Auxin-induced signaling protein nanoclustering contributes to cell polarity formation

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Cell polarity is fundamental to the development of both eukaryotes and prokaryotes, yet the mechanisms behind its formation are not well understood. Here we found that, phytohormone auxin-induced, sterol-dependent nanoclustering of cell surface transmembrane receptor kinase 1 (TMK1) is critical for the formation of polarized domains at the plasma membrane (PM) during the morphogenesis of cotyledon pavement cells (PC) in Arabidopsis. Auxin-induced TMK1 nanoclustering stabilizes flotillin1-associated ordered nanodomains, which in turn promote the nanoclustering of ROP6 GTPase that acts downstream of TMK1 to regulate cortical microtubule organization. In turn, cortical microtubules further stabilize TMK1- and flotillin1-containing nanoclusters at the PM. Hence, we propose a new paradigm for polarity formation: A diffusive signal triggers cell polarization by promoting cell surface receptor-mediated nanoclustering of signaling components and cytoskeleton-mediated positive feedback that reinforces these nanodomains into polarized domains.
Cell polarity is a fundamental cellular property required for the specialization and function of essentially all cells. It is now widely accepted that the plasma membrane (PM) is highly compartmentalized into specialized functional domains with distinct protein and lipid compositions. The formation of cell polarity depends on breaking of the PM symmetry to form spatially segregated domains at the cell surface, How the spatiotemporal organization of signaling components is initiated, especially by uniform signals, remains unclear, despite decades of studies on the mechanisms behind the establishment and maintenance of cell polarity. To date, two prevalent models have been proposed to explain self-organizing cellular symmetry breaking or that induced by spatial cues: asymmetric recruitment of polarity signaling proteins or endomembrane-linked trafficking of these proteins. However, these models have difficulty in explaining how lateral segregation of signaling components into polarized membrane domains is achieved, especially by a uniform field of highly diffusible small signaling molecules. Furthermore, although the importance of lipid environment for the polarized sorting of signaling proteins is well-established in yeast and animal epithelial cells, the mechanisms by which membrane lipids contribute to the formation of polarized domains remain poorly understood.

Unequivocal evidence shows that many cell surface-signaling proteins are not uniformly mixed with membrane lipids via free diffusion-based equilibrium, but laterally segregated into highly dynamic signaling nanoclusters with specific lipids on the PM. Furthermore, the spatiotemporal distribution of membrane lipids is also known to play a critical role in regulating signaling events. Among these lipids, cholesterol and related sterols often appear organized in clusters and have the ability to form tightly ordered domains together with sphingolipids. These domains, known as “membrane rafts,” have long been suggested as a crucial platform for protein sorting and signal transduction at the cell surface, particularly in the spatial regulation of key cellular processes, such as cell polarization and immune response. Notably, members of the Rho family GTPases, such as Ras, Cdc42, and ROP6 (a Rho-like GTPase from plants), appear to form transient nanoclusters and have the ability to form tightly ordered domains with interlocking lobes and indentations, which are conserved in plants and found in PM nanodomains in various organisms, including plants. PCs exhibited a patchy distribution of sterols in PCs by staining cotyledons with otillin1 as a live molecular marker for sterol-rich lipid domains. Here, we tested this hypothesis via genetic, biochemical, and chemical approaches. On the basis of our findings, we propose a working model for auxin-triggered polarized domain formation at the PM via auxin-induced nanoclustering of sterol lipids and PM-associated signaling proteins. Auxin first induces the appearance of asymmetric distribution of signaling components by promoting the formation of interdependent TMK1 nanoclusters and ordered lipid nanodomains, and then this initial asymmetry is reinforced to form the polarized domains by microtubule-mediated diffusion restriction of these TMK1/ordered lipid nanodomains.

Results
Sterol-rich domains define indentations of pavement cells. To assess the role of PM nanodomains in the morphogenesis of the puzzle-piece-shaped PCs, we first analyzed the distribution of ordered lipid domains in cotyledon PCs using dI-4-AANEPPDHQ, a lipid order-sensitive probe. Based on the fluorescence intensity of the probe in two different spectral channels, green (ordered) and red (disordered), a ratiometric general polarized image (GP image) was generated. High GP value (increased green fluorescence compared with red) represents high order of membrane lipids (Fig. 1a–e). Plasmolysis with 0.8 M mannitol showed that the peripheral staining was associated with the PM, but not with the cell wall (Supplementary Fig. 1). The optical resolution limit of confocal microscopy prevents the mid-plane distinction of complementary lobe and indenting regions of the PM in two adjacent PCs. As previously described, therefore, we used optical sections just above (1–1.5 µm) the cell mid-plane to separate the complementary PM regions of the adjacent cells. As shown in Fig. 1a–e, the GP images and quantitative analyses revealed that the localization of ordered lipid domains was biased toward the indenting region.

Sterols are known as a key component of ordered domains in the PM of yeast and mammalian cells. We thus visualized the distribution of sterols in PCs by staining cotyledons with filipin, a sterol-binding probe commonly used for in situ sterol localization in various organisms, including plants. PCs exhibited a patchy pattern of filipin–sterol complexes along the PM, which are preferentially distributed to indenting regions (Supplementary Fig. 2A–D and Supplementary Movie 1). To complement these staining methods, we also attempted fluorescent protein-tagged flotillin1 as a live molecular marker for sterol-rich lipid domains. In mammalian cells, flotillin has been shown to co-localize and associate with cholesterol-rich microdomains using both biochemical and microscopy methods. Flotillin is conserved in plants and found in PM nanodomains in Arabidopsis. Arabidopsis flotillin1 is also enriched in detergent-resistant membrane (DRM) fractions, which likely contain membrane raft components, indicating its potential association with sterol-rich domains. To further test whether flotillin1 is a useful marker for sterol-rich nanodomains in plants, we generated a transgenic line expressing flotillin1-mVenus under its native promoter. As shown in Supplementary Movies 2 and 3, flotillin1 proteins form dynamic nanoclusters in the PM. These flotillin1 nanodomains strongly colocalized with the patched filipin–sterol complexes in the PM of PCs (Supplementary Fig. 2E–H), but not the general PM marker LIT6b (Supplementary Fig. 2I–L). These results suggest that flotillin1 is a conserved marker for sterol-rich nanodomains. Consistent with the staining results, in the majority of cases, flotillin1 proteins were more abundant in indentations than in lobes...
Fig. 1 Ordered membrane domains are preferentially localized to indenting regions. a, e Plasma membrane order visualization using di-4-ANEPPDHQ staining in the pavement cells of 2-3-day-old Arabidopsis cotyledons (Col-0). a–d Representative images obtained after di-4-ANEPPDHQ staining. a Di-4-ANEPPDHQ fluorescence recorded between 500 and 580 nm, representing high lipid ordering. b Di-4-ANEPPDHQ fluorescence recorded between 620 and 750 nm, representing low lipid ordering. c A radiometric color-coded GP image generated from a and b. d An enlarged GP image corresponding to the boxed areas in c. The GP image is a false-color image, which runs over the range indicated by the color bar. Color bar values represent GP values ascend from bottom to top, with red colors indicating high membrane ordering, whereas blue colors indicating low membrane ordering. Scale bars = 15 μm. e Quantitative analysis of mean GP values obtained from the complementary lobing and indenting regions of 161 sites of 56 cells from three independent experiments. GP values at indenting regions are significantly higher than that at lobing regions. f-h Flotillin1-mVenus shows a polar distribution toward indenting regions. f Representative image showing the distribution of flotillin1-mVenus in PCs of 2–3-day-old cotyledons. The region highlighted in the dotted-line box is further analyzed in g. Scale bars = 15 μm. g Fluorescent intensity values scanned along the indicated region in f. h Quantitative analysis of fluorescence intensity at the complementary lobing and indenting regions of 138 sites of 45 cells from three independent experiments. i, j The sterol biosynthesis mutant jocket-J79 (fk-J79), which is defective in gene encoding a sterol C14 reductase, shows altered PC shape. i Representative images of cotyledon pavement cells in fk-J79 mutant and its wild type. Scale bars = 30 μm. j Quantitative analysis of the number of lobes and indentation widths in PCs of fk-J79 mutant and its wild type. n represents the number of cells. Data are representative of three independent experiments which have the same pattern. ***P ≤ 0.001.

To evaluate the roles of sterols in PC morphogenesis, we examined PC phenotypes of sterol biosynthesis mutants (fk-J79, hyd1-E508, cpi1-1) with an overall reduction in the major sterols (sitosterol and stigmasterol) found in the PM38–40. All these mutants display greatly altered PC shapes with reduced lobe numbers and increased indentation width compared with their wild-type control (Fig. 1i, j; Supplementary Fig. 3A, B). Importantly, the depletion of sterol from the PM by methyl-β-cyclodextrin (mβCD)31,41, a sterol-depleting agent, also caused a similar defect in PC morphogenesis and compromised auxin-promoted PC interdigitation (Supplementary Fig. 3C, D). These defects resemble those induced by the disruption of the ROP6 pathway that promotes the organization of cortical microtubules and the formation of PC indentation24,26. Because sterol perturbation either by genetic or pharmacological approaches may generate pleiotropic effects apart from the disruption of sterol-rich nanodomains, one cannot exclude the possibility that PC morphogenesis is not directly linked to these nanodomains. However, the similar PC shape changes (i.e., increased indentation widths and reduced lobe numbers) resulting from defects in ROP6 signaling and sterol accumulation, together with the preferential distribution of both ROP6 and sterol-rich ordered lipid nanodomains to the indenting regions, strongly support a role for the sterol nanodomains in defining and establishing the indenting regions, herein termed the indentation polarity.

Auxin induces lipid ordering required for ROP6 activation. Our previous studies show that auxin promotes the formation of the puzzle-piece PC shape, suggesting that auxin is a signal that initiates the formation of the PC polarity21,25. Thus, we asked whether auxin regulates the generation of ordered lipid domains in PCs. The auxin biosynthesis mutant we18-1tar2-1 with reduced auxin levels in cotyledons43 exhibited greatly reduced lipid ordering in PCs, as indicated by di-4-ANEPPDHQ staining (Fig. 2a, b). This defect in lipid ordering was rescued by exogenous auxin (Fig. 2a, b). Similar to the we18-1tar2-1 mutant, the sterol biosynthesis mutant fk-J79 also exhibited reduced lipid ordering (Fig. 2c, d). However, unlike the we18-1tar2-1 mutant, the fk-J79 mutant was completely insensitive to exogenous auxin in the promotion of lipid ordering (Fig. 2a–d). Furthermore, the auxin-induced increase in the number of polar sites (as indicated by the lobe number) in PCs was completely abolished in the fk-J79 mutant (Fig. 2e, f).
To understand how ordered lipid domains contribute to the formation of the indentation polarity, we examined the effect of sterols on the activity of ROP6, an indentation polarity marker critical for indentation formation. Previously we showed that the activity of ROP6 is promoted by auxin. We further found that reducing PM sterols by mβCD treatment greatly hindered auxin-induced increase in ROP6 activity (Fig. 2g, h), suggesting that ordered lipid domains are important for the auxin promotion of ROP6 activity. Previous biochemical evidence showed that S-acylation contributes to the recruitment of ROP6 into lipid-raft-like, detergent-resistant membrane fraction. We further showed that auxin increased the amount of S-acylated ROP6 in a dose-dependent manner (Fig. 2i, j), consistent with the notion that auxin promotes the association of ROP6 with sterol-rich ordered lipid domains. Therefore, we propose that auxin promotes the formation of ordered lipid domains required for the ROP6-dependent indentation formation.
Auxin promotes the formation of TMK1 nanoclusters. We next investigated how auxin promotes the formation and polarization of ordered lipid domains to the indenting region. Auxin signaling is mediated by at least two major pathways: the nuclear-based TIR1/AFB pathway that regulates the expression of auxin-induced genes\(^{19,20}\) and the PM-based TMK pathway involved in both transcriptional and non-transcriptional auxin responses\(^{21,22}\). Because auxin promotes ROP6 activation in a TMK-dependent manner\(^{21,25}\), we speculated that the TMK-dependent pathway regulates lipid ordering and its polarity. In addition, the tmkl-tmkr double mutant showed reduced lipid ordering in the PM of cells and was insensitive to the auxin-induced lipid ordering (Supplementary Fig. 4). As an initial step in testing our hypothesis, we analyzed the spatiotemporal dynamics of TMK1, a major member of the TMK clade with four functionally overlapping members\(^{25}\). To study dynamics of TMK1, time-lapse images were collected on a Zeiss microscopy equipped with a total internal reflection fluorescence (TIRF) module for 2–3-day-old cotyledon PCs followed by single-particle-tracking method, which is commonly used to visualize the dynamics of PM-localized nanoscale compartments including protein nanoclusters\(^{45–47}\). As illustrated in Fig. 3a, anticalin surfaces of PCs form interlocking lobes and indent, but it is technically impossible to image these surfaces using TIRF or other single-molecule imaging methods. Therefore, we investigated how auxin affects the dynamic behaviors of nanoparticles on the peripheral surface of PCs. The results showed that TMK1-GFP, expressed under TMK1’s native promoter, was laterally segregated as nanoparticles at the PM (Supplementary Movie 5 and Fig. 3b). Treatment with 100 nM IAA resulted in a dramatic decrease in the density of TMK1-GFP particles (Fig. 3c), accompanied with an increase in the particle size (Fig. 3d, e). These results suggest that auxin promotes the formation of larger TMK1 nanoclusters, possibly by coalescing single proteins or small and unstable nanoclusters. Further diffusion analyses showed that a two-component model describes the distribution of diffusion coefficients much better than a one-component model (Supplementary Fig. 5A), indicating TMK1-GFP particles on the membrane consist of at least two populations, one with slow and one with fast diffusion. IAA treatment (100 nM, 10 min) resulted in a significant reduction in diffusion coefficients of both populations (Fig. 3f; Supplementary Table 1). These results support the hypothesis that auxin specifically promotes the formation of the TMK1 nanoclusters with larger size and slower diffusion.

TMK1 nanoclustering and lipid ordering are interdependent. Because ligand-induced receptor clustering may modulate the local lipid environment and stabilize ordered lipid domains around the receptor\(^{48,49}\), we hypothesize that auxin-triggered TMK1 nanoclustering stabilizes a local ordered lipid environment. To test the hypothesis, we first studied the dynamic relation between TMK1 and flotilin1 proteins at the PM. As shown in Fig. 3g–i, TMK1-tagRFP particles are partially colocalized with flotilin1-mVenus particles. As a protein associated with ordered lipid domains, flotilin1 is expected to be present in diverse populations of nanoclusters, and thus its partial colocalization with TMK1 is anticipated. To functionally test the role of ordered lipid domains in TMK1 nanoclustering, we analyzed TMK1 dynamics in the presence of the sterol-disrupting agent methyl-β-cyclodextrin. A treatment with NaCl instead of HYD was used as the negative control. Quantitative analysis of the relative S-acylated ROP6 level from three independent experiments. The relative active ROP6 level was determined as the amount of S-acylated ROP6 divided by the amount of total GFP-ROP6. The relative active ROP6 level in different treatments was normalized to that from the mock-treated control as standard. Data are presented as mean ± SD. n in b, d, f represents the number of independent cells. WT, wild type. CTR, control. Scale bars: 15 μm in a and c, and 30 μm in e. *P < 0.05; ****P ≤ 0.0001; ns, not significant.

TMKs are required for auxin-mediated nanoclustering of ROP6. TMKs are required for auxin-induced changes in ordered lipid nanodomains (Fig. 4) and increases in ROP6 activity\(^{21,25}\), while auxin-induced lipid ordering is critical for auxin-induced increase in ROP6 activity (Fig. 2). Thus, we hypothesize that auxin promotes the nanoclustering of active ROP6 in a TMK- and sterol-dependent manner. Indeed, single-particle-tracking analysis showed that the constitutively active (CA) form of ROP6 (YFP-Carop6\(^{36,44}\)) was preferentially distributed in the particles with larger size and slower diffusion properties in comparison with the inactive form of ROP6 (dominant negative,
Fig. 3 Auxin promotes TMK1 nanoclustering in a sterol-dependent manner. a The 3D view of the pavement cell (PC). b Representative TIRF images of PCs expressing TMK1::TMK1-GFP with or without the IAA (100 nM, 10 min) treatment and the methyl-β-cyclodextrin (mβCD, 10 mM, 30 min) pretreatment. c Quantitative comparison of the TMK1-particle density in different treatments. d Histogram of TMK1-particle size distribution in control and IAA-treated cells. e Relative frequency of TMK1 particles with size larger than 300 nm. Data in c and e are presented as mean ± SD, and n represents the number of independent cells. f Comparison of diffusion coefficients of TMK1 particles in different treatments. The violin plot shows the distribution of the original diffusion coefficients without the log transformation. The distribution of log-transformed diffusion coefficients is shown in Supplementary Fig. 5. The number of particles analyzed in each treatment (from left to right) are 156,480, 131,187, 98,267, and 107,269, respectively. Statistical significance of treatment effects was evaluated on the overall diffusion coefficient of each treatment. CTR, control. Data in c, d, e, and f) are representative of three independent experiments with the same pattern. g–i Colocalization analysis of PCs co-expressing flotillin1::flotillin1-mVenus and TMK1::TMK1-tagRFP. g Representative Airyscan images. The yellow dashed boxes indicate the regions depicted in h. To test the specific colocalization, the merged image was generated by rotating the image from the red channel with respect to the image from the green channel by 90°, a condition in which only random colocalization can be observed16. i A statistical analysis comparing Spearman’s rank-correlation coefficients in nonrotated (0°) and 90° rotated images. The significantly higher correlation coefficients obtained in nonrotated images than in 90° rotated images suggests that the observed colocalization is not due to random chance. Data were obtained from 58 regions of 20 cells in two independent experiments. Scale bars: 2 μm b, 3 μm g, and 0.5 μm h. **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001; ns, not significant.
DNrop6<sup>D121A</sup> (Fig. 5a–c; Supplementary Fig. 5D and Supplementary Table 1). The DNrop6<sup>D121A</sup> line is different from the line (DNrop6<sup>T30N</sup>) used by Porat-Gavra et al. Unlike DNrop6<sup>T30N</sup>, the recruitment of DNrop6<sup>D121A</sup> to the PM is not compromised and does not accumulate in nuclei (Supplementary Fig. 6). To test the effect of auxin on ROP6 nanoclustering, we performed single-particle tracking of mEGFP-ROP6 in a ROP6p::mEGFP-ROP6 line. mEGFP-ROP6 showed clear lateral segregation on the PM (Fig. 5d; Supplementary Movie 6). Treatment with 100 nM IAA, which induces ROP6 activation by fourfolds<sup>21</sup>, significantly increased ROP6 particle sizes (Figs. 5d, e). Further analyses showed that, like TMK1 particles, the distribution of diffusion coefficients of ROP6 particles was better described by a two-component model, indicating the existence of at least two populations of ROP6 particles, one slow- and one fast-diffusing populations (Supplementary Fig. 5C). Consistently, IAA treatment significantly reduced the ROP6 diffusion coefficient with an increase in the fraction of the slow-diffusion population (Fig. 5f; Supplementary Fig. 5E and Supplementary Table 1). In addition, the effect of auxin on the size increase and diffusion decrease for ROP6 particles was completely abolished in the presence of sterol-depleting agent mβCD (Fig. 5g, h). Furthermore, ROP6 and flotillin1 particles partially reside in the same nanodomains upon the auxin treatment (Supplementary Fig. 7). Together, these data suggest that auxin-triggered ROP6 activation induces ROP6 stabilization in sterol-rich nanodomains with increased size and reduced diffusion. Similar auxin-induced formation of ROP6 nanoclusters were recently observed in roots by both photo-activated localization microscopy and TIRF imaging<sup>16</sup>. We next asked whether TMK1/4 also regulates the formation of ROP6 nanoclusters. Indeed, in the tmk1tmk4 double mutant, the ROP6 particles are much smaller in size (Fig. 5d, e) and have a higher mobility (Fig. 5f). In addition, the auxin-triggered ROP6 particle size increase (Fig. 5d, e) and diffusion decrease (Fig. 5f) were greatly compromised in the tmk1tmk4 double mutant, indicating that TMK1/4 are critical for auxin-induced changes in the dynamic behavior of ROP6 particles. Taken together, these data strongly support the hypothesis that TMK1/4 regulates auxin-induced ROP6 nanoclustering via shaping the sterol-rich lipid environment at the PM. Interestingly, Platre et al. showed that phosphatidylserine lipid is also important for auxin-mediated ROP6 nanoclustering in roots, and phosphatidylserine biosynthesis mutants exhibit severe PC morphogenesis defect. Thus, ROP6 nanoclusters in PCs are likely enriched in both sterols and phosphatidylserine.

CMTs stabilize TMK1/ordered lipid nanodomains. Our data described above show that auxin induces both the formation of the indentation polarity and sterol-dependent nanoclustering of TMK1 and ROP6, which promote cortical microtubule (CMT) organization during PC development<sup>24–26</sup>. Interestingly, we found that a portion of constitutively active (CA) ROP6 particles was localized along CMTs (Supplementary Fig. 8A–C), suggesting...
a potential connection between CMTs and active ROP6s. Thus, we hypothesize that CMTs may stabilize TMK1 nanoclusters and ordered lipid nanodomains at the PM. CMTs are physically attached to the inner leaflet of the PM, and thus could presumably hinder the mobility of TMK1 nanoclusters and ordered lipid nanodomains. To investigate whether CMTs regulate the TMK1 nanoclusters, we observed an immediate effect of microtubule disruption on TMK1 nanoclusters by treating the cotyledons expressing TMK1-GFP or flotillin1-mVenus with 5 μM oryzalin for 30 min. The effectiveness of oryzalin treatment in disrupting CMTs was demonstrated by treating cotyledons expressing the 35S::GFP-TUB construct with the corresponding drug (Supplementary Fig. 8D, E). As shown in Fig. 6a–c, CMT disruption with oryzalin greatly decreased the size and increased the diffusion of TMK1 particles on the PC. Flotillin1 particles became highly diffusive upon oryzalin treatment, even though no significant changes in particle size were observed (Fig. 6d–f). In addition, TMK1 and flotillin1 particles displayed largest particle sizes and slowest diffusion rates in the presence of auxin and intact CMTs, and depolymerizing CMTs did not completely block the auxin-induced diffusion decrease (Fig. 6a–f), suggesting that the stabilization of TMK1 and flotillin1 particles is regulated by auxin-induced nanoclustering and the subsequent CMT-mediated diffusion restriction. To complement the pharmacological approach, we further studied the dynamics of TMK1 and flotillin1 particles in the ktn1 mutant, which has altered microtubule organization and PC shape.27 As shown in Supplementary Fig. 9A–D, the particles of TMK1 and flotillin1 displayed smaller size and faster diffusion rate in the ktn1 mutant than in the wild type. In addition, the dynamics of TMK1 particles were studied in the temperature-sensitive mor1-1 mutant, in which CMTs become shortened and disorganized when grown at the restrictive temperature (29 °C) for 2 h.31 The diffusion rate of TMK1 particles increased in both wild-type and mor1-1 mutant

Fig. 5 TMK contributes to the auxin-promoted nanoclustering and stabilization of ROP6 particles. a–c CArop6 formed larger particles with slower diffusion rate than DNrop6 at the plasma membrane. a Representative TIRF images of pavement cells (PCs) expressing 35S::YFP-CArop6 and 35S::YFP-DNrop6. b Relative frequency of YFP-CArop6 and YFP-DNrop6 particles with size larger than 350 nm. c Diffusion coefficients of YFP-CArop6 and YFP-DNrop6 particles. The number of particles analyzed for YFP-DNrop6 and YFP-CArop6 lines are 97,390 and 86,712, respectively. d–f Auxin-induced increase in size and decrease in diffusion rate of ROP6 particles was compromised in the presence of the sterol-depleting agent methyl-β-cyclodextrin (mβCD). g Relative frequency of ROP6 particles with size larger than 350 nm in control and IAA-treated cells with or without mβCD pretreatment. h Comparison of diffusion coefficients of ROP6 particles in different treatments. The number of particles analyzed in each treatment (from left to right) are 354,303, 355,561, 458,949, and 411,675, respectively. CTR, control. Data in b, e, g are presented as mean ± SD, and n represents the number of independent cells. All data are representative of three independent experiments with the same pattern. Scale bars = 2 μm. *P < 0.05, **P ≤ 0.0001; ns, not significant.
after treatment with the restrictive temperature for 2 h. However, the relative change of diffusion rate was much higher in the mor1-1 mutant than in the wild type, and the temperature-triggered decrease in particle sizes was only significant in the mor1-1 mutant (Supplementary Fig. 9E–G). Moreover, introducing the CArop6 into the TMK1-GFP background increased the size and reduced the diffusion rate of TMK1 particles (Supplementary Fig. 9H, I). Importantly, the effect of CArop6 expression was compromised by oryzalin treatments, indicating that intact CMTs are required for the CArop6-induced dynamic changes of TMK1 particles. These results demonstrate a critical role for ROP6-dependent CMTs in stabilizing TMK1 nanoclusters.

Taken together, we propose the following model for the formation of the PC polarity (Fig. 6g): (1) Upon auxin signaling, ordered lipid nanodomains and TMK1 proteins are clustered. TMK1 nanoclustering further promotes the formation of larger, stabilized ordered lipid nanodomains, probably by coalescing small, unstable lipid nanodomains. (2) The newly formed large lipid nanodomains provide an ideal lipid environment for ROP6-mediated MT clustering and activation. (3) The active ROP6 interacts with downstream effectors and promotes CMT ordering, which further restrict the diffusion of TMK1 nanoclusters and their surrounding ordered lipid nanodomains. (4) The initial asymmetric distribution of active ROP6 is further amplified by the positive feedback loop of ordered lipid nanodomain-ROP6-MT signaling-CMT ordering, and eventually leads to the formation of the multiple alternating polarized ROP6 domains prior to the indentation formation.

Discussion

Our findings reveal a nanodomain-centric principle for the formation of cell polarity and for auxin signaling at the PM. These
findings demonstrate a pivotal role of nanoclustering in controlling cell polarity formation, expanding its emerging roles in the regulation of fundamental processes, including cell spreading and endocytosis. Importantly based on our findings, we proposed a new paradigm for cell polarity formation that can explain cell polarization that is self-organizing or induced by uniform or localized signals. Local protein recruitment and/or endomembrane trafficking have been prevailing models to explain cell polarization. Here, we show that the coordination of nanoclustering of cell surface-signaling components with microtubule-dependent feedback regulation of nanocluster diffusion restriction is essential for auxin-mediated PC formation. This new paradigm likely provides a common design principle underlying polarity formation in different cell systems. A number of cell polarity proteins such as Rac1 and Cdc42 are found in nanoclusters, but it is unclear whether their nanoclustering is functionally linked to cell polarity formation. ROP6 acts downstream of TMKs and promotes the organization of ordered CMTs, which in turn slow down the diffusive movement of TMK1 and flotillin1 particles at the PM. This feedback loop-dependent regulation of nanocluster is distinct from the TMK-based ROP6 nanoclustering directly triggered by auxin and is essential for the PC formation. The CMT regulation of ROP6 nanoclustering appears to be at least partially mediated by the direct association of active ROP6 (in the nanoclusters) with CMTs, as we found a portion of active ROP6 is associated with CMTs as nanoclusters. This differs from the regulatory mechanism of flotillin1 particles imposed by CMTs as the majority of the flotillin1 particles do not associate with CMTs, but rather localize close to or within CMT corrals (Supplementary Fig. 8F–K). It is possible that sterol-rich nanodomains provide the microenvironment for converting inactive ROP6 to active ROP6, which then interacts with its downstream effectors and promotes the CMT ordering. The association of ROP6 with CMTs may make them leave sterol-rich nanodomains, presumably because of steric-hindrance effects between CMTs and sterol-rich nanodomains. This could also explain the observed partial colocalization of flotillin1 and ROP6 nanoclusters (Supplementary Fig. 7). A critical role for microtubules in cell polarization is well recognized in eukaryotic cells, and their association with Rho GTPase signaling proteins (ICR1, MDD1, RhoGEFs, etc.) is widely documented. CMTs have been implicated in regulating PIN protein polarization, and ROP6 nanoclustering may also impact PIN polarity. During symmetry breaking at starvation exit in fission yeast cells, microtubules are important for the formation of polarized sterol-rich membrane domains. Previous studies have also implicated CMTs in restricting the diffusion of PM-associated signaling molecules, thus, promoting their clustering during signaling. All these observations potentially link cytoskeletal modulation of signaling protein nanoclusters to cell polarity formation in various systems. Therefore, future studies should determine whether the coordination of ligand-activated nanoclustering and cytoskeleton-based diffusion restriction of nanoclusters serves as a general principle behind the formation of cell polarity. In addition, considering the impact of cell walls on the dynamics of PM-localized protein nanoclusters and PC morphogenesis revealed by recent studies, our future work will focus on how the interplay between cell wall components, PM nanodomains and the cytoskeleton regulates PC formation. Emerging evidence suggests that ligand-induced formation of protein-lipid nanodomains at the PM serves as pivotal signaling platforms throughout eukaryotic cells. Here, we show that auxin-induced formation of TMK1 nanoclusters and ordered lipid nanodomains are interdependent. We propose that this ligand-mediated self-organizing lipid–protein interaction provides an important mechanism to generate a nanodomain signaling platform. This is in line with the ligand-induced transmembrane receptor sculpting of the surrounding ordered lipid environment proposed for animal cells. Such clustering of ordered lipid-associate proteins modulates the activity of signaling proteins associated with the inner leaflet of the PM, such as ROP6 GTPase as we showed here and other GTPases in animal systems. Notably, auxin-induced ROP6 nanoclustering also requires phosphatidylinerse lipid (negatively charged) and positively charged residues located at the C-terminus of ROP6 in root cells. Because phosphatidylinerse synthesis defects alter PC shape, it is likely phosphatidylinerse-dependent ROP6 nanoclustering provides another layer of modulation to promote ROP6 nanoclustering. In mammalian cells, Ras nanoclustering is regulated by the ratio of cholesterol and phosphatidylinerse on the PM. In addition, phosphatidylinerse coalesces with cholesterol to support phase separation in the model membrane and is essential for the generation and stabilization of cholesterol-dependent nanoclusters in the live-cell membrane. It is also worth noting that, different Ras isoforms integrate into non-overlapping isoform-specific nanodomains containing distinct lipid composition. Two functionally distinct ROPs, ROP2 and ROP6, though both are activated by auxin, are differently localized to the lobes and indenting regions of the PM to promote lobe and indentation formation, respectively. Spatial separation of ROP2 and ROP6 into distinct nanodomains may ensure robust signaling and functional specificity for each ROP. Thus, it will be interesting to investigate whether ROP2 and ROP6 are indeed segregated into two distinct nanodomains with differential dynamic behaviors, and to understand how these potentially distinct nanoclustering behaviors contribute to their specific functions.

Recently, Platte et al. showed that auxin induces phosphatidylinerse-mediated ROP6 nanoclustering in Arabidopsis roots. Our findings here and those reported by Platte et al. together show that auxin-induced nanoclustering of cell surface-signaling components plays an important role in the regulation of auxin responses. This PM-localized auxin signaling system enables rapid regulation of cytoplasmic activities and complements the TIR1/AFB-based signaling that mainly controls nuclear gene expression, thus, greatly expanding the toolbox for auxin signaling machinery that is needed for the wide range of auxin responses. Our results show that auxin-induced ROP6 acylation and TMK1-modulated lipid ordering are critical for the nanoclustering of the cell surface auxin-signaling components. These findings together have firmly established a new auxin signaling mechanism at the cell surface that depends on a localized lipid–protein interaction platform as signaling “hot spot.” Interestingly, auxin also promotes the cleavage of intracellular kinase domain from the cell surface TMKs to regulate nuclear gene expression. Therefore, the PM-originated signaling mediated by TMKs is emerging as an important auxin signaling mechanism, and thus it would be interesting to determine whether all TMK-dependent auxin signaling pathways are mediated via nanoclustering.

Methods

Growth conditions and plant materials. A. thaliana ecotypes Columbia-0 (Col-0) was used as the wild type in this study. The transgenic plants containing 35S::YFP-Coprop6, 35S::YPF-DNop6, pTMK1::TMK1-GFP, or pFER::FER-GFP were reported previously. The hyd1-ES08 seeds were kindly given by Dr. Kathrin Schrick (Kansas State University, USA). The fck179 seeds were gifts from Dr. Iyan-Chyun Jang (The Ohio State University, USA). The cp1-1 seeds were kindly shared by Dr. Markus Grebe (Swedish University of Agricultural Sciences, Sweden). The we8-1nar2-1 seeds were kindly shared by Dr. Jaimie Van Norman (University of California, Riverside, USA). The flotillin1-mVenus x tmklmm4, mEGFP-ROP6 x tmklmm4, flotillin1-mVenus x kint1, TMK1-GFP x kint1, TMK1-GFP x mok1 mutants were generated by genetic crosses and confirmed by genotyping. Plants were grown in soil or on half-strength Murashige and Skoog agar Petri dishes.
supplemented with 1% (v/v) sucrose. Controlled environmental conditions were provided in the growth room at 22 °C with 16 h light/8 h dark photoperiod.

DNA constructs and plant transformation. All constructs were made using the primers listed in Supplementary Table 2. The pGW66/100,R0P6:mgFEGP-R0P6 plant expression vector was constructed as follows. The R0P6 promoter (1081 bp upstream of the translation start of R0P6) was amplified by PCR from Col-0 genomic DNA using the following primer pairs: R0P6prePro/R0P6preR and R0P6F/R0P6G, respectively. The mgFEGP coding sequence was amplified from the plasmid kindly shared by Dr. Xuemei Chen (University of California, Riverside, USA) with the primers mgFEGP-F and mgFEGP-R. Those three PCR fragments, R0P6 promoter, mgFEGP, and R0P6 genomic sequence, were fused together by the overlapping PCR. The fused PCR products were recombined into the pDONOR207 vector using BP recombination (Invitrogen), and the resulting entry vector was then transferred into the Gateway binary vector pGW66/100 via the LR reaction. The construct with similar design (ROP6:GFP-ROP6) was able to fully complement the rop6-1 knock-out mutant29. Because a previous publication reported that the native promoter-driven GFP-tagged AtFlotillin1 had very weak to no fluorescence37, we generated the native promoter-driven line with the otmR and subcloned into the pCAMBIA1300 vector via HindIII and BamHI sites. The genomic sequence (without stop codon) of otmR was amplified using the primers flogFR/oSlogF and the mVenus coding sequence was amplified using the primers mVenusF and mVenusR. Those two PCR fragments were further fused by the overlapping PCR. The fused PCR products were subcloned into the pCAMBIA1300 vector containing the 35S promoter using BamRI and KpnI sites. The pCAMBIA1300/otmR::LIT6b-mVenus construct was generation as follows. The otmR genomic DNA was amplified using the primers TMK1F/TMK1R, and then inserted into the pDO-ScarletR, MAP4F/MAP4R and NOSF/NOSR, respectively, and then were assembled using the builder HiFi DNA Assembly Master Mix. All constructs were sequence-verified.

Chemicals. Stock solutions of indole-3-acetic acid (IAA, Sigma-Aldrich) and oryzalin (Sigma-Aldrich) were prepared in dimethyl sulfoxide (DMSO). The stock solution of methyl-β-cyclodextrin (mCD, Sigma-Aldrich) was prepared in deionized water.

Analysis of Arabidopsis cotyledon pavement (PC) cell shape. For visualizing PC shape, adaxial cotyledon epidermis of 2–3-day-old seedlings was stained in propidium iodide (2 mg/ml) solution for 20 min and then imaged using a Leica TCS SP5 confocal microscope with the following settings: excitation 553 nm and emission 600–630 nm. PC images were consistently taken at the mid-age of cotyledons with the same developmental stage. We applied a blind analysis for quantifying PC phenotypes. The researcher who analyzed the shape had no knowledge of the identity of the samples. The number of lobes was counted manually or by previous method described for the Arabidopsis cotyledon pavement growth with that has a curvature with the depth of at least 1 µm was considered as a lobe, and tricellular junctions were excluded from the lobe counting. The indentation width was determined by measuring the shortest distance across the cell between two indenting regions using ImageJ as previously described27. The data shown in Figs. 2, 3, and Supplementary Fig. 3 are representative of three independent experiments with the same pattern. Note that the homogamous sterol biosynthesis mutants germinate and grow much slower than their wild types, PC phenotypes of mutants and their wild types were quantified at 7 days after seed plating (Fig. 1j, j; Supplementary Fig. 3A, B). The quantification of autofluorescence and mCD effects (Supplementary Fig. 3C, 3D, 3E, 3F), seeds were germinated on solid half-strength Murashige and Skoog (½ MS) medium containing 50 nM IAA, 10 mM mCD, 50 mM IAA plus 10 mM mCD, or 0.1% DMSO (mock treatment). A consistent DMSO concentration (0.1%, v/v) was used in all treatments, including mock and mCD treatments. After germination, liquid medium was replaced every 3 days. Solid medium to soak the germinated seeds for additional 2 days. The liquid medium containing the chemicals was refreshed for every 12 h.

Polarization measurements of ordered lipid domains (di-4-ANEPPDHQ staining), filipin–sterol complexes and otmR1 proteins between lobing and indenting regions, the optical section above the cell mid-plane (1–1.5 µm) was imaged at which the PM membranes of two adjacent cells were optically separated. The same visualization method was used in Fu et al.28 and Li et al.30. 3D reconstruction of confocal z stacks was carried out in Imaris 9.1.2 (Bitplane).

ROP6 activity assays. ROP6 activity assays were performed as described previously29. Briefly, protoplasts were isolated from leaves of 3-week-old wild-type plants. Isolated protoplasts were treated with half-strength liquid MS media containing 100 nM IAA for 10 min or 10 mM mCD for 30 min. Where mCD was used, a pretreatment of 10 mM mCD was used for 30 min, followed by a 10 min co-treatment with mCD and 100 nM IAA. After treatments, total proteins were extracted from 107–108 treated protoplasts using extraction buffer (25 mM HEPES, pH 7.4, 10 mM MgCl2, 10 mM KCl, 5 mM dithiothreitol, 5 mM Na2VO4, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1x COMPLETE, Mini EDTA-free Protease Inhibitor Cocktail (Roche)). Twenty micrograms of MBP-RCI-conjugated agrose beads were added into the protein extracts and incubated at 4 °C for 3 h. The beads were washed four times by washing buffer at 4 °C. GFP-bound ROP proteins associated with the MBP-RCI beads were washed in the washing buffer with rabbit polyclonal anti-ROP6 antibodies (Abicodie) and anti-rabbit IgG-HRP antibodies (GE Healthcare). Prior to the pull-down assay, a fraction of total proteins was analyzed by immunoblot assay to determine total ROP6. The experiments were repeated three times.

ROP6 S-acylation assays. ROP6 S-acylation assays were performed according to the method described by Hemsley et al.31. IAA treatments were performed on 3-week-old transgenic plants expressing 3S5-GFP-ROP6 by spraying. Leaf tissues were collected 10 min after spraying and immediately flash frozen in liquid nitrogen. Collected samples were ground in liquid nitrogen to a fine powder and then mixed with 1.5 ml lysis buffer (100 mM Tris pH 7.2, 150 mM NaCl, 25 mM EDTA, 2.5% SDS, 25 mM N-Ethylmaleimide) with 1x COMPLETE, Mini EDTA-free Protease Inhibitor Cocktail (Roche). After infiltration through a 200-µm nylon mesh, samples were centrifuged at 16,000 × g for 5 min. The supernatant was collected and protein concentrations was determined using the 260 nm absorbance. 10 µg of protein was incubated in the lysis buffer for 2 h at room temperature with gentle mixing and then precipitated using the methanol/chloroform extraction method26. The pellets were resuspended in 1 ml binding buffer (100 mM Tris pH 7.2, 150 mM NaCl, 25 mM EDTA, 2% SDS, 6 M urea with protease inhibitors), and the solution was further divided into two equal aliquots. One aliquot was combined with an equal volume of 1 M hydroxylamine. As a control, the other aliquot was treated identically but hydroxylamine was replaced with 1 M NaCl. After mixing, 50 µl aliquots were taken and used as a loading control. The remaining samples were incubated with 100 µg of a 50% suspension of prewashed Thiopropyl Sepharose CL-6b beads (GE Healthcare) at room temperature for 1 h. The beads were washed three times with 1 ml of binding buffer. Proteins were eluted by incubating the beads at 37 °C for 30 min in 35 µl 2x SDS sample buffer containing 6 M urea and DTT. Samples were analyzed by SDS/PAGE and western blotting using rabbit polyclonal anti-ROP6 antibodies (Abicodie) and anti-rabbit IgG-HRP antibodies (GE Healthcare). The experiments were repeated three times.

Di-4-ANEPPDHQ probe-based membrane ordering measurements. The di-4-ANEPPDHQ staining was performed according to the procedure previously described24. The 2–3-day-old seedlings were incubated in 5 µl di-4-ANEPPDHQ dissolved in DMSO for 1 h, washed three times in water, then imaged with a Leica SP5 confocal laser-scanning microscope (CLSM). The live samples were excited with a 488-nm laser, and fluorescence was detected in two emission windows at 500–580 nm and 620–750 nm. Fluorescence signals were collected by a 40× oil-immersion objective. Before the dye was added to seedlings, no autofluorescence was detected in these two spectral regions when excited with the 488-nm laser light. Photobleaching with 0.8 M mannitol revealed that the peripheral staining is associated with the plasma membrane, but not with the cell wall (Supplementary Fig. 1). After imaging, we followed the published protocol29 to
generate pseudo-colored ratiometric general polarization (GP) images. Briefly, the ImageJ macro for GP analysis was applied with the following settings: the threshold value was fixed at 15, the color scale for the output GP images was set to “grays”, and no immunofluorescence mask was selected. The GP values were calculated according to the following equations:

\[
GP = (I_{500-580} - I_{520-750}) / (I_{500-580} + I_{520-750}).
\]

(1)

\[
G = (GP_{\text{ref}} + GP_{\text{mix}}) / (GP_{\text{mix}} - 1) / (GP_{\text{ref}} + GP_{\text{mix}}) - GP_{\text{ref}} - 1.
\]

(2)

where \(G\) represents the intensity in each pixel in the images collected from two spectral channels of CLSM, 500–580 nm and 620–750 nm; \(G\) is a calibration factor, which is used to compensate for different collection efficiency between two channels and is calculated according to Eq. (2); \(GP_{\text{mix}}\) is the GP value of di-4-ANEPPDHQ in pure DMSO solution with the same microscopy settings as those used for co-detection of flippin and lipin. \(GP_{\text{ref}}\) is calculated using the following equation: \(GP_{\text{ref}} = \left(\frac{I_{\text{lipin1-mVenus/LIT6b-mVenus}}}{I_{\text{fl}}\text{otillin1-tagRFP, YFP-CArop6 and mScarlet-MAP4, flotillin1-tagRFP, YFP-CARop6 and mScarlet-MAP4}}\right)\), a Zeiss LSM880 equipped with an Airyscan detector was used.

**Detection of flippin–sterol fluorescence in pavement cells.** The flippin staining was performed according to the procedure previously described. The 2–3-day-old seedlings (wild type or expressing mVenus-tagged proteins) were submersed to 300 µl of microtubule-stabilizing buffer (50 mM PIPES, 5 mM EGTA, 5 mM MgSO4 [pH 7.0]) containing 4% paraformaldehyde (PFA) and 225 µM flippin III (Sigma-Aldrich). Samples were then placed into a microwave oven and pulsed six times for 15 s at 90 W with a 1-min pause in between each pulse. Sample staining/fixation was continued for 1 h at room temperature in the dark. Samples were then washed three times in 1 ml of dH2O and mounted in Citifluor AF1 antifade reagent for observation by a Zeiss LSM880 confocal microscopy. Flippin and mVenus were excited using 364-nm and 488-nm laser lines, respectively. The emission windows were set to 405–480 (for flippin) and 520–550 nm for mVenus. Sequences acquisition was used to avoid fluorescence bleed-through during co-detection of flippin–sterol complexes and flotillin1-mVenus/LIT6b-mVenus.

**Variable-angle total internal reflection fluorescence imaging.** Cotyledons of 2–3-day-old seedlings were imaged on a Zeiss Elyra PS1 system (Zeiss, Germany) with a ×100 Apo (numerical aperture 1.46) oil-immersion objective, in total internal reflection fluorescence (TIRF) mode. The optimum critical angles ranging from 66.8° to 67.5° were used to provide the best signal-to-noise ratio during imaging. Pixel size was 0.107 µm. The development of Arabidopsis cotyledon pavement cells is separated into three stages. To minimize developmental differences, stage II cells with shallow lobes were selected for imaging and the focus was set at 100 µm below the margin of pavement cells. Movies were recorded at a rate of ten frames per second (100 ms exposure time) on a 256 × 256-pixel region of interest for 200 frames. Over this imaging period photobleaching was negligible. GFP/mVenus was excited using a 488 nm solid-state laser diode, and the emission was collected with a 495 nm band-pass and a 570-nm long-pass filter. Airyscan imaging was performed using 488 nm and 561 nm excitation for mVenus and tagRFP, respectively. Fluorescence emission was collected with a dual 495–550nm band-pass and 570-nm long-pass filter in combination with a 630 nm short-pass filter. A ×100 Apo (numerical aperture 1.46) oil-immersion objective was used for all imaging. Spearman’s rank correlation coefficients were calculated using the Coloc2 plug-in from Fiji software. The negative control was provided by calculating the Spearman’s rank correlation coefficient for the same image but without rotating the image of the red channel with respect to the image from the green channel by 90°, a condition in which only random colocalization should be identified. Same confocal microscope settings were used to collect data of mEGFP-RoP6 and flotillin1-tagRFP, YFP-CARop6 and mScarlet-MAP4, flotillin1-mVenus and mScarlet-MAP4.

**Spot size measurement and single-particle tracking.** Particle-tracking and trajectory analysis were performed using a custom MATLAB R2018a extension code with the Spots Detection module in Imaris 9.1.2 (Bitplane) software. Prior to spot estimation, background subtraction was performed on all images. Estimated spots were modeled with a minimal XY diameter spot size to 0.3 µm, followed by further filtering using the default “quality” setting automatically calculated by Imaris. Filtered spots were served as seed points for calculating spot sizes using a region growing method. The spot region growth was halted when the local contrast around the border of the spot region met the automatic threshold calculated by Imaris. Spot diameters were then recalculated based on the region volume detected in the previous step. No manual editing was performed.

An autoregressive motion algorithm was used for particle tracking. A maximum distance was set to 0.5 µm. This setting disallows connections between a spot and a candidate match if the distance between the predicted future position of the spot and the candidate position exceeds 0.5 µm. A gap-closing algorithm was used to compensate undetected spots in one or more of the consecutive frames along a spot trajectory to prevent inaccurately fragmented tracks. A maximum gap size, i.e., the allowed maximum number of consecutive time frames with missing spots, was set to 8. Single-particle-tracking trajectories in Supplementary Movies 2, 3, 5, and 6 are shown as dragontail visualization with ten time points of the track length.

**Trajectory analyses.** The mean-square displacement (MSD) and diffusion coefficients were analyzed following a method developed earlier. Trajectories with length >5 frames were selected for MSD and diffusion coefficient analyses. For each track, the MSD (\(t\)) was calculated from the following formula:

\[
\text{MSD}(t) = \frac{1}{L} \sum_{i=0}^{L-t} (r(i + t) - r(i))^2.
\]

where \(n = t/\Delta t, L\) is the length of the trajectory (number of frames), and \(r(t)\) is the two-dimensional position of the particle in frame \(s (s = 0)\) corresponds to the start of the trajectory). The diffusion coefficient for a spot was determined by fitting a line to MSD (\(t\)) with \(r\) ranging from 1 to the largest integer \(\leq 4/\Delta t\).

The natural logarithm of diffusion coefficients for all tracks (>8 frames) in each condition were pooled to obtain the log(D) density histogram with the number of bins of 800. The data were then fitted into one component and two-component Gaussian mixture (univariate, unequal variance) models using the R package mclust with default settings. The package provides the Bayesian Information Criterion (BIC) to assess how well the model explains the data. For all density distributions of TMK1, flotillin1 and RoP6 particles that were obtained and analyzed in this study, the two-component model yielded significantly higher BIC values than the one-component model, indicating the better fit. Each fit to the two-component model outputs a set of parameters: mean (\(\mu_1, \mu_2\)), standard deviation (\(\sigma_1, \sigma_2\)), and variance (\(\nu_1, \nu_2\)) for each component in the model. The mean (\(\mu_1, \mu_2\)) and variance (\(\nu_1, \nu_2\)) of the lognormal distribution for each component is calculated based on the following equations:

\[
m = \exp(\mu - \sigma^2/2)
\]

\[
v = \exp(2\mu + \sigma^2) / (\exp(\sigma^2) - 1).
\]

The overall average of diffusion coefficient (\(m_{\text{overall}}\)) and variance (\(v_{\text{overall}}\)) is calculated based on the following equations:

\[
m_{\text{overall}} = m_1 + m_2, \quad v_{\text{overall}} = v_1 + v_2 + \frac{m_1 - m_2}{m_1 m_2} + \frac{v_1 - v_2}{m_1 m_2} + \frac{v_1 v_2}{m_1 m_2} - v_{\text{overall}}.
\]

The calculated \(m_1, m_2, v_1, v_2\) are overall and \(v_{\text{overall}}\) are related to \(D_1, D_2\) and \(D_{\text{overall}}\) in Supplementary Table 1. Violin plots in the main and Supplementary Figs. (Figs. 3f, 4c, 5c, 5h, 6c, 6f) show the distribution of the overall diffusion coefficients without the log transformation. Distributions of log-transformed datasets are shown in Supplementary Fig. 5. The overall diffusion coefficients were used to determine statistical significance of treatment effects.

**Quantification and statistical analyses.** All data analyses were performed with PRISM software (GraphPad Software Inc., La Jolla, CA). Each sample was subjected to three different normality tests (D’Agostino & Pearson, Shapiro–Wilk, and Kolmogorov–Smirnov) to determine whether the sample is normally distributed. Samples were considered as a normal distribution as long as they passed one of the three normality tests (\(P < 0.05\)). Accordingly, parametric (\(P < 0.05\)) or nonparametric tests (\(P < 0.05\)) were performed. For parametric test, the paired \(t\) test or the unpaired \(t\) test with Welch’s correction was used to assess statistical difference. For parametric test, with paired or unpaired \(t\) was only two samples, while analysis of variance (ANOVA) coupled with multiple comparison was used for datasets containing more than two samples. For nonparametric test, Wilcoxon
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Competing interests
The authors declare no competing interests.

Additional information
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