive interactions were detected between GNOM_C with PRAF4_FYVE-CC but not with PRAF4_PH-RCC1, whereas no interactions were detected for GNOM_N (Fig. 6b). However, further narrowing down the PRAF4_FYVE-CC fragment did not allow us to detect interactions between GNOM_C with PRAF4_FYVE or PRAF4_CC (Fig. 6b). In the in vitro pull-down assays using recombinant proteins produced by E. coli, we further confirmed the physical association between GNOM_C and PRAF4_C that contains FYVE-CC and BRX (Supplementary Fig. 8b). Taken together, our in vitro data suggest that PRAF may physically interact with GNOM via the FYVE-CC subdomain.

Next, we tested the PRAF-GNOM interaction in plant cells by the BiFC assay in N. benthamiana epidermis. While the negative controls did not produce detectable signals, the complimented YFP signals for PRAF4/5/8-GNOM appeared as punctate compartments in the cytoplasm (Fig. 6c and Supplementary Fig. 8c, PRAF9 showed strong autoactivation in the BiFC, thus was excluded from the assay). To test the in vivo interaction of PRAF-GNOM, we performed co-IP experiment by co-expressing the ubiquitous 35S promoter-driven GNOM-Myc together with the native promoter-driven YFP-tagged PRAF proteins in Arabidopsis plants. Because of the relatively low expression levels of PRAF4/5/8-YFP (Supplementary Fig. 8d), we relied on the plants expressing PRAF9-YFP. The co-IP results show that when PRAF9-YFP was pulled down by GFP-trap agarose beads, GNOM-Myc was identified to co-immunoprecipitate with PRAF9-YFP (Fig. 6d), supporting the physical association of GNOM with PRAF9 in vivo. To further visualize the PRAF-GNOM interaction in vivo, we examined the subcellular localization of GNOM-GFP alone (driven by the native promoter), mCherry-PRAF8 alone (driven by the BASL promoter), and the co-expression pattern when both proteins were present. Interestingly, when co-expressed, the two proteins became highly overlapping (PCC around 0.67) (Fig. 6e). Similar results were obtained when the two proteins co-expressed in N. benthamiana epidermal cells (Supplementary Fig. 8e, f). Lastly, we examined whether PRAFs and GNOM may impact each other’s endogenous localization in Arabidopsis. Indeed, the native promoter driven PRAF8-YFP or GNOM-GFP both showed abnormal aggregations in gnom or in praf4t;5c;8c;9c mutants, respectively (yellow arrowheads in Supplementary Fig. 9a, b), indicating PRAF and GNOM are mutually influential for their subcellular localizations. Thus, our biochemical, cell biological, and genetic data collectively supported that the PRAF and GNOM proteins may physically interact in vivo.

GNOM was determined to mainly localize to the Golgi apparatus in Arabidopsis52. Our results above suggested PRAF8 associates with the Golgi, TGN/EE, and endosomes (Fig. 4b-d and Supplementary Fig. 6a-c). We thus further investigate which subcellular compartments PRAFs may possibly interact with GNOM by expressing BiFC PRAF8-GNOM protein pairs with the mCherry WAVE markers50 in N. benthamiana epidermal cells. Overall, the distribution of PRAF8-GNOM BiFC signals showed a mixed population of membrane structures with varying sizes (Fig. 6c and quantification of 3-D volume in Supplementary Fig. 10a). Aided by the WAVE markers, we detected the larger-sized vesicles (10–110 μm2) partially overlapped with RabE1d (Golgi and endosomes) and RabC1 (uncharacterized compartments) (white circles and insets in Fig. 6f), and no co-localizations were detected for other markers (Fig. 6f and Supplementary Fig. 10b). The results supported the possible interactions of PRAF8-GNOM may occur at the Golgi and RabC1-decorated structures in plant cells.

PRAF and GNOM proteins physically interact. The highly resembling phenotypes of praf and gnom mutants inspired us to further investigate whether the PRAF and GNOM proteins directly interact. In the yeast two-hybrid assays, because the expression of full-length GNOM kills the yeast cells, we split GNOM into two halves (GNOM_N and GNOM_C, Fig. 6a), and no interactions were detected with the four full-length PRAF proteins (Supplementary Fig. 8a). We then tested whether GNOM_N or GNOM_C may interact with the subdomain of PRAFs. By using PRAF4 as a representing member, we tested PRAF4_PH-RCC1 (the fragment containing PH and RCC1) and PRAF4_FYVE-CC (containing FYVE and CC). Positive interactions were detected between GNOM_C with PRAF4_FYVE-CC but not with PRAF4_PH-RCC1, whereas no interactions were detected for GNOM_N (Fig. 6b). However, further narrowing down the PRAF4_FYVE-CC fragment did not allow us to detect interactions between GNOM_C with PRAF4_FYVE or PRAF4_CC (Fig. 6b). In the in vitro pull-down assays using recombinant proteins produced by E. coli, we further confirmed the physical association between GNOM_C and PRAF4_C that contains FYVE-CC and BRX (Supplementary Fig. 8b). Taken together, our in vitro data suggest that PRAF may physically interact with GNOM via the FYVE-CC subdomain.

The abundance and activity of plasma membrane proteins are regulated by endocytosis, endosomal sorting, endocytic recycling, and vacuolar degradation, etc.35. We first used FM4-64 internalization to measure endocytosis in the wild type, praf-quad, and gnom mutants. Results of FM4-64 internalization at sequential time points (5-, 10-, 20-, 30-, and 40-min treatment) suggested that the wild-type cells taking up FM4-64 was maximized at 15- to 20-min, whereas neither praf-quad nor gnom mutants showed obvious FM4-64 internalization until 30- to 40-min (white arrowheads in Fig. 7b and Supplementary Fig. 11b, quantification in Fig. 7c). Therefore, both PRAF and GNOM positively regulate endocytosis, consistent with the early study demonstrating the positive role of GNOM in endocytosis56.

In the FM4-64 staining experiments, surprisingly, we identified a population of enlarged membrane aggregations in the cytoplasm of praf-quad and gnom mutants (yellow arrowheads in Fig. 7b and Supplementary Fig. 11b). These large compartments appeared very early when FM4-64 just became detectable in leaf epidermal cells (within 5-min) (Supplementary Fig. 11b) and the size and shape of these membrane aggregations resemble those of the “BFA-bodies” in wild-type plants (Fig. 7b and quantification in 7d). We suspect that the organization of the endomembrane system is disturbed in praf-quad and gnom mutants. To test this hypothesis, we examined a few YFP-tagged
endomembrane makers, including RabE1d (Golgi/endosomal), endosomal RabD1, RabD2a, RabA1e, RabF2a, uncharacterized RabC1, and vacuolar VAMP711. Interestingly, most of these endosomal makers were more or less changed by praf-quad or gnom mutations and most of these changes were commonly found in both mutants (Fig. 7e and Supplementary Fig. 11c). Overall, the membrane compartments decorated by RabA1e, RabC1, RabD1, RabD2a, or RabE1d became smaller in both mutants, though the vacuolar marker VAMP711 remained unchanged in both mutants (Fig. 7e, f, and Supplementary Fig. 11c). More specifically, in both praf-quad and gnom mutants, RabC1 appeared more diffused in the cytoplasm, RabD1 showed abundant accumulation at the cell cortical region, whilst RabE1d became more associated intracellular filamentous structures, likely the cytoskeletal elements (Fig. 7e and Supplementary Fig. 11c). Thus, our data suggested that PRAF and GNOM are required to maintain the morphology, organization, and function of many endomembrane compartments, in particular the Golgi, post-Golgi secretory and recycling pathways towards the plasma membrane in plant cells.
Fig. 6 PRAF and GNOM proteins physically interact. a Diagrams depict the domain structure of PRAF4 and GNOM, respectively. “N”, the N-terminus; “C”, the C-terminus. Dashed red box, identified PRAF-GNOM interacting domains. b Pairwise yeast two-hybrid assays show PRAF\_FYVE\_CC interacts with GNOM\_C. Bait, GNOM\_N (left) or GNOM\_C (right) fused with BD. Prey, subdomains of PRAF4 fused with AD and “\_” indicates AD only. “Test” means interaction assays on synthetic dropout media (-LTH). “Control” means yeast growth in rich media (-L). Auto-activity of the bait protein fusions were suppressed by the addition of 3-AT. The result represents three biological repeats. c BiFC assays in N. benthamiana leaf epidermal cells show interactions of PRAF4 (left) or PRAF8 (right) with GNOM. nYFP N-terminal YFP, cYFP C-terminal YFP. Complemented YFP signals were converted to the ImageJ’s Fire LUT mode. Data represent results of three independent experiments. d In vivo co-IP assays test the interaction between PRAF9 and GNOM. 5- to 7-day-old seedlings co-expressing 3SS\_GNOM\_Myc with PRAF9-GFP, PRAF9-YFP or BASLp\_GFP were used for the co-IP experiment. The numbers indicate protein sizes (kDa). The result represents three biological repeats. e Individual protein expression and co-localization of mCherry-PRAF8 (magenta) and GNOM-GFP (green) in stomatal lineage cells. Note the changes of GNOM alone vs. when co-expressed with PRAF8. Protein co-localization is calculated as PCC values (cyan). n = 29 stomatal lineage cells counted. f Co-localization of the BiFC PRAF8\_GNOM interaction signals (green) with the WAVE endomembrane markers (magenta) in N. benthamiana leaf epidermal cells. Data represent the results of three independent experiments. Overlapping signals (white circles) were identified between PRAF8\_GNOM BiFC with RabC1 (uncharacterized membrane compartments) and RabEld (Golgi/endosomes) but not with the LE/PVC marker RabF2b. Insets show enlarged views of overlapping signals between BiFC and the WAVE marker. (2), z-stacked confocal images. Scale bars are as indicated (\(\mu\text{m}\)).

Discussion

In this study, we identify members of the PRAF/RLD protein family as physical partners of the intrinsic polarity protein BASL in the stomatal lineage cells. Mutating the four PRAF genes (4, 5, 8, and 9) in Arabidopsis results in defects in cell polarity, stomatal lineage division, and general plant development. We further show that the PRAF proteins are localized to the plasma membrane, and partly associate with Golgi, TGN/EE, and post-Golgi endosomes in plant cells. The interaction between PRAF and BASL is required for BASL polarization at the plasma membrane in stomatal lineage cells. On the other hand, PRAFs become polarized in the presence of BASL in Arabidopsis stomatal lineage cells, suggesting a positive feedback relationship between BASL and PRAF for maintaining cell polarity. Furthermore, we show that PRAF protein physically interacts with the Arf GEF GNOM and praf and gnom mutants share common defects of disturbed protein distribution at the plasma membrane, abnormal endomembrane morphogenesis and trafficking in Arabidopsis. Thus, we propose that PRAF proteins function in the network of GNOM signaling to regulate endosomal trafficking to contribute to the cell polarity formation at the plasma membrane in plants (Fig. 7g).

The initial symmetry breaking of stomatal lineage cell appears to be intrinsic because the polarization axes of BASL crescents are largely random in a developing leaf in Arabidopsis and BASL itself could spontaneously form a polarity site in cultured plant cells. On the other hand, the BASL polarity is also regulated by external cues. Regeneration and reorientation of BASL polarity during reiterative stomatal ACDs are guided by both chemical external cues. Regeneration and reorientation of BASL polarity during reiterative stomatal ACDs are guided by both chemical and mechanical signaling-driven processes. While previous work suggested protein phosphorylation-code serves as one of the mechanisms for BASL to polarize and function, our work here provides new mechanistic insights that endosomal activities mediated by the PRAF and GNOM proteins play a crucial role in establishing the BASL polarity domain in Arabidopsis stomatal lineage cells.

Polarization of membrane-associated proteins, such as the small GTPase Cdc42 in budding yeast and the PAR proteins in C. elegans zygote, requires positive feedback signaling loops. In plants, GNOM as an activator of Arf GTPases is an endosomal regulator of vesicle budding and controls the basally (rootward) localized integral membrane PIN proteins. In this study, we show that the PRAF proteins may function together with GNOM and regulate endosomal trafficking, thus cell polarization (Fig. 7g). Indeed, the polarized PIN proteins were found normally localized in same praf/rld quadruple mutants. In a previous study that examined the polarization orientation of BASL protein in non-stomatal lineage cells, Mansfield et al. proposed a common mechanism that defines a proximodistal field throughout the leaf epidermis may determine the polarization orientation of PIN1 and BASL. Here, our demonstration of GNOM and PRAFs required for BASL polarization was a surprise but indeed supports that, regardless of the cargo proteins being membrane integral (PIN1) or peripheral (BASL), GNOM and PRAF can promote both types of proteins to polarize at the plasma membrane.

In the cube-shaped root cells, opposing polarity domains are suggested by preferentially apical and basal localization of auxin transporters at the plasma membrane, such as apical AUX1, basal PIN1, and apical/basal PIN2 depending on cell types in Arabidopsis. GNOM, possibly functioning with Arf1 and RabA members, plays a unique role in mediating endocytic recycling of the PIN proteins to the basal side. The apical side polarization appears to be GNOM-independent and may require concerted activities of small RabA GTPases and the BIG clade Arf GEFs. In the leaf epidermal cells, it remains unknown whether a complementary membrane domain opposing the BASL polarity site is actively maintained by certain landmark proteins in stomatal stem cells. Whilst intrinsic phosphorylation codes within the PIN proteins appear to direct their polarization orientation in a cell-type-specific manner, no evidence has been shown yet for BASL polarity orientation to flip to the other side caused by the differential phosphorylation status of the protein. The identified components of the polarity module, including the scaffolding proteins POLAR9 and BRX families, the MAPKKK YODA, GSK3-like BIN2 kinases and the BSL phosphatases, all overlap with the BASL crescent and require the presence of BASL for their polarization. It would be intriguing to test whether the compound endosidin 16 (ES16), which disturbs non-basal PM trafficking in the Arabidopsis root via interfering with the RabA GTPase-dependent pathway, has impacts on the BASL polarity module. Insights from such studies would further inform whether and how PRAF and GNOM contribute to the common regulatory theme underlying planar cell polarity in the leaf epidermis.

We demonstrated that the intracellular PRAF8 proteins are localized to the vesicular compartments that partially overlap with the Golgi, TGN/EE, and some endosomes in N. benthamiana leaf epidermal cells (Fig. 4 and Supplementary Fig. 6). Interestingly, the interactions between PRAF8 and GNOM were identified to mainly occur at the RabC1- and RabE1d-labeled endomembrane compartments (Fig. 6 and Supplementary Fig. 10), hinting the relevance or potential importance of the secretion/endosomal recycling in establishing cell polarity. RabC1 has not been well characterized in Arabidopsis yet, though its homolog Rab18 in mammals was found to associate with the...
vesicles near the apical surface in polarized epithelial cells to promote targeted secretion. The plant RabE GTPases, homologs of Sec4/Rab8 (yeast/mammals), were found to localize to the Golgi and promote polarized exocytosis and secretion in both Arabidopsis and tobacco. Thus, we propose that the connected function of PRAF-GNOM may regulate Golgi and post-Golgi endosomal activities, particularly the RabC- and RabE-mediated pathways, to promote directional exocytosis and secretion. This hypothesis is supported by the previous observation of cell wall defects in gnom mutants and our observation of abnormal morphogenesis and distribution of RabE1d and other organelle markers in both gnom and praf mutants (Fig. 7e and Supplementary Fig. 11c). Surprisingly, we did not detect a significant connection between RabA GTPases (RabA1e and RabA5d) and PRAF proteins (Supplementary Figs. 6 and 10b). The RabA GTPases are the homologs of Rab11 in animals that function in secretion and endocytic recycling of PM proteins. The Arabidopsis genome encodes an expanded RabA group comprising 26 members. The possible functional connection between PRAF and RabA GTPases
Fig. 7 PRAFs and GNOM are required for endomembrane trafficking. a Localization of PIN3-GFP (green) in the designated genetic backgrounds. White dashed arrows indicate uneven distribution of PIN3 at the plasma membrane. Yellow arrowheads mark abnormal aggregations of PIN3 in the cytoplasm. Three independent experiments were performed. b FM-64 (red) dye distribution (8 μM, 40 min) in stomatal lineage cells of WT, praf4t;5c;8c;9c, and gnom (segregated from gnomB4049) to compare with WT treated with BFA (70 μM, 60 min). White arrowheads indicate internalized FM-64 in WT. Yellow arrowheads indicate abnormal FM-64 aggregations in praf4t;5c;8c;9c, gnom, or in WT treated with BFA. Data represent results of three independent experiments. c Quantification of endocytosis rates based on FM-64 internalization. Box plots show numbers of FM-64 positive endosomes per cell in WT, praf4t;5c;8c;9c and gnom after 5, 10, 15, 30, and 40 min of 8 μM FM-64 treatment, respectively. Box plots show first and third quartile (box), median (line) and (mean (cross)). n, # cells measured. Student’s unpaired t tests were used to compare with the wild type. Two-sided P values are 0.0001 (for WT vs. praf4t;5c;8c;9c) and 0.0004 (for WT vs. gnom) with 30-min FM-64 treatment, respectively. All other two-sided P values are <0.0001. ***P < 0.001; ****P < 0.0001. d Quantification of sizes of FM-64-positive compartments in designated backgrounds. Box plots show first and third quartile (box), median (line) and mean (cross). n, # FM-64 positive compartments measured. Student’s unpaired t tests were used in (c) and (d) to compare with the wild type. All two-sided P values are <0.0001. ***P < 0.001. e Localization of RabC1 (green, left) and RabE1d (green, right) in designated backgrounds. Red arrowheads mark more diffused RabC1 in the mutants. Cyan arrowheads mark filamentous distribution of RabE1d. Three independent experiments were performed. Scale bars in (a), (b), and (c) are as indicated (μm). f Quantification of vesicular sizes for RabA1e, RabC1, RabD1, RabD2a, RabE1d, and RabF2b in WT, praf4t;5c;8c;9c, and gnom, respectively. Box plots show first and third quartile (box), median (line) and mean (cross). n, # vesicles measured. Student’s unpaired t tests were used. Two-sided P values are 0.0053 (for RabC1 vs. RabC1;gnom) and 0.0112 (for RabF2b vs. RabF2b;praf4t;5c;8c;9c). All other two-sided P values are <0.0001. *P < 0.05; **P < 0.005; ****P < 0.0001. g Proposed working models for the subcellular localization of PRAF8 proteins (left) and trafficking pathways possibly interfered by the absence of the four PRAF genes (PRAF4, PRAF5, PRAF8 and PRAF9) or GNOM (right). In wild-type plants, the polarization of BASL protein (green) in the stomatal lineage cell requires the physical partner, four PRAF proteins (orange), as well as the Golgi-localized Arf GEF GNOM (blue). The PRAF8 proteins are predominantly localized to the plasma membrane, where they may polarize together with BASL. The PRAF8 proteins may also partially associate with the Golgi, TGN/EE and a subset of endosomes/vesicles decorated by RabC1 and RabF2b. Furthermore, the PRAF proteins physically interact with GNOM, possibly leading to the association of GNOM to the RabC1- and RabE1d-decorated membrane structures. In the absence of the four PRAF genes or GNOM, multiple endomembrane markers are similarly defective in morphology and/or distribution (red crosses), suggesting that multiple endocytosis, secretion, and recycling etc., are commonly disturbed in praf and gnom mutants. We propose that the connected function of PRAF and GNOM plays important roles in endomembrane trafficking and is required for the establishment of BASL polarization in the stomatal lineage cells. PM plasma membrane, NE nuclear envelope, ER endomembrane reticulum, G Golgi, TGN/EE trans-Golgi network/early endosome, SV secretory vesicle, RE recycling endosome, MVB multivesicular body, LV lytic vacuole.

deserves more detailed investigation for their in vivo localization, genetic and biochemical interactions. Considering the fact that GNOM predominantly localizes to the Golgi apparatus22, whilst PRAF8 localizes to both the Golgi, TGN/EE and post-Golgi endosomes, it is likely that PRAF proteins function as mediators of GNOM-driven Golgi-to-PM membrane trafficking that is required for the establishment of polarized membrane domains in Arabidopsis.

In responding to gravity signaling in columella cells of Arabidopsis lateral roots, the PRAF/RLD proteins become polarized to the plasma membrane by interacting with AtLAZY1/1-LAZY1-like (LZY) proteins. Polarized PRAF/RLD proteins may then direct PIN3 relocalization to modulate auxin flow for making changes in root growth angle29. In stomatal lineage cells, we showed that members of PRAF proteins interact with BASL and, when highly expressed in the stomatal lineage cells, become polarized in a BASL-dependent manner (Fig. 3d, e). Furthermore, myristoylated PRAF8 that is predominantly localized to the plasma membrane partially rescues mutant phenotypes, supporting the functional location of PRAF at least partly at the plasma membrane (Fig. 3h).

Then, what are the possible functions of PRAF at the plasma membrane in the stomatal lineage cells? Our hypothesis is that PRAFs might (1) through physical binding, stabilize BASL polarization at the plasma membrane, (2) promote regional cell expansion8, (3) regulate the PIN proteins and auxin signaling in stomatal lineage cells53. Considering the mild phenotype of the pin mutants in stomatal development (Supplementary Fig. 11d), it is likely the BASL polarity pathway was most significantly affected by the praf or gnom mutations. With regards to BASL polarization, the previously identified BASL partners, the BRX proteins, localize to the plasma membrane by palmitoylation, through which BASL can be stabilized at the plasma membrane10. The BRX domain in PRAFs can mediate homotypic and heterotypic interactions between and within the BRX and PRAF family members81,23, therefore PRAF may associate with the plasma membrane via its BRX domain to bind to BRX proteins and/or via its PH domain to bind to PtdIns(4,5)P2. As GFP-BASL also requires PRAFs to polarize (Fig. 2d, e and Supplementary Fig. 3c, d), the interdependence of PRAF and BASL for polarization suggested a positive feedback loop between the two proteins for the establishment of cell polarity in stomatal lineage cells. Furthermore, when BASL is ectopically expressed, overaccumulation of BASL protein at the polarity site induces local cell expansion5. It is likely that PRAFs are recruited to the BASL polarity module, where PRAFs may crosstalk with phos- phoinositide signaling, alter membrane property, and/or enrich regulators in membrane trafficking. The Rat GTPases in exocytosis and secretory pathways are promising candidates bridging polarized BASL and PRAF to enforced local deposition of cell wall materials in plant cells.

Methods

Plant materials and growth conditions. The Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type unless otherwise noted. The sequence data of the proteins reported in this study can be found in TAIR (www.arabidopsis.org) with the following accession numbers: BASL (At5g60880), GNOM (At1g39810), PRAF1 (At1g65920), PRAF2 (At3g47660), PRAF3 (At1g69710), PRAF4 (At1g67950), PRAF5 (At4g21240), PRAF6 (At3g23270), PRAF7 (At1g43570), PRAF8 (RflD2, At5g23300) and PRAF9 (RflD3, At3g19420).

Arabidopsis mutants and marker lines used in this study were basil-2, GFP-BASL8, TMM-GFP33, MUTE markers34, and gnom beige (gnom beige) is a trans-heterozygous complementing line harboring gnom54 and gnom beige. The T-DNA insertion mutants were obtained from the Arabidopsis Biological Resource Center, ABRC, including praf4 (SALK_067605), praf4 (::GABI_225B01), praf8 (SALK_061699), and praf9 (SALK_091363). The alleles of the triple mutant praf4;6t-1;9t-1 were described in ref. 29. The Gnom-related reagents, including the endogenous promoter driven GNOM-GFP/RFP and gnom GFP (SALK_103014) were reported in ref. 29. The Wave endomembrane markers (DNA and seeds) were described in ref. 29 and obtained from the ABRC.

To grow plants, in general, Arabidopsis thaliana seeds were surface sterilized and stratified for at least 1 day in the dark at 4 °C before transferred to the light. Seedlings were grown at 22 °C on half-strength Murashige & Skoog (MS) media in

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Molecular cloning and transgenic plants. In general, the LR Clonase II-based gateway cloning (GVPAM) was used to generate constructs. All primer sequences are provided in Supplementary Table 1.

To generate C-terminal YFP-tagged PRAF protein fusion, the genomic coding regions of PRAF genes (4, 5, 8, and 9) (from ATG to immediately ahead of the stop codon) were amplified and cloned into pENTR/D-TOPO (Invitrogen), respectively. Next, the promoter regions were amplified and inserted into the NotI site of the pENTR/D-TOPO carrying genomic regions, respectively. To generate myr-gPRAF8-YFP, the myristoylation lipidation site was synthesized in the amplifying primers to attach to the genomic region of PRAF8 for subcloning into pENTR/D. Then, the entry clone was recombined by the Clonase II enzyme mix (Invitrogen) into the destination vector p-mLC for expression in plants.

To create N-terminal fluorescent-tagged Venus/mCherry-PRAF driven by the stomatal-lineage-specific TMM promoter, modified RtpGW4B433 vectors containing the TMM promoter were used. To introduce a marker line or another mutation into plants simultaneously. A Cas9-free BIA2300 containing PRAF8-sgRNA, PRAF9-sgRNA, and Cas9 into the wild-type N. benthamiana plants were then screened out from the F2 populations.

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To express recombinant proteins in E. coli, the coding region of GNO1 was cloned into pET28a vector to generate His-tagged GNO1 C. The coding region of PRAF4/5/8/9_C was cloned into pGEX-4T-1 vector by a single LR reaction.

Recombinant protein production and pull-down assay. For recombinant protein expression and purification, the recombinant BAC1 protein tagged by His-sumo and His-tagged GNO1_C were purified using Ni-NTA agarose (QIAGEN) according to the manufacturer’s protocol. To perform the pull-down assay, GST or GST-PRAF3 protein was immobilized on Pierce™ Glutathione Superflow Agarose (Thermo Scientific™), which were then incubated with equal amount of purified His-sumo-BASL or His-GNO1_C, respectively, on a rotating wheel at 4 °C –40 min supplemented with 8 μM BFA and 50 µM CHX or DMSO. For BFA wash-out experiments, seedlings were then washed in ddH2O and Confocal imaged post-washing. For FM4-64 internalization experiments, 3-day-old seedlings were incubated for 5–10 min supplemented with 8 µM FM4-64 before mounting for imaging. Forwortmannin treatment, 3- to 4-day-old seedlings were treated with water containing 2 μM wortmannin, 3 μM chemical or 3 μM DMSO (mock) for 2 or 3 h. For the plasmolysis experiments, 4-day-old seedlings were first incubated with PI for 10 min and then 20% sucrose for 30 min prior to mounting for imaging.

Yeast two-hybrid assay. A genome-wide yeast two-hybrid screen for BASL physical interactors was performed as described in ref. 39. To further confirm the interaction of yeast two-hybrid assays were described previously in85,88. Specifically, prior to leaf infiltration, Agrobacterium tumefaciens GV3101 and yeast transformation mixture were co-transformed into the yeast strain AH109 and positive transformants were selected with SD/-Leu/-Trp medium after 2 days of yeast growth at 30 °C. The interactions were tested on SD/-Leu/-Trp/-His medium and observed after 3 days of yeast growth at 30 °C. To inhibit self-activation of certain protein fusions with DNA-binding domain (DBD), specific concentrations of 3-Amino-1,2,4-triazole (3-AT) was used as indicated in the figures.

Transient protein expression, co-localization, and BiFC in Nicotiana benthamiana. The vectors and protocol of using N. benthamiana epidermal cells for transient protein expression, co-localization and Bimolecular fluorescence complementation (BiFC) assays were described previously in36,88. Specifically, prior to leaf infiltration, Agrobacterium tumefaciens GV3101 harboring the expression vector was co-cultivated over 10 ml of Loría Bertani (LB) medium containing appropriate antibiotics. Bacterial cells were then harvested at 4000 x g for 10 min and resuspended in 10 ml of 10 mM MgCl2, followed by another step of 10 mM MgCl2 washing. Cells then remained in the medium for 3 h at room temperature prior to infiltration. Equal volumes of cell culture expressing the protein-of-interest and the strain expression the p19 protein (to suppress gene silencing)87 were mixed to reach an optical density 600 (OD600) of 0.5 and infiltrated into the 4-week-old N. benthamiana leaves. Three to five days after infiltration, leaf disks were excised and mounted onto slides for confocal imaging.

1% agar plates supplied with 16-h light/8-h dark cycles for 6–10 days. Seedlings were then transferred to the soil for growth in a 22 °C growth chamber with 16-h light/8-h dark cycles. The wild-type Nicotiana benthamiana plants were grown at 25 °C and supplied with 14-h light/10-h dark cycles.

RNA extraction and RT-PCR. Total RNAs were extracted from the whole Arabidopsis seedlings (4- to 5-day-old) using a RNeasy Plant Mini Kit (Qiagen). The cDNA reverse transcription of the RNAs were performed using the oligo dT primer. For reverse transcription (RT)-PCR, the ribosomal S18 (RPSS18) gene was used as an internal standard for normalization of gene expression levels. PRAF genes and S18 were amplified for 28 and 25 cycles, respectively, with the primers listed in Supplementary Table 1.
Co-IP and MS. To identify BASL-interacting proteins through co-IP MS, 5 g of seedlings (expressing 35S:GFP-BASL) in Col-0, or 35S::GFP in Col-0 at 3-dpg) were grown in liquid nitrogen. Proteins were extracted with the extraction buffer (100 mM Tris-HCl at pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na3VO4, 20 mM NaF, 50 mM β-glycerophosphate, 10% glycerol, 1 mM PMSF, protease inhibitor cocktail (Millipore, Sigma, P 9599), 1% (v/v) NP-40). The homogenates were sonicated for 10 s, then diluted to NP-40 (10%); the extraction buffer with 0.2% (v/v) NP-40. Finally, 5× SDS sample buffer was added to the beads and boiled for 5 min. The protein samples were separated by 10% SDS–PAGE by a short distance, followed by gel reduction, alkylation, and digestion with trypsin (sequencing grade, Thermo Scientific Cat # 90058). The peptides were extracted twice with 5% formic acid, 60% acetonitrile, and dried under a vacuum.

LC-MS/MS analysis was performed at the Biological Mass Spectrometry facility of Rutgers University. Samples were analyzed by LC-MS using Nano LC-MS/MS ( Dionex Ultimate 3000 RSLC nano system) interfaced with Q Exactive HF (Thermo Fisher). Peptides were loaded on to a fused silica capillary column Acclaim PepMap 100, 75 µm × 2 cm (Thermo Fisher). After washing for 5 min at 5 µl/min with 0.1% TFA, the trap column was brought in-line with an analytical column (Nanoacaze MZ peptide BEH C18, 130 A. 1.7 µm, 75 µm × 250 mm, Waters) for LC-MS/MS. Peptides were fractionated at 300 nL/min using a segmented linear gradient 4–15% B in 30 min (where A: 0.2% formic acid, and B: 0.16% formic acid, 80% acetonitrile) at 25–50% B in 35 min, and 50–99% B in 11 min. MS data were acquired using a data-dependent acquisition procedure with a cyclic series of a full scan acquired in Orbitrap with resolution of 120,000 followed by MS/MS (HCD relative collision energy 27%) of the 20 most intense ions and a dynamic exclusion duration of 20 s. The peak list of the LC-MS/MS were generated by Thermo Proteome Discoverer (v. 2.1) into Mascot Generic Format (MGF) and searched against Arabidopsis (TAIR v. 10), plus a database comprised of the common lab contaminants using an in house version of XTandem (GPM Furry, Craig and Beavis, 2004). Search parameters are as follows: fragment mass error: 20 ppm, parent mass error: ±7 ppm; fixed modification: carbamidomethylation on cysteine; flexible modifications: Oxidation on Methionine; protease specificity: trypsin (C-terminus following RS, with 1 miss-cuts). MS/MS spectra were searched against the Arabidopsis (TAIR v. 10), plus a database comprised of the common lab contaminants using an in house version of XTandem (GPM Furry, Craig and Beavis, 2004). Search parameters are as follows: fragment mass error: 20 ppm, parent mass error: ±7 ppm; fixed modification: carbamidomethylation on cysteine; flexible modifications: Oxidation on Methionine; protease specificity: trypsin (C-terminus following RS, with 1 miss-cuts). The ratios of high fluorescence intensity values over low fluorescence intensity values obtained from equal lengths along the cell periphery and within the same cell. To quantify the number of endosomal compartments (proteins or FM4-64 staining) per cell, 10–15 z-projected confocal images were captured, and the endosomes were counted by Fiji. To quantify the size of vesicles, z-projected confocal images were first processed by “FIFT-Bandpass Filter” and “Image-Adjust-Threshold” in Fiji. The sizes of individual vesicles were measured by the function of “Analyze Particles”.

For colocalization analysis between PRAF8 and organellar markers in N. benthamiana, 3–5 day of epidermal cells, 3–5 days after infiltration, z-stacks of sequential scanning images (thickness ~50 µm, with each optical section distanced by 0.5 µm) were captured from the abaxial side of the infiltrated leaves by the Leica SP5 II confocal microscope. The Fiji Coloc2 plugin was used to obtain the Pearson correlation coefficient (PCC) values using automated thresholding, combined with PSF = 3.00 pixel. Costes randomizations ≥ 25. Register of interest (ROI) were circled (r = 17.05 µm for 40 smaller ROIs or r = 34.10 µm for 10 larger ROIs) from each z-projected image. Only when Costes P-value ≥ 0.95, PCC values above the threshold were recorded and analyzed. To measure the volume size of the BiFC particles, z-projected images (16 images for each sample) were first deconvoluted by Leica Application Suite Advanced Fluorescence (LAS AF) for 3D analysis (blind deconvolution, 10 iterations). The volume of each particle was measured by Fiji’s 3D Object Counter after automatic stack thresholding was applied. The histograms for particle volumes (above 0.01445 µm3) were generated by the GraphPad Prism.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that support the findings of this study are available in the main text or the supplementary information. Source data are provided with this paper. The MS dataset is deposited into the MassIVE website with an accession number MSV000088441. All unique biological materials (e.g., plant lines, DNA constructs) are available from the corresponding author upon request. Source data are provided with this paper.

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Confocal imaging, data processing, and quantification analysis. Confocal images were captured with the Leica TCS SP5 II Confocal microscope (40× or 63×). Unless otherwise noted, the adaxial epidermis from cotyledons of 3- or 4-day-old seedlings or mutants were stained with PI and imaged on the Confocal to obtain daughter cells. To quantify stomatal divisional asymmetry, 4-day-old seedlings of wild type or mutants were stained with PI and imaged on the Confocal to obtain daughter cells. To quantify the number of endosomal compartments (proteins or FM4-64 staining) per cell, 10–15 z-projected confocal images were captured, and the endosomes were counted by Fiji. To quantify the size of vesicles, z-projected confocal images were first processed by “FIFT-Bandpass Filter” and “Image-Adjust-Threshold” in Fiji. The sizes of individual vesicles were measured by the function of “Analyze Particles”.

For colocalization analysis between PRAF8 and organellar markers in N. benthamiana, 3–5 day of epidermal cells, 3–5 days after infiltration, z-stacks of sequential scanning images (thickness ~50 µm, with each optical section distanced by 0.5 µm) were captured from the abaxial side of the infiltrated leaves by the Leica SP5 II confocal microscope. The Fiji Coloc2 plugin was used to obtain the Pearson correlation coefficient (PCC) values using automated thresholding, combined with PSF = 3.00 pixel. Costes randomizations ≥ 25. Register of interest (ROI) were circled (r = 17.05 µm for 40 smaller ROIs or r = 34.10 µm for 10 larger ROIs) from each z-projected image. Only when Costes P-value ≥ 0.95, PCC values above the threshold were recorded and analyzed. To measure the volume size of the BiFC particles, z-projected images (16 images for each sample) were first deconvoluted by Leica Application Suite Advanced Fluorescence (LAS AF) for 3D analysis (blind deconvolution, 10 iterations). The volume of each particle was measured by Fiji’s 3D Object Counter after automatic stack thresholding was applied. The histograms for particle volumes (above 0.01445 µm3) were generated by the GraphPad Prism.

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Author contributions

L.W., D.L., K.Y., and J.D. designed the research. L.W., D.L., and K.Y. performed most of the experiments. X.G. performed co-IP MS of BASL. C.B. contributed to the yeast two-hybrid assays. T.N. assisted with mutant analysis. L.W., D.L., K.Y., J.L., M.T.M., D.C.B., and J.D. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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