Lateral distribution of phosphatidylinositol 4,5-bisphosphate in membranes regulates formin- and ARP2/3-mediated actin nucleation

Received for publication, August 24, 2018, and in revised form, January 13, 2019 Published, Papers in Press, January 28, 2019, DOI 10.1074/jbc.RA118.005552

Robert Bucki‡1,2, Yu-Hsii Wang†5,1, Changsong Yan‡2, Sreeja Kutty Kandy∗1, Ololade Fatunmbi∗1, Ryan Bradley∗∗, Katarzyna Pogoda‡2,3, Tatjana Svitkina, Ravi Radhakrishnan∗∗ and Paul A. Janmey∗∗∗

From the Departments of †Physiology, ‡Chemistry, §Biology, and **Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia, Pennsylvania 19104, the ‡Department of Microbiological and Nanobiomedical Engineering, Medical University of Białystok, 15-089 Białystok, Poland, the ‡‡Institute of Nuclear Physics, Polish Academy of Sciences, PL-31342 Kraków, Poland, and the **∗Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Edited by Alex Toker

Spatial and temporal control of actin polymerization is fundamental for many cellular processes, including cell migration, division, vesicle trafficking, and response to agonists. Many actin-regulatory proteins interact with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) and are either activated or inactivated by local PI(4,5)P2 concentrations that form transiently at the cytoplasmic face of cell membranes. The molecular mechanisms of these interactions and how the dozens of PI(4,5)P2-sensitive actin-binding proteins are selectively recruited to membrane PI(4,5)P2 pools remains undefined. Using a combination of biochemical, imaging, and cell biologic studies, combined with molecular dynamics and analytical theory, we test the hypothesis that the lateral distribution of PI(4,5)P2 within lipid membranes and native plasma membranes alters the capacity of PI(4,5)P2 to nucleate actin assembly in brain and neutrophil extracts and show that activities of formins and the Arp2/3 complex respond to PI(4,5)P2 lateral distribution. Simulations and analytical theory show that cholesterol promotes the cooperative interaction of formins with multiple PI(4,5)P2 headgroups in the membrane to initiate actin nucleation. Masking PI(4,5)P2 with neomycin or disrupting PI(4,5)P2 domains in the plasma membrane by removing cholesterol decreases the ability of these membranes to nucleate actin assembly in cytoplasmic extracts.

Although it constitutes less than 1% of the total phospholipid of the cell, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) is implicated in control of many protein functions and, as a result, many different cellular tasks ranging from vesicle trafficking and ion flux at the plasma membrane to chromatin remodeling within the nucleus. One of the earliest and most thoroughly documented effects of this lipid in eukaryotic cells is the control of the actin-based cytoskeleton. Because the same pool of cytoplasmic actin needs to be arranged differently to support the spectrum of its cellular functions, a dynamic lipid-based regulation at the cytoplasm/membrane interface provides a unique mechanism to control and modify actin assembly with spatial and temporal specificity.

Relevance of PI(4,5)P2 to cytoskeletal assembly was first suggested by biochemical studies of its interaction with actin-binding proteins (1), including those that sever actin filaments, nucleate actin assembly, and attach actin filaments to each other and to transmembrane complexes (2). Subsequently, manipulation of enzymes involved in PI(4,5)P2 production showed that increasing cellular PI(4,5)P2 levels massively increased actin assembly (3) and stress fiber formation (4), whereas increasing PI(4,5)P2 degradation globally (5) or locally (6) destabilized actin assembly and actin-dependent processes. Targeted delivery of lipid vesicles containing PI(4,5)P2 or PI(3,4,5)P3 into a Xenopus egg extract is sufficient to cause actin assembly at the vesicle that drives its motility through the extract, whereas vesicles with phosphatidylinositol had no effect (7). Similar studies show that filopodial structures form when Xenopus extracts are added to supported bilayers containing PI(4,5)P2 (8). Such studies have identified scores of proteins involved in actin remodeling that are affected by PI(4,5)P2 but have not yet led to a clear understanding of how cellular PI(4,5)P2 distribution is controlled in the plasma membrane or how the proteins that are potentially regulated by PI(4,5)P2 compete for this scarce lipid.

The importance of cholesterol in arranging plasma membrane PI(4,5)P2 and the role of PI(4,5)P2 in organizing the cytoskeleton have been previously reported (9). PI(4,5)P2 levels and lateral mobility of plasma membrane proteins are reduced after

VMD, visual molecular dynamics; MβCD, methyl-β-cyclodextrin; Lc, liquid-ordered; Ld, liquid-disordered; GTPγS, guanosine 5′-O-(γ-thio)triphosphate; PS, phosphatidylserine; PE, phosphatidylethanolamine; MD, molecular dynamics; mN, millinewton; PIP2, phosphatidylinositol 4,5-bisphosphate.
cholesterol depletion, suggesting links between PI(4,5)P2-mediated control of actin assembly (9) and lateral mobility of membrane proteins.

Dozens of actin-binding proteins bind with high specificity to PI(4,5)P2 (10, 11). In many cases, the domain of the protein responsible for its regulation by the lipid consists largely of multiple basic amino acids interspersed with some hydrophobic residues, rather than a specific folded structure characteristic of a tight binding pocket within a protein for a specific soluble ligand. Measurement of PI(4,5)P2 diffusion shows that most of the plasma membrane PI(4,5)P2 pool is bound or sequestered to some extent (12). A major unresolved question is how PI(4,5)P2 distributes laterally within the plasma membrane and whether all PI(4,5)P2 molecules are equally effective at binding their targets.

Among other hypotheses for how a relatively scarce small molecule like PI(4,5)P2 can control the function of hundreds of its target proteins with fidelity is the idea that specific proteins bind PI(4,5)P2 only when PI(4,5)P2 is appropriately distributed within the membrane bilayer. For example, in vitro, phospholipid vesicles containing PI(4,5)P2 inhibit the function of the actin-severing protein gelsolin much more strongly when the vesicles undergo a cholesterol-dependent redistribution into liquid-ordered (L\textsubscript{o}) and liquid-disordered (L\textsubscript{d}) domains (13). Other actin-binding proteins such as N-WASP, talin, and several others bind with different affinities to lipid bilayers containing constant amounts of PI(4,5)P2 but various amounts of other lipids to which the protein does not bind directly. In a cellular context, the demixing of PI(4,5)P2 into nanoscale domains that are highly enriched for this minor lipid has been observed using either fluorescent analogs of the lipid or fluorescently labeled lipid-binding proteins, and the targeting of proteins to PI(4,5)P2 often leads to their distinct localization within the cell. Recent studies show the relevance of nanoscale PI(4,5)P2 clusters to critical PI(4,5)P2-dependent cellular functions (14–18). Potential mechanisms that explain how local concentration fluctuations of PI(4,5)P2 might regulate cellular functions were summarized in a recent review, which combines studies from both experiments and simulations (11).

Here, we test the hypothesis that the lateral distribution of PI(4,5)P2 within lipid membranes alters its ability to nucleate actin in cell extracts using a combination of purified lipid monolayers, bilayer vesicles, and cell-derived membrane sheets that retain the complexity of the cells’ plasma membrane. In all cases, incorporation of PI(4,5)P2 into these membranes is required for them to nucleate actin assembly. Masking PI(4,5)P2 within a cell membrane by competitive binding of exogenous ligands or disrupting PI(4,5)P2 domains within the plasma membrane by removing cholesterol destroys the ability of these membranes to nucleate actin assembly in cell extracts derived from bovine brain and human neutrophils.

Pharmacologic inhibition of the two major actin-nucleating factors, formins and Arp2/3 complex, showed that formins and Arp2/3 were the dominant factors responsible for PI(4,5)P2-mediated activation of actin assembly in brain and neutrophil extracts. A quantitative analysis of changes in actin assembly caused by increasing concentrations of PI(4,5)P2, delivered by vesicles of either uniform or demixed composition, showed that the binding kinetics could be best described by a two-state mechanism in which the nucleating factor, presumably a formin, first docks electrostatically to the membrane surface, and then it cooperatively binds three or more PI(4,5)P2 molecules to acquire actin-nucleating activity. The requirement for simultaneous binding of multiple PI(4,5)P2 is consistent with a greater effect of PI(4,5)P2 when it is locally concentrated. The effect of cholesterol (CHOL) on augmenting the effect of PI(4,5)P2 is supported by a molecular dynamics simulation of the docking of the PI(4,5)P2-binding site of mDia2 on a lipid bilayer with a variable composition.

Results

Actin assembly on phase-demixed monolayers with Ca\textsuperscript{2+}-induced perturbations

The lateral distribution of actin assembled on a supported lipid monolayer reflects the lateral distribution of PI(4,5)P2 at the membrane/extract interface as PI(4,5)P2 serves as a membrane anchor/activator for nucleation-promoting factors such as N-WASP, WAVE2, and formins. The actin assembly assay on supported monolayers therefore provides an imaging-based platform for examining PI(4,5)P2–protein interactions. To investigate Ca\textsuperscript{2+}-mediated perturbation of PI(4,5)P2–protein interactions, we adopted an actin assembly assay using supported lipid monolayers. For phase-demixed monolayers that were transferred at 20 mN/m in the absence of Ca\textsuperscript{2+}, the assembled actin filaments were found in both L\textsubscript{o} and L\textsubscript{d} phases (Fig. 1, A and B). In contrast, when Ca\textsuperscript{2+} was added to the monolayer prior to its deposition on the glass support, actin filaments were concentrated in one of the phases, namely the L\textsubscript{d} phase, as indicated by the partitioning of Rho-DOPE (Fig. 1C). The uneven distribution of assembled actin seen by fluorescent phalloidin staining (Fig. 1D) and its corresponding quantification showed that the amount of polymerized actin per unit area within the L\textsubscript{o} domains was 80% less than that measured in the L\textsubscript{d} phase (Fig. 1E).

Actin assembly on supported monolayers with Ca\textsuperscript{2+}-induced PI(4,5)P2 clusters

To further define how actin assembly was affected by the change of the PI(4,5)P2 lateral structure induced by Ca\textsuperscript{2+}, we examined the efficacy and the localization of actin filament nucleation from cytoplasmic extracts on nondemixed supported monolayers (Fig. 2). For this purpose, lipids from free-standing lipid monolayers formed with or without Ca\textsuperscript{2+} in the subphase were transferred onto coverslips to fix the lipid distribution. The resulting supported monolayers were used for actin assembly and analyzed by both fluorescence and electron microscopy (EM). As revealed by the fluorescence micrographs following phalloidin staining, the densities of actin filaments assembled on supported monolayers transferred in the presence of Ca\textsuperscript{2+} were significantly greater than those transferred in the presence of 5 μM EGTA, although the overall PI(4,5)P2 mole fraction was the same in both conditions (Fig. 2, A and B). Brain extracts used in these experiments contained 5 mM EGTA, a large excess over the Ca\textsuperscript{2+} concentration used during lipid transferring, suggesting that it was PI(4,5)P2 lateral distribution, rather than free Ca\textsuperscript{2+} ions, acting on cytoplasmic pro-
teins that triggered actin polymerization. Further analysis of similar samples by platinum replica EM showed that actin fila-
ments assembled on Ca^{2+}/H11001-treated monolayers were attached to round disk-like structures (Fig. 2, C–E), which resembled Ca^{2+}-induced nano-sized PI(4,5)P2 clusters (16). Disk-bound actin filaments were frequently found in Ca^{2+}-treated mono-
layers but not in Ca^{2+}-free monolayers. These disk-bound actin filaments were mostly long and unbranched. As detected by EM, short branches were found only occasionally (Fig. 2F, arrows).

**Actin assembly induced by PI(4,5)P2 lipid bilayer vesicles with different lateral structure**

We next evaluated actin assembly in cytoplasmic extracts triggered by large unilamellar vesicles (LUVs) that contained PI(4,5)P2 in the context of mixed or demixed lipid biflayers.
Three sets of LUVs prepared by lipid extrusion were tested in this experiment. LUVs A contained 15% PI(4,5)P$_2$ with 10% DOPC and were phase-demixed by incorporating 30% dihydrocholesterol (dCHOL) in a DPPC background. LUVs B contained the same amount of PI(4,5)P$_2$ but with a uniform distribution by replacing both dCHOL and DPPC with DOPC (13). LUVs C, which contained no PI(4,5)P$_2$, served as a negative control and were made out of 15% DOPC and 85% DPPC. The extracts were supplemented with pyrenyl-actin to monitor actin polymerization. When LUVs A or B were added into the cytoplasmic extract, the actin polymerization rate increased significantly by 61 and 20%, respectively, relative to basal actin polymerization rate of the extract alone (Fig. 3A). The addition of LUVs C did not promote actin polymerization confirming
the requirement for PI(4,5)P₂ in promoting actin polymerization. The fact that LUVs A were more effective than LUVs B in accelerating actin polymerization suggests that it is not only the global concentration but also the lateral distribution of PI(4,5)P₂ in the membrane that matters for its interaction with cytosolic proteins. We then sought the cytosolic proteins responsible for promoting actin polymerization in the presence of LUVs A and B. The obvious targets were formins and Arp2/3 complex, whose roles in this assay were examined by applying specific inhibitors. SMIFH2 at 50 μM, which inhibits formin-mediated actin assembly (19), greatly suppressed the accelerated actin polymerization mediated by both LUVs A and B. Under the same conditions, the ARP2/3 inhibitor CK-666 (20) at 100 μM did not produce a significant change in the rate of actin polymerization (data not shown). This result suggested that formin-nucleated actin assembly was activated by PI(4,5)P₂ more effectively than nucleation by Arp2/3.

**EM analysis of LUV-induced actin filaments**

We characterized actin filaments formed in a LUV A-dependent manner by negative staining EM (Fig. 3, B–I). The reaction mixture that contained LUVs, actin monomers (G-actin) and the cytoplasmic extract, was applied to EM grids and after a short incubation (1–5 min) prepared for EM (Fig. 3F). As a control, we incubated grids with incomplete mixtures containing G-actin only (Fig. 3B), LUVs A only (Fig. 3C), neutrophil extract only (Fig. 3D), or LUVs A and G-actin, but no extract (Fig. 3E). G-actin alone or in a mixture with LUVs A produced very few long actin filaments, presumably from spontaneous nucleation, whereas LUVs A alone and extract alone produced no detectable actin filaments. In contrast, when all three ingredients of the reaction were combined, many more actin filaments were observed by EM (Fig. 3F). Complete mixtures containing LUVs B or C instead of LUVs A produced significantly fewer but longer actin filaments (Fig. 3 H and I), suggesting lower frequencies of actin nucleation and a consumption of available monomers by these few nuclei, which led to their more extensive elongation.

Most filaments nucleated in the LUV A-containing reactions were long and unbranched, suggesting a formin-mediated nucleation. We were able to detect only very few reliably identified actin filament branches that might be consistent with nucleation by an Arp2/3-dependent mechanism. Some actin filaments were associated with the vesicles, but many filaments did not contact LUV A vesicles or intersected them, probably because actin filaments were easily released from LUVs after nucleation. We used decoration of actin filaments with myosin subfragment 1 (S1) to determine actin filament polarity relative to LUVs A in EM samples (Fig. 3G). These data showed that 64% of the actin filaments attached to LUVs contacted the vesicle by their pointed ends and had their barbed ends oriented away from the vesicle, whereas the remaining 36% had the opposite orientation. These data suggest that after being nucleated at LUVs A, an actin filament is not anchored to the vesicle but elongates freely into the surrounding volume. Because barbed ends grow much faster, the filaments nucleated at the LUVs A on average become oriented with barbed ends away from the vesicle. Despite this fact, a significant fraction of filaments did retain an association of their barbed ends with LUVs A, consistent with formin-mediated nucleation or perhaps capture by a PI(4,5)P₂-activated F-actin–binding protein like α-actinin or talin.

**PI(4,5)P₂-dependent actin assembly on plasma membrane sheets**

Plasma membranes represent physiologically relevant protein-enriched lipid bilayers at which actin filament polymerization occurs in cells. To evaluate a role of PI(4,5)P₂ in this process, we first developed an actin polymerization assay using isolated plasma membranes from cultured cells. Plasma membranes of attached PtK2 cells expressing membrane-targeted GFP (GFP-CAAX, where AA is aliphatic amino acid) were isolated by sonication-mediated unroofing. Immunofluorescence staining of PI(4,5)P₂ in these membrane sheets showed numerous bright spots on a background of more uniform staining (Fig. S1A). These PI(4,5)P₂ puncta generally did not colocalize with local GFP-CAAX enrichments suggesting that they are not membrane folds, but more likely reflect formation of PI(4,5)P₂ clusters in the plasma membrane. Because the anti-PI(4,5)P₂ antibody also recognizes phosphatidylinositol-4-phosphate and PI(3,4,5)P₃, we stained plasma membranes prepared from cells expressing a membrane-targeted catalytic domain of the polyphosphoinositide 5-phosphatase synaptojanin-1 (mRFP-IP1-CAAX) (13). The membranes derived from mRFP-IP1-CAAX–expressing cells showed significantly lower levels of PI(4,5)P₂ immunostaining (Fig. S1). Although synaptojanin-1 can dephosphorylate both PI(4,5)P₂ and PI(3,4,5)P₃, the abundance of PI(3,4,5)P₃ in plasma membranes is much lower than that of PI(4,5)P₂, suggesting that the antibody recognizes PI(4,5)P₂ in the plasma membrane sheets.

Isolated plasma membranes were incubated with brain cytoplasmic extract supplemented with fluorescently labeled G-actin and guanosine 5’-O-(γ-thio)triphosphate (GTPγS). Preliminary experiments showed that in contrast to experiments with LUVs in suspension, GTPγS was required to induce robust actin assembly on plasma membrane sheets. In time-course experiments, significant actin assembly occurred after incubation for ~15 min or longer at 37 °C (Fig. S2). Rhodamine-actin assembly in the form of small foci or polymorphic aggregates was clearly detectable by confocal microscopy on GFP-CAAX–labeled plasma membranes after 20 min of incubation with the extract (Fig. 4A). Immunostaining of PI(4,5)P₂ in these samples revealed some degree of colocalization between the PI(4,5)P₂ signal and sites of actin assembly (Fig. 4A, top). By plotting the mean fluorescence intensities of PI(4,5)P₂ and rhodamine-actin against each other for individual membrane sheets, we observed a substantial positive correlation between these two values (Fig. 4B, top).

To evaluate a role of PI(4,5)P₂ in actin assembly on isolated plasma membranes, we incubated membranes with the extract in the presence of neomycin, which binds PI(4,5)P₂ and blocks access of PI(4,5)P₂-interacting partners (21, 22). These experiments showed that neomycin did not significantly decrease the PI(4,5)P₂ immunofluorescence signal (Fig. 4C, left), presumably being unable to prevent PI(4,5)P₂ interaction with the antibody. However, actin assembly on neomycin-treated plasma mem-
brane sheets was significantly decreased (Fig. 4, A and C). The remaining low levels of actin assembly still positively correlated with the PI(4,5)P₂ immunoreactivity (Fig. 4A).

Cholesterol is important for organizing lipid domains in the plasma membrane and can affect lateral organization of PI(4,5)P₂ within the plasma membrane. To assess a role of cholesterol in lateral organization of PI(4,5)P₂ for actin assembly on plasma membranes, we extracted cholesterol from the membranes by treating membranes with methyl-β-cyclodextrin (MβCD). Staining with filipin (23) confirmed a significant decrease in the cholesterol content in the membrane sheets (Fig. S3). This treatment did not affect the overall immunofluorescence intensity of PI(4,5)P₂ (Fig. 4C), and bright PI(4,5)P₂ puncta were still preserved, probably because PI(4,5)P₂ might also be clustered by cholesterol-independent means, for example, within clathrin-coated structures. Importantly, actin assembly on MβCD-treated plasma membranes was significantly reduced (Fig. 4, A and C) and its levels positively correlated with the PI(4,5)P₂ levels (Fig. 4B). These data suggest that membrane structures formed in the presence of cholesterol stimulate actin assembly.

Two major classes of actin nucleators, Arp2/3 complex and formins, are likely candidates to stimulate actin polymerization downstream of PI(4,5)P₂. To determine roles of these nucleators for actin assembly on isolated plasma membranes, we performed the experiments in the presence of an Arp2/3 complex inhibitor CK-666 or a formin inhibitor SMIFH2 (Fig. 5). The results showed that rhodamine-actin incorporation was ~10-fold greater in the presence of the extract than with the buffer alone. The levels of extract-induced actin assembly were reduced by ~80% in the presence of CK-666 and by ~40% in the presence of SMIFH2, indicating that both formins and the Arp2/3 complex contribute to actin assembly on plasma membranes in the presence of the extract supplemented with GTPγS.

Ultrastructure of the PI(4,5)P₂-dependent actin cytoskeleton

We next examined ultrastructural organization of PI(4,5)P₂ and actin filaments using platinum replica EM combined with immunogold labeling of PI(4,5)P₂. Untreated plasma membranes (Fig. 6A) were associated with flat or curved clathrin lattices, as commonly observed in unroofed cells (24). However, actin filaments and other cytoskeletal elements were mostly removed in our unroofing conditions. The abundance of PI(4,5)P₂ immunogold labeling was highly variable among cells. However, gold particles typically formed sizable clusters (Fig. 6, A and F).

When plasma membranes were incubated for 20 min with bovine brain extract, abundant actin filaments were found in association with the membranes by EM (Fig. 6B and C). Most actin filaments were relatively long, although branched filaments were also observed. We also noticed significantly more clathrin-coated vesicles and caveolae associated with the membranes after their incubation with the extract, as compared with control membranes. Clusters of PI(4,5)P₂ immunogold in extract-treated samples were often associated with clathrin-coated vesicles (Fig. 6B), but even more frequently they colocalized with caveolae (Fig. 6C). Importantly, clusters of PI(4,5)P₂ immunogold were observed to associate with actin filaments at the surface of clathrin-coated structures or caveolae. These
Figure 5. Both Arp2/3 complex and formins are required for actin polymerization on plasma membrane sheets. A, fluorescence microscopy of plasma membranes from unroofed Ptk2 cells expressing GFP-CAAX (green) after incubation at 37 °C for 20 min with solutions containing 0.2 μM rhodamine-actin (red), ATP, and GTP. Conditions: buffer only (No extract); extract only (Control); extract containing 50 μM CK-666, and extract containing 100 μM SMIFH2. B, mean fluorescence intensities of assembled rhodamine-actin in indicated conditions. Error bars, S.E. n (regions of interest) are indicated; **, p < 0.01; ***, p < 0.001. Scale bar, 5 μm.

Figure 6. Platinum replica EM of plasma membrane sheets from Ptk2 cells stained with PI(4,5)P2 antibody (yellow). A, control plasma membrane sheets lack associated cytoskeleton. B and C, after incubation with bovine brain extract, sheets are associated with abundant actin filaments, clathrin-coated vesicles (green) and caveolae (blue). D and E, plasma membrane sheets incubated with the bovine brain extract containing 2 mM neomycin (D) or 2.5 mM MJCD (E). F, mean number of immunogold particles per cluster in plasma membrane sheets labeled with PI(4,5)P2 antibody. Error bars, S.E. N (clusters) = 81 (control), 30 (neomycin), and 27 (MJCD); n.s., nonsignificant; ***, p < 0.001.

PI(4,5)P2- and cholesterol-mediated control of actin assembly
data suggest that the cytoplasmic extract induces assembly of actin filaments, clathrin-coated vesicles, and caveolae on plasma membrane sheets at PI(4,5)P2-positive sites.

When reactions were performed in the presence of neomycin (Fig. 6D) or MβCD (Fig. 6E), actin filaments were hardly detectable on the extract-treated plasma membranes by EM. PI(4,5)P2 immunogold labeling after treatment with neomycin or MβCD was decreased relative to control samples. The results contrast with immunofluorescence data that showed no significant differences in PI(4,5)P2 signals after treatment with these inhibitors. This discrepancy could be explained by lower accessibility of epitopes for the much larger immunogold-conjugated antibodies, as compared with fluorophore-labeled ones. The PI(4,5)P2 immunogold in neomycin-treated samples was still clustered with no significant differences in the cluster size, whereas the PI(4,5)P2 labeling in MβCD-treated samples was usually observed in the form of individual particles or small clusters of 2–3 particles (Fig. 6, D–F). This scattered distribution of PI(4,5)P2 immunogold after treatment with MβCD is consistent with a role of cholesterol in the formation of PI(4,5)P2-rich domains. Together, these data suggest that de novo assembly of actin filaments on plasma membranes in the presence of the cytoplasmic extract depends on the presence of PI(4,5)P2, as well as on the lateral PI(4,5)P2 distribution. We also show a connection between PI(4,5)P2, actin filaments, clathrin, and caveolae.

Cholesterol enhances PI(4,5)P2-mediated formin activation in a molecular model

To identify the molecular features of enhanced formin binding to vesicles, and to corroborate the mechanism proposed above, we constructed atomistic molecular dynamics (MD) simulations of the protein mDia2 bound to bilayers (see under “Computational methods”) containing 20% CHOL in both leaflets, POPC on the outer leaflet and 10, 20, or 30% PI(4,5)P2 on the inner leaflet along with a PE and PS mixture adjusted to control the surface charge density across conditions. A simulation without cholesterol was performed with 20% PI(4,5)P2. A typical simulation is depicted in Fig. 7. The number of hydrogen bonds between the protein and the lipids serves as a proxy for binding affinity. We find that the presence of cholesterol enhances binding of mDia2 to PI(4,5)P2 (Fig. 8). Moreover, the hydrogen bond valence to PI(4,5)P2 increases with cholesterol, particularly from Arg-25 and Arg-35 (Fig. 9). These residues are incidentally also the most sensitive to the highest PI(4,5)P2 concentration we studied, suggesting that both cholesterol and increased PI(4,5)P2 concentration cause the same particular protein bonds to form. Although increased availability of PI(4,5)P2 may explain the enhanced binding at higher concentrations, the binding enhancement due to cholesterol is observed at a fixed PI(4,5)P2 concentration. The results of our molecular model support the view that multivalency of the PI(4,5)P2 recognition peptide binding to the membrane is enhanced by the presence of CHOL at a fixed PI(4,5)P2 concentration and that the average multivalency is 3–4 under intermediate-to-high PI(4,5)P2 concentrations of 20–30%.

Quantitative model to describe PI(4,5)P2-dependent actin filament formation

The symbols in Fig. 10 show the initial actin formation rate as a function of PI(4,5)P2 concentration for LUVs A and LUVs B added to different extract concentrations. There is a background spontaneous nucleation of actin filaments in the extract. Our model describes the formin-induced nucleation (above and beyond the observed spontaneous nucleation), as described below. For a given number of formin nucleation sites, \( N_\alpha \), the number of actin monomer units incorporated in filaments in the initial time regime is given by Equation 1,
\[ n(t) = N_t(k_{on}c_0 - k_{off})t \]  
\[ \text{rate} = \frac{d}{dt}(N_t(k_{on}c_0 - k_{off})t) \]  
\[ \text{rate} = N_t k_{on}c_0 \]

where, \( k_{on} \) is the polymerization rate; \( k_{off} \) is the de-polymerization rate, and \( c_0 \) is the actin monomer concentration. Hence, the initial polymerization rate can be written as shown in Equation 2.

Now, assuming that formation of \( N_t \) is instantaneous, and \( k_{off} \) is negligible, we have Equation 3.

In the proposed two-step reaction, formin (\( F \)) first binds to any membrane site (\( M \)) as shown in Equation 4, and then the bound formin (\( FM \)) can be activated depending on the PI(4,5)P2 concentration as shown in Equation 5.

\[ F + M \rightleftharpoons FM \]  
\[ FM + nP \rightleftharpoons FMP_n \]

Here, \( P \) represents PI(4,5)P2, and \( n \) is the Hill coefficient of the reaction. The equilibrium association constants of the first and second reactions are \( K_d = ([FM])/(\text{[FM]}[M]) \) and \( K_{d} = ([FMP_n])/(\text{[FM]}[P]^n) \), respectively. Here, we assume a fraction \( x \)
of FM and all of FMPn can act as nucleation sites for actin. Hence, the concentration of nucleation sites is given by Equation 6.

\[
[N_n] = x[FM] + [FMP_n]
\]

\[
= K_a[F][M] + K_pK_F[M][P]^n
\]

\[
= (x + K_p[P]^n)K_F[M]
\]  

(Eq. 6)

Because the total membrane sites \([M]_{\text{tot}}\) can be written as shown in Equation 7,

\[
[M]_{\text{tot}} = [M] + [FM] + [FMP_n] = [M](1 + K_F[F] + K_pK_F[F][P]^n)
\]

(Eq. 7)

we can rewrite \([M]\) in terms of total membrane sites as shown in Equation 8.

\[
[M] = \frac{[M]_{\text{tot}}}{(1+K_F[F] + K_pK_F[F][P]^n)}
\]

(Eq. 8)

Hence, the concentration of nucleation sites is given by Equation 9.

\[
[N_n] = \frac{(x + K_p[P]^n)K_F[M]_{\text{tot}}}{(1+K_F[F] + K_pK_F[F][P]^n)}
\]

(Eq. 9)

A comparison of actin formation rate in the proposed reaction with the experimental data is shown in Fig. 10. This mechanism captures all four of the observations obtained from the experiments.

**Model to describe actin filament length distribution**

As described in Equation 9 and depicted in Fig. 10, the number of actin nucleation sites on the membrane depends on PI(4,5)P2 and cholesterol concentration. To obtain the length distribution of actin filaments as a function of the number of
nucleation sites, we utilized a spatial model for describing actin-filament formation on the membrane, as described under “Computational methods.” In this model, actin monomers can polymerize at the nucleation sites on the membrane surface. A snapshot of the simulation is shown in Fig. 11A. Length distributions of actin filaments for different concentrations of nucleation sites obtained from simulations are shown in Fig. 11B. Our results are in striking agreement with the experimental observations of the length distributions upon adding the different LUVs as determined from EM studies, see Fig. 11C. The length distributions of actin filaments in LUVs A, LUVs B, and LUVs C show LUVs A having a large number of short filaments and LUVs C with the least. The observed trends in the length distributions of actin filaments are consistent with the expectation of exponential distribution at equilibrium (long times) and peaked distributions at intermediate times.

**Discussion**

**Role of cholesterol in PI(4,5)P<sub>2</sub>-containing monolayers**

Actin assembly on supported monolayers allows a direct observation of the spatial correlation between PI(4,5)P<sub>2</sub>-regulated actin-binding proteins and PI(4,5)P<sub>2</sub> nanoclusters. PI(4,5)P<sub>2</sub> serves as a membrane anchor/activator for actin nucleation–promoting factors such as N-WASP (25) and WAVE2 (26) and the formins mDia1 and mDia2 (27, 28). The distribution of actin assembled on a supported lipid monolayer reflects the lateral distribution of the membrane lipids capable of activating actin nucleators. Such a functional assay is independent of fluorescent PI(4,5)P<sub>2</sub> analogs and therefore free from potential artifacts arising from modifying the physical chemistry of the phospholipid. To perturb PI(4,5)P<sub>2</sub> distribution in the membrane, we introduced two elements in our experimental settings. The first component is dCHOL, which was used instead of CHOL to avoid photooxidation. The incorporation of dCHOL, similar to that of CHOL, promotes the lipid-phase separation in both monolayer and bilayer membranes, although with a slightly different phase behavior (29). In a typical ternary phase diagram for bilayer membranes composed of CHOL, saturated and unsaturated lipids such as DPPC and DOPC, the tie-line suggests that the mole fraction of unsaturated lipids can reach 70–75 mol % in the L<sub>d</sub> phase, but it is only 10–15 mol % in the L<sub>α</sub> phase upon demixing (30). Because the naturally occurring brain PI(4,5)P<sub>2</sub> species 1-stearyl-2-arachidonoyl-PI(4,5)P<sub>2</sub> and dioleoyl-PI(4,5)P<sub>2</sub> (31) used here both have unsaturated acyl chains, their distribution is similar to that of the unsaturated DOPC used in our monolayers. These compositions predict a nearly 3-fold increase of the PI(4,5)P<sub>2</sub> surface charge density in the L<sub>d</sub> phase upon phase demixing when the total mole fraction of unsaturated lipids in LUVs A was controlled at 25 mol % experimentally. The resulting increase in the local charge density as well as the surface potential leads to an enhanced electrostatic interaction (32). Such an effect of PI(4,5)P<sub>2</sub> lateral segregation was demonstrated to be very effective in mediating gelsolin activity in vitro (13).

**Role of calcium**

Another important factor is Ca<sup>2+</sup>-induced perturbation of PI(4,5)P<sub>2</sub> lateral distribution (33). The formation of PI(4,5)P<sub>2</sub> nanoclusters induced by Ca<sup>2+</sup> has two consequences: 1) increased chemical potential of PI(4,5)P<sub>2</sub> within the cluster, and 2) the silencing effect of Ca<sup>2+</sup> due to charge neutralization. Depending on the interaction with PI(4,5)P<sub>2</sub> whether it is a receptor–ligand type of interaction such as a PLCδPH domain or a pure electrostatic interaction such as MARCKS peptide, the net effect of Ca<sup>2+</sup> for a PI(4,5)P<sub>2</sub>–protein interaction might be different. The former has been investigated recently in both in vitro (34) and in cellular studies (18) in which the recognition of PI(4,5)P<sub>2</sub> by PLCδPH domain was suppressed by Ca<sup>2+</sup> through forming Ca<sup>2+</sup>–PI(4,5)P<sub>2</sub> complexes. The latter case was investigated in this study by an actin assembly assay sensitive to actin nucleation–promoting factors that interact with PI(4,5)P<sub>2</sub> through an unstructured polyanionic motif. The effect of Ca<sup>2+</sup> and CHOL are not mutually exclusive, and we therefore also considered scenarios where both were present in the system.

**Effect of PI(4,5)P<sub>2</sub> clustering**

Starting with supported lipid monolayers, we noticed that dCHOL-mediated phase demixing and changes in PI(4,5)P<sub>2</sub> lateral distribution affected actin assembly only when there was Ca<sup>2+</sup>. The assembled actin filaments distributed evenly among L<sub>α</sub> and L<sub>d</sub> phases in the absence of Ca<sup>2+</sup> and associated primarily with the L<sub>d</sub> phase when there was Ca<sup>2+</sup>. The fact that actin assembly was excluded from the L<sub>α</sub> microdomain in the pres-
ence of Ca\(^{2+}\) leads to two possible explanations: 1) PI(4,5)P\(_2\) is either excluded from the L\(_0\) phase in the presence of Ca\(^{2+}\) or 2) PI(4,5)P\(_2\) in the L\(_0\) domain is “silenced” upon Ca\(^{2+}\) adsorption. Because the brain extract contains 5 mM EGTA, trace amounts of Ca\(^{2+}\) carried over from lipid transferring were removed by EGTA. The differences in the assembled actin distribution could then only result from Ca\(^{2+}\)-induced perturbation in PI(4,5)P\(_2\), lateral distribution, as the “silencing” effect mediated by the formation of Ca\(^{2+}\)–PI(4,5)P\(_2\) complexes should be eliminated by EGTA. Although EM provides no direct evidence that these round disk-like structures are PI(4,5)P\(_2\)-enriched clusters, the sizes of such structures (~70–120 nm in diameter as shown in Fig. 2, C–E) fall within the same size distribution of Ca\(^{2+}\)-induced PI(4,5)P\(_2\) clusters under the same conditions (84 ± 24 nm in diameter) that were measured by atomic force microscopy (33, 35).

**Effect of PI(4,5)P\(_2\) local concentration and spatial organization in bilayer vesicles**

In the experimental system employing LUVs, in which lateral mobility of lipids is not restricted by adsorption to glass, the presence of cholesterol efficiently enhanced the ability of PI(4,5)P\(_2\) to stimulate actin polymerization in the presence of cytoplasmic extracts. Because the amount of PI(4,5)P\(_2\) was the same in cholesterol-containing LUVs A and in cholesterol-lacking LUVs B, these results suggest that lateral demixing of PI(4,5)P\(_2\), induced by cholesterol is responsible for the increased PI(4,5)P\(_2\) ability to stimulate actin assembly.

**Role of actin nucleation factors**

The increased actin polymerization in extracts containing LUVs A can be explained by additional nucleation and/or enhanced elongation of actin filaments. Because our extracts exhibited basal levels of actin polymerization even in the absence of LUVs, we cannot strictly distinguish between these possibilities. However, using specific inhibitors of the Arp2/3 complex and formins, we found that LUV A–mediated enhancement of actin assembly largely depends on formins, but not significantly on the Arp2/3 complex. Similar conclusions can be derived from our EM data that show predominantly unbranched actin filaments in LUV A-containing reaction mixtures. An apparent conflict with other studies that reported a role of Arp2/3 complex in PI(4,5)P\(_2\)-dependent actin assembly in cytoplasmic extracts (7, 8) can be explained by the fact that in contrast to other studies, we did not add GTPγS to the reaction mixture in these experiments. GTPγS helps to maintain Rho family GTPases in the extract in an active state. In turn, active Cdc42 and Rac GTPases are necessary to activate nucleation-promoting factors N-WASP and WAVE complex, respectively, for stimulation of Arp2/3 complex activity. Our results obtained with the Arp2/3 complex inhibitor are therefore consistent with this notion.

**Effect of formins**

The role of formins in this assay can include both nucleation and elongation of actin filaments. Interestingly, although activation of formins also depends on Rho GTPases (36), our experiments suggest that formins can be activated in the absence of a GTPγS-mediated boost of Rho GTPase activity, if LUVs A with demixed PI(4,5)P\(_2\) are provided. The formins mDia1 (27) and mDia2 (28, 37) can interact with acidic phospholipids through their N-terminal basic domains. This interaction contributes to proper localization of these formins in cells (27, 29). Our data suggest that phospholipids contribute not only to localization of formins, but also to their activation. It is likely that in normal endogenous conditions in cells Rho GTPases and phospholipids cooperate for activation of formins.

In cells, polymerizing barbed ends of actin filaments are typically oriented toward and anchored to the plasma membrane by barbed end-associated proteins, such as formins and Ena/VASP family proteins. The proteins of both families directly interact with the barbed ends, whereas their association with the plasma membrane partially or completely depends on other membrane-anchored proteins. This property of the plasma membrane apparently is not reproduced in the mixture of LUVs and the cytoplasmic extract, which can explain the frequent release of actin filaments from LUVs A observed in our experimental system.

**Cell-derived plasma membrane sheets as PI(4,5)P\(_2\)-dependent actin-nucleating surfaces**

As a more physiological assay to test the role of PI(4,5)P\(_2\) in actin assembly, we used isolated plasma membrane sheets as a lipid interface, which we exposed to cytoplasmic extracts. In these assays, we supplemented the cytoplasmic extracts with GTPγS, because we were not able to obtain robust actin assembly on the plasma membrane in the absence of GTPγS. Accordingly, we found that both the Arp2/3 complex and formins contributed to actin assembly in these conditions. Quantitative data suggest some cooperation between the two assembly mechanisms, because the effects of the two inhibitors were not simply additive. We envision two nonexclusive ways of cooperation between formin and Arp2/3-dependent polymerization. Formins might nucleate initial “mother” filaments to enable subsequent Arp2/3 complex–dependent branched nucleation. In contrast, formins may capture barbed ends of Arp2/3 complex–nucleated actin filaments and promote their elongation. Our EM data showing predominantly long actin filaments with infrequent branches are more consistent with the latter scenario.

Our evaluation of the roles of PI(4,5)P\(_2\) for actin assembly on plasma membranes included two complementary assays. Using neomycin, we tested a role of PI(4,5)P\(_2\) availability to proteins in the extract. Using MβCD, we tested whether the lateral organization of PI(4,5)P\(_2\) in the plasma membrane is important. Our data show that both availability and distribution of PI(4,5)P\(_2\) in the plasma membrane are important for actin assembly. This conclusion is further validated by strong positive correlation between the degree of actin assembly and the presence of PI(4,5)P\(_2\) in individual membrane sheets, both in control samples and samples treated with the inhibitors, in which the overall levels of actin polymerization were significantly diminished by neomycin or MβCD treatment. Moreover, the availability and distribution of PI(4,5)P\(_2\) were similarly important for the assembly of other membrane-associated structures, clathrin-coated pits and caveolae, suggesting that our findings may be
**PL(4,5)P2- and cholesterol-mediated control of actin assembly**

applicable to a much broader range of PL(4,5)P2-interacting proteins.

**Molecular model of formin-membrane binding**

We constructed a molecular model of a peptide derived from the mDia2 basic domain interacting with bilayers of different compositions and conducted molecular dynamics simulations to infer the molecular interactions, mechanisms, and dynamics of formin recruitment to the membrane and the role of cholesterol in mediating the interactions. At the atomic scale, we designed computational models of peptide–bilayer interactions with and without cholesterol and at varying concentrations of PL(4,5)P2. We performed all-atom MD simulations for 150 ns to study the interactions. Through these studies, we could validate the hypothesis that interaction between the basic domain peptide of mDia2 and PL(4,5)P2, as tracked by the temporal evolution of hydrogen bonds between peptide residues and lipid headgroups, is highly dependent on bilayer composition, and specifically the concentration of PL(4,5)P2. Interestingly, we found that cholesterol plays a significant role in defining the multivalency of the cooperative PL(4,5)P2–peptide-binding interactions, with a characteristic Hill coefficient between 3 and 4. The experimental results for polymerization rate show cooperativity in formin binding to PL(4,5)P2 with a Hill coefficient of 3, consistent with the finding of molecular dynamics simulations.

**Kinetic model of PL(4,5)P2-stimulated actin assembly**

Complementing the molecular models, we described the kinetics of actin filament formation using kinetic models defining the peptide protein cooperative interactions as well as spatial models of actin polymerization on the membrane. These models describe the interactions at the microscale, in which the formin–PL(4,5)P2 interactions and the effect of cholesterol were considered through the binding constants. We showed that the effect of PL(4,5)P2 concentration, cholesterol, and extract concentration are simultaneously captured by a single unified model whose parameters are consistent with the findings of the molecular dynamics simulations. The salient findings include the following. (a) The presence of cholesterol enhances formin binding to PL(4,5)P2 resulting in lower effective $K_{a}$ values for LUVs A compared with LUVs B; this lowering of the effective $K_{a}$ is consistent with the enhancement in multivalent interactions observed in the molecular dynamics simulations when cholesterol was included in the bilayers. (b) The change in $K_{a}$ with increase in formin, PL(4,5)P2, and cholesterol concentration can be explained by a two-step reaction mechanism of formin recruitment to the membrane and multivalent binding with PL(4,5)P2; moreover, the parameters of the kinetic model are consistent with the findings of molecular simulations. (c) Our results suggest that as formin (extract) concentration increases, the number of filament nucleation sites $N_{f}$ can saturate at a lower concentration of PL(4,5)P2. This behavior mimics a change in $K_{a}$ values as seen in the experimental data. This model enables us to simultaneously describe the filament formation rates versus PL(4,5)P2 concentration under low and high extract concentrations with a single (unified) set of parameters. (d) The spatial model of actin polymerization successfully explains the experimentally observed length distribution of actin filaments for the different LUVs.

**Interpretation of molecular dynamics and modeling**

Although the agreement of the molecular model and the kinetic model with the reported experiments is intriguing, it raises the important question as to the mechanism behind cholesterol-mediated enhancement of binding of the peptide to the bilayer. In Fig. 12, we show four snapshots of lipid clustering at the site of the peptide (different rows) from our molecular dynamics simulations for systems without cholesterol (1st and 2nd columns) and with CHOL (3rd and 4th columns). The snapshots indicate that in the presence of cholesterol, there is more PL(4,5)P2 being recruited, which is consistent with the enhancement in multivalent interactions as reported in Fig. 9. We reiterate that the interactions are primarily electrostatic involving the charged residues of the peptide as noted earlier; this finding is also consistent with protein–lipid interactions observed in molecular dynamics simulations of other related systems reported recently (37, 38). However, the altered presentation of PL(4,5)P2 to the peptide mediated by the presence of cholesterol does not have an electrostatic origin because there is no significant hydrogen bonding between cholesterol and PL(4,5)P2 or PS (Fig. S4). Instead, the excluded volume and van der Waals interactions between the CHOL and the acyl chains of the lipids (i.e., lipid without the headgroup) are responsible for the CHOL-mediated interactions. In support of this claim, we report the radial distribution functions between lipids (Fig. 13). We find that in general CHOL reduces lipid–lipid distances slightly (see Fig. 13, upper left). Ordering between PS and PL(4,5)P2 disappears above 10%, and both CHOL and high PL(4,5)P2 concentrations cause higher PS–PS ordering. The high PL(4,5)P2 concentration shows the most ordering between CHOL and PS. These observations lead us to conclude that there is enhanced CHOL–PS and PS–PS structuring at the nearest neighbor level, and this CHOL-mediated reconfiguration facilitates a better presentation of the PL(4,5)P2 local cluster to interact with the peptide as observed in the snapshots of Fig. 12. We hypothesize that compositions that encourage formin–PL(4,5)P2 binding also result in PL(4,5)P2–PS repulsion. Analysis of lipid–lipid hydrogen bonding and salt bridges (Fig. S4) shows that the reordering observed in the radial distribution functions is not visible in the inter-lipid bonding, which appears to be mostly opportunistic. An abstract measurement of lipid-binding partners (Fig. S4) shows that most lipids have no significant preference for particular lipid neighbors. This suggests that cholesterol and PL(4,5)P2 concentration effects observed here are not due to lateral rearrangements of these lipids but to subtle differences in both packing and the particular molecular conformations of the PL(4,5)P2.

**Conclusion**

A combination of biochemical and computational studies shows that the lateral distribution of PL(4,5)P2 within the lipid bilayer of the plasma membrane is an essential element in the control of actin assembly at the cell cortex. PL(4,5)P2-dependent nucleation of actin polymerization involves the function of both formin and nucleation-promoting factors that activate the
The greater activity of PI(4,5)P₂ to stimulate formin-dependent activation when the lipid is in a phase-separated membrane is consistent with molecular dynamics simulations and analytical models supporting a mechanism in which formin first binds in an inactive state to the membrane and then cooperatively binds 3 eq of PI(4,5)P₂ to initiate actin nucleation. The computational models we have presented at the molecular and microscales collectively provide an integrated framework for the mechanism of actin filament formation induced by nucleating factors such as formin and quantitatively define the roles played by the lipid composition, namely PI(4,5)P₂ and cholesterol, on the filament formation rates.

**Experimental procedures**

*Preparation of supported lipid monolayers*

Synthetic PI(4,5)P₂ with uniform acyl chain (1,2-dioleoyl-sn-glycero-3-phosphatidylinositol 4,5-bisphosphate), other neutral phospholipids, such as 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and fluorescently labeled L₄₄ marker,
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine-DOPE) were from Avanti (Alabaster, AL). Dihydrocholesterol (dCHOL, also known as cholestanol) was purchased from Sigma. Subphase reagents (HEPES, EDTA, CaCl2, MgCl2, NaCl, KCl) were purchased from ThermoFisher Scientific (Hampton, NH) and centrifuged at 160,000 g for 2 hours at 4° C and then sonicated in a water bath sonicator for 10 min and extruded through a polycarbonate membrane with an average pore size of 200 nm (Avestin, Ottawa, CA) using a mini-extruder (Avanti, Alabaster, AL) on a hot plate at 60 °C. The lipid resuspension was extruded 31 times to ensure proper mixing. The effective PI(4,5)P2 concentration for LUVs A and B stock solutions was ~250 μM, which considers only the PI(4,5)P2 in the outer leaflet assuming equal distribution of PI(4,5)P2 between the two leaflets. LUVs C were prepared in the same way starting from 565 μg of DOPC. The hydrodynamic diameters of the LUVs were determined by dynamic light scattering using a DynaPro99 instrument (Wyatt Technology, formerly Protein Solutions) (39).

**Preparation of bovine brain extract**

Bovine brain tissue was collected from a nearby slaughterhouse (Bringhurst Meats, Berlin, NJ) and snap-frozen in liquid nitrogen for future use. The brain extract was prepared according to published methods (40). In brief, a piece of flash-frozen bovine brain (10 g) was homogenized on ice with a mortar and pestle in the presence of Complete protease inhibitor mixture (Roche Applied Science, Mannheim, Germany) in a 20-ml breaking buffer containing 25 mM Tris, pH 8.0, 500 mM KCl, 250 mM sucrose, 2 mM EGTA, 1 mM DTT. The cell extract was further homogenized with a Dounce homogenizer (Kontes Co., Vineland, NJ) and centrifuged at 160,000 g for 2 h at 4 °C using a Beckman OptimaTM LE-80K ultracentrifuge and a Ti 70.1 rotor to remove any insoluble debris. The cell extract was desalted on HiTrap Desalting Column (GE Healthcare) at 4 °C into a cytosolic buffer containing 25 mM HEPES, 120 mM potas-
sium glutamate, 20 mM KCl, 2.5 mM MgCl₂, and 5 mM EGTA, pH 7.4.

**Preparation of neutrophil extract**

Supernatant from lysed neutrophils was prepared as described previously (41, 42). Briefly, neutrophils isolated from human blood were resuspended at ~3 × 10⁸ cells/ml in intracellular physiological buffer (135 mM KCl, 10 mM NaCl, 2 mM MgCl₂, 2 mM EGTA, 10 mM HEPES, pH 7.1) containing protease inhibitors (1 μg/ml leupeptin, 1 μg/ml benzamidine, 10 μg/ml aprotinin, 10 μg/ml N-α-p-tosyl-L-arginine methyl ester). Then, cells were lysed by sonication (three times 2-s pulse on setting 40 of Dynatech Sonic Dismembrator 150; Dynatech Laboratories, Inc.). To obtain supernatant, the neutrophils’ lysed suspension was subjected to centrifugation (first, 14,000 rpm (~1.5 × 10⁵ g) for 5 min; second, 80,000 rpm for 20 min (~5.6 × 10⁶ g). The protein concentration was assessed using a total protein kit (Micro Lowry TP0300, Sigma) and was adjusted to 3 mg/ml. The PI(4,5)P₂ concentration in obtained supernatant was 4.15 pmol/μl as assessed by a PI(4,5)P₂ mass ELISA kit (K-4500, Echelon).

**Actin assembly on supported lipid monolayers**

Thawed cell extract was supplemented with 1 mM ATP and 20 μM GTP before use. Freshly prepared supported lipid monolayer was retrieved from PBS solution. The solution remaining on the coverslip was carefully removed by tissue paper from the side followed by blow-drying briefly to remove tiny droplets that remained attached before applying the cell extract. A cell extract of 50 μl was applied on top of the supported lipid monolayer and incubated on a pre-warmed heating metal block at 37 °C for the indicated periods of time. The samples were fixed by the gentle addition of 50 μl of 1.5% glutaraldehyde solution into the cell extract to avoid actin filament detachment and incubated at room temperature for 40 min. The samples were rinsed with a 10 mM HEPES buffer at pH 7.4 and stained with Alexa Fluor 633-phalloidin (Invitrogen) with a 1:500 or 1:1000 dilution for 30 min. The samples were gently rinsed three times to avoid actin filament detachment, air-dried, and kept away from light for imaging studies. For image analysis, fluorescent micrographs were segmented based on the lipid phases using ImageJ. Average phalloidin intensity within L₀ and L₄ phases was quantified by randomly selecting fields of view from multiple samples.

**EM of supported lipid monolayers**

Air-dried supported lipid monolayer samples, with or without assembled actin, were rotary coated with a ~1-nm layer of platinum at a 20° angle and an ~5-nm layer of carbon at an 80° angle using Auto306 vacuum evaporator (Edwards, UK). The coated sample was floated on a diluted hydrofluoric acid solution to separate replica from the coverslip and transferred onto Formvar-coated EM grids. Samples were analyzed using a JEM-1011 transmission electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 100 kV. Images were captured by an ORIUS 832.10W CCD camera from samples that were presented in inverse contrast (Gatan, Warrendale, PA).

**Actin polymerization assay with LUVs**

To induce actin polymerization, a concentrated solution of KCl and MgCl₂ was added to G-actin containing ~50% of pyrenyl-actin to obtain 150 mM KCl and 2 mM MgCl₂ final concentration. LUVs (0–30 μl) and human neutrophil extract (0–5 μl) were sequentially added. The sample was then topped up to 300 μl with water so that the final G-actin concentration reached 1.7–2 μM, whereas the G-actin concentration in the neutrophil extract was estimated to be around 12 μM. Changes of pyrene fluorescence were monitored for 3–30 min (λₘₐₓ 365 nm and λₑₘₐₓ 386 nm) using an SL-50B spectrofluorimeter (PerkinElmer Life Sciences). Actin polymerization rate was calculated from the initial slope of the fluorescence increase in the first 30 s. To determine whether PI(4,5)P₂-promoted actin polymerization is mediated by formins or Arp2/3 complex, SMIFH2 (Sigma, S4826) or CK-666 (Sigma, SML0006) was added to the reaction from stock solutions in DMSO. For negative staining EM, a mixture containing G-actin, neutrophil extract, and/or LUVs was loaded onto a carbon-coated EM grid and following incubation for 1–5 min was stained with aqueous 1% uranyl acetate for 1 min before draining and drying. For S1 labeling, after incubation of samples on grids, the grid was passed through 1 drop of S1 (0.25 mg/ml in actin polymerization buffer without ATP), incubated with the second drop of S1 for 5 min, and stained with 1% uranyl acetate.

**Actin assembly on plasma membrane sheets**

The plasma membrane sheets for actin assembly were prepared based on a published protocol (40). Briefly, plasma membranes of PtK2 cells stably expressing membrane-targeted GFP (GFP-CAAX) (gift of Dr. W. Guo, University of Pennsylvania) were isolated by sonication-mediated unroofing. The membrane sheets were incubated at 37 °C with bovine brain extracts supplemented with 0.2 μM rhodamine-actin, 1.5 mM ATP, and 150 μM GTPγS and, optionally, with various pharmacological inhibitors, such as SMIFH2, CK-666, 2 mM neomycin (Sigma, N1876), or MβCD (Sigma, 332615). The samples then were fixed with 0.2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3, for 20 min at room temperature. After washing three times with PBS, they were quenched by incubation with NaBH₄ (three times for 15 min total), blocked for 30 min with 10% normal goat serum diluted in PBS, and stained for 1 h with mouse monoclonal IgM antibody to PIP₂ (Abcam, clone 2C11; ab11039) at a 1:60 dilution for fluorescence microscopy or a 1:20 dilution for EM, then with goat anti-mouse IgM CCL-647–conjugated secondary antibody (Santa Cruz Biotechnology, SC-395787, 1:150) for fluorescence microscopy or 18-nm colloidal gold–conjugated secondary antibody (EM Sciences, 25149; 1:10) for EM. To evaluate efficiency of cholesterol depletion from plasma membrane sheets after incubation with MβCD, the sheets were fixed for 20 min with 4% formaldehyde in PBS, quenched with 0.3 M glycine for 10 min, and with 50 μg/ml filipin III (F4767, Sigma) in PBS containing 0.2% saponin and 0.1% BSA for 1 h at room temperature. Samples were washed three times with PBS, mounted into Prolong Gold antifade mountant (P36930, ThermoFisher Scientific), and imaged. Fluorescence imaging was performed using an Eclipse TiE
inverted microscope (Nikon) equipped with a CSUX1 spinning disk (Yokogawa Electric Corp.). For filipin imaging, 405-nm laser and ET455/50M DAPI-ET emission filter was used. EM images were obtained using a JEM 1011 transmission electron microscope (JEOL) operated at 100 kV with an ORIUS 832.10W charge-coupled device camera (Gatan) and presented in inverted contrast.

For quantification of rhodamine-actin incorporation, PI(4,5)P2 immunofluorescence, and filipin staining, plasma membrane sheets were identified based on GFP-CaAX fluorescence, and the mean fluorescence intensities of actin, anti-PI(4,5)P2, or filipin within the regions of interest chosen away from the membrane sheet margins were measured using ImageJ (FIJI, National Institutes of Health). For quantification of PI(4,5)P2 immunogold clustering in platinum replica EM images, a number of gold particles per cluster was counted. Gold particles were considered to belong to the same cluster if they were separated by less than twice the diameter of gold particles (36 nm). Statistical significance was determined by Tukey-Kramer multiple comparisons test after evaluating data distribution normality by Kolmogorov-Smirnov normality test using Instat software (GraphPad Software).

**Computational methods**

**Homology modeling of mDia2**

Previous co-sedimentation assays conducted on mDia1 demonstrated that peptides spanning basic acid clusters (amino acids 12–21) within the basic domain of mDia1 (amino acids 1–60) are sufficient to bind PI(4,5)P2 (27). The basic amino acid cluster in mDia1 shares 90% homology with the basic amino acid cluster in mDia2 (amino acids 25–40) (27). We created models of this domain (amino acids 25–40) of mDia2. Molecular models of mDia2 were constructed using both the *ab initio* and homology modeling methods Robetta (43) and Modeler (44), respectively, and the highest quality structures yielded from the methods were selected.

To create homology models of the basic mDia2 (amino acids 25–40), the X-ray structure of the human Cdc37 N-terminal domain (Protein Data Bank code 2NCA, amino acids 54–62) was used as a template. The sequences of human Cdc37 N-terminal and human mDia2 were obtained from Uniprot (accessions codes Q16543 and Q9NSV4, respectively). The Clustal Omega (45) program was used to align sequences of the human CDC37 N-terminal domain with the sequence of the human mDia2 indicating the sequences shared 66% identity. This alignment was then used to construct 10 molecular models using Modeler (44). In Robetta (43), the sequence of the basic peptide region was input to the web server, and the resulting structures were analyzed. The top Modeler (44) and Robetta (43) models were selected based on the discrete optimized protein energy score and then relaxed using all-atom molecular dynamics simulations using the molecular dynamics package GROMACS (46), and the CHARMM force field (47). The qualities of the minimized and simulated models were assessed through PROCHECK (48) and SWISS MODEL SERVER (49). PyMOL (50) and Visual Molecular Dynamics (VMD) (51) were used to analyze and visualize the resulting structures. The highest quality selected model based on the assessment methods was selected for the simulations of mDia2 on bilayers.

**Bilayer construction and protein adhesion**

To decipher the effect of cholesterol and the importance of PI(4,5)P2 concentration in PI(4,5)P2—protein—bilayer interactions, bilayers containing varying concentrations of PI(4,5)P2 and cholesterol were constructed. We constructed six bilayers containing 10, 20, and 30% PI(4,5)P2 concentrations and two bilayers containing no cholesterol. Table 1 lists the bilayer composition of the described bilayers. The inner leaflet contained PI(4,5)P2, DOPE, DOPS, and CHOL, and the outer leaflet contained POPC and CHOL (Fig. 8). DOPE concentrations were adjusted to account for area change when varying the PI(4,5)P2 and CHOL concentrations. The resulting areas of the bilayers were ~62 nm². The simulations were solvated with ~4459 water molecules and neutralized with 239 Na⁺ and 71 Cl⁻ ions. An energy minimization of the solvated bilayer and neutralized structures was performed to correct the inappropriate geometry.

**Molecular dynamics of mDia2 on PI(4,5)P2-containing asymmetric bilayers**

In the past, molecular dynamics simulations have revealed unique atomistic resolution characteristics important in understanding protein–membrane interactions and dynamics (52). To decipher the effect and the importance of PI(4,5)P2 concentration, we performed six all-atom simulations of mDia2 adhered to the bilayers containing varying concentrations of PI(4,5)P2 listed in Table 1. Then to understand the role of cholesterol, we carried out two simulations mDia2 adhered to the bilayers containing no CHOL.

All simulations were performed using GROMACS version 5.1.2 Charmm36 force field for all standard protein and lipids parameters (53). Long-range interactions were considered through the particle-mesh–Ewald method. mDia2 was placed on a previously equilibrated bilayer containing water molecules and counter sodium and chloride ions and ~252 lipids. mDia2 was adhered to at least one PI(4,5)P2 molecule at the center of the bilayer with a distance of 3 Å. The simulations were solvated with ~4459 water molecules. The Linear Constraint Solver (LINCS) algorithm was used to constrain bond lengths. Each system was simulated for 150 ns in the semi-isotropic NPT ensemble, with constant particle number, normal pressure of 1 atm, and constant temperature of 300 K. A time step of 1 fs was used in all simulations. The resulting simulations were viewed and analyzed using VMD and in-house analysis codes.

![Table 1](image-url)

**Table 1** Bilayer compositions utilized in the molecular modeling.

| Bilayer | Leaflet | PI(4,5)P2 | DOPE | DOPS | POPC | CHOL |
|---------|---------|-----------|------|------|------|------|
| 10% PIP2 | Inner  | 10        | 50   | 20   | 0    | 20   |
|         | Outer   | -         | -    | -    | -    | -    |
| 20% PIP2 | Inner   | 20        | 40   | 20   | 0    | 20   |
|         | Outer   | -         | -    | -    | 80   | 20   |
| 30% PIP2 | Inner   | 30        | 30   | 20   | 0    | 20   |
|         | Outer   | -         | -    | -    | -    | -    |
| w/o CHOL| Inner   | 22        | 55   | 23   | 0    | 0    |
|         | Outer   | -         | -    | -    | 80   | 20   |

*Fig. 8*
Continuum spatial model of actin filament formation on the membrane

The spatial model for actin nucleation consists of the following: (i) a patch of the cell membrane of 500 × 500-nm dimension simulated using the dynamically triangulated Monte Carlo method (54); and (ii) actin monomers that can polymerize at the nucleation sites on the membrane surface. A snapshot of the simulation is shown in Fig. 11A. The membrane is placed in a periodic box, and the direction perpendicular to the membrane plane is taken to be the z direction. Actin nucleation sites are allocated at a random position on the membrane. Monomers of actin are taken to be coarse-grained beads and are allowed to reside and diffuse in the box, on one side membrane, with periodic boundary conditions in the x and y directions. New actin filaments are nucleated from actin nucleation complexes on the membrane with the filament barbed end toward the membrane. After nucleation, filaments polymerize on the barbed end and depolymerize at the pointed end. Filament growth is modeled as diffusion-limited aggregation and de-polymerization as a stochastic event for each filament (55). The ratio of polymerization to depolymerization is set to be comparable with experimental values (8 μM). Actin filaments are treated as semi-flexible filaments with persistence length (l_p) = 10 μm. The bound monomers in a filament interact with the connected neighbors via a spring potential \( E_{\text{spring}} = k_{\text{spring}}(r - a)^2/2 \) and an angle potential \( E_{\text{bend}} = k_{\text{bend}}(1 - \cos \theta)/2 \). Here \( a \), the diameter of the monomers, is taken to be 7 nm, comparable with the diameter of actin filaments. We take \( k_{\text{bend}} = k_{\text{spring}}/a \) and \( k_{\text{bend}} = k_{\text{spring}} \). The concentration of actin monomers is taken to be 100 μM.

Author contributions—R. Bucki, Y.-H. W., C. Y., S. K. K., R. Bradley, T. S., R. R., and P. A. J. conceptualization; R. Bucki, T. S., R. R., and P. A. J. funding acquisition; R. Bucki, Y.-H. W., C. Y., S. K. K., O. F., R. Bradley, K. P., T. S., R. R., and P. A. J. writing-original draft; Y.-H. W., C. Y., S. K. K., and P. A. J. investigation; Y.-H. W., C. Y., O. F., R. Bradley, K. P., T. S., R. R., and P. A. J. methodology; C. Y., T. S., and R. R. visualization; S. K. K., R. Bradley, R. R., and P. A. J. formal analysis; K. P. data curation; T. S. and P. A. J. supervision; T. S. and P. A. J. project administration; T. S. and P. A. J. writing-review and editing; R. R. and P. A. J. validation; P. A. J. resources.

Acknowledgments—We thank Dr. Michael Marks and Yueyao Zhu for help with filipin staining and imaging.

References

1. Lassing, I., and Lindberg, U. (1985) Specific interaction between phosphatidylinositol-4,5-bisphosphate and profilin. Nature 314, 472–474 CrossRef Medline
2. Yin, H. L., and Janmey, P. A. (2003) Phosphoinositide regulation of the actin cytoskeleton. Annu. Rev. Physiol. 65, 761–789 CrossRef Medline
3. Shibasaki, Y., Ishihara, H., Kizuki, N., Asano, T., Oka, Y., and Yazaki, Y. (1997) Massive actin polymerization induced by phosphatidylinositol-4-phosphate 5-kinase in vivo. J. Biol. Chem. 272, 7578–7581 CrossRef Medline
4. Yamamoto, M., Hilgemann, D. H., Feng, S., Bito, H., Ishihara, H., Shibasaki, Y., and Yin, H. L. (2001) Phosphatidylinositol 4,5-bisphosphate induces actin stress-fiber formation and inhibits membrane ruffling in cv 1 cells. J. Cell Biol. 152, 867–876 CrossRef Medline
5. Sakisaka, T., Itoh, T., Miura, K., and Takenawa, T. (1997) Phosphatidylinositol 4,5-bisphosphate phosphatase regulates the rearrangement of actin filaments. Mol. Cell. Biol. 17, 3841–3849 CrossRef Medline
6. Varnai, P., Thyagarajan, B., Rohacs, T., and Balla, T. (2006) Rapidly inducible changes in phosphatidylinositol 4,5-bisphosphate levels influence multiple regulatory functions of the lipid in intact living cells. J. Cell Biol. 175, 377–382 CrossRef Medline
7. Ma, L., Cantley, L. C., Janmey, P. A., and Kirschner, M. W. (1998) Core-requirement of specific phosphoinositides and small GTP-binding protein cdc42 in inducing actin assembly in Xenopus egg extracts. J. Cell Biol. 140, 1125–1136 CrossRef Medline
8. Lee, K., Gallop, J. L., Rambani, K., and Kirschner, M. W. (2010) Self-assembly of filopodia-like structures on supported lipid bilayers. Science 329, 1341–1345 CrossRef Medline
9. Kwik, J., Boyle, S., Fookson, D., Margolis, L., Sheetz, M. P., and Eddin, M. (2003) Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. Proc. Natl. Acad. Sci. U.S.A. 100, 13964–13969 CrossRef Medline
10. Jungmichel, S., Sylvester, K. B., Choudhary, C., Nguyen, S., Mann, M., and Nielsen, M. L. (2014) Specificity and commonality of the phosphoinositide-binding proteome analyzed by quantitative mass spectrometry. Cell Rep. 6, 578–591 CrossRef Medline
11. Janmey, P. A., Bucki, R., and Radhakrishnan, R. (2018) Regulation of actin assembly by PI(4,5)P2 and other inositol phospholipids; an update on possible mechanisms. Biochem. Biophys. Res. Commun. 506, 307–314 CrossRef Medline
12. Golebiowska, U., Nyako, M., Woturski, W., Zaitseva, I., and McLaughlin, S. (2008) Diffusion coefficient of fluorescent phosphatidylinositol 4,5-bisphosphate in the plasma membrane of cells. Mol. Biol. Cell 19, 1663–1669 CrossRef Medline
13. Wang, Y. H., Bucki, R., and Janmey, P. A. (2016) Cholesterol-dependent phase-demixing in lipid bilayers as a switch for the activity of the phosphoinositide-binding cytoskeletal protein gelsolin. Biochemistry 55, 3361–3369 CrossRef Medline
14. Honigmann, A., van den Bogaart, G., Iraheta, E., Risselada, H. J., Mi- lovanovic, D., Mueller, V., Mullar, S., Diederichsen, U., Fasshauer, D., Grubmuller, H., Hell, S. W., Eggeling, C., Kuhn, K., and Jahn, R. (2013) Phosphatidylinositol 4,5-bisphosphate clusters act as molecular beacons for vesicle recruitment. Nat. Struct. Mol. Biol. 20, 679–686 CrossRef Medline
15. van den Bogaart, G., Meyenberg, K., Risselada, H. J., Amin, H., Willig, K. L., Hubrich, B. E., Dier, M., Hell, S. W., Grubmuller, H., Diederichsen, U., and Jahn, R. (2011) Phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. J. Cell Biol. 191, 7578–7581 CrossRef Medline
16. Wang, J., and Richards, D. A. (2012) Segregation of PI2P2 and PI3P into distinct nanoscale regions within the plasma membrane. Biol. Open 1, 857–862 CrossRef Medline
17. Chierico, L., Joseph, A. S., Lewis, A. L., and Battaglia, G. (2014) Live cell imaging of membrane/cytoskeleton interactions and membrane topology. Sci. Rep. 4, 6056 Medline
18. Kang, I. K., Kim, O. H., Hur, J., Yu, S. H., Lamichhane, S., Lee, J. W., Ohja, U., Hong, J. H., Lee, C. S., Cha, J. Y., Lee, Y. I., Im, S. S., Park, Y. J., Choi, C. S., Lee, D. H., et al. (2017) Increased intracellular Ca2+ concentrations prevent membrane localization of pH domains through the formation of Ca2+-phosphoinositides. Proc. Natl. Acad. Sci. U.S.A. 114, 11926–11931 CrossRef Medline
19. Rizvi, S. A., Neidt, E. M., Cui, J., Feiger, Z., Skau, C. T., Gardel, M. L., Kozmin, S. A., and Kvar, D. R. (2009) Identification and characterization of a small molecule inhibitor of formin-mediated actin assembly. Chem. Biol. 16, 1158–1168 CrossRef Medline

4 Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.
Pl(4,5)P2- and cholesterol-mediated control of actin assembly

20. Nolen, B. J., Tomasevic, N., Russell, A., Pierce, D. W., Jia, Z., McCormick, C. D., Hartman, J., Sakowicz, R., and Pollard, T. D. (2009) Characterization of two classes of small molecule inhibitors of arp2/3 complex. *Nature* 460, 1031–1034 CrossRef Medline

21. Lassing, I. and Lindberg, U. (1988) Evidence that the phosphatidylinositol cycle is linked to cell motility. *Exp. Cell Res.* 174, 1–15 CrossRef Medline

22. Lewis, A. E., Sommer, L., Arntzen, M. O., Strahym, Y., Morrice, N. A., Divecha, N., and D’Antonis, C. S. (2011) Identification of nuclear phosphatidylinositol 4,5-bisphosphate-interacting proteins by neomycin extraction. *Mol. Cell. Proteomics* 10, M110.003376 CrossRef Medline

23. Mahammad, S. and Parmryd, I. (2015) Cholesterol depletion using methyl-β-cyclodextrin. Methods Mol. Biol. 1232, 91–102 CrossRef Medline

24. Collins, A., Warrington, A., Taylor, K. A., and Svitkina, T. (2011) Structural organization of the actin cytoskeleton at sites of clathrin-mediated endocytosis. *Curr. Biol.* 21, 1167–1175 CrossRef Medline

25. Tomasevic, N., Jia, Z., Russell, A., Fujii, T., Hartman, J. J., Clancy, S., Wang, M., Beraud, C., Wood, K. W., and Sakowicz, R. (2007) Differential regulation of WASP and N-WASP by Cdc42, Rac1, Nck, and PI(4,5)P2. *Biochemistry* 46, 3494–3502 CrossRef Medline

26. Suetsugu, S., Miki, H., Yamaguchi, H., Obinata, T., and Takenawa, T. (2001) Enhancement of branching efficiency by the actin filament-binding activity of N-WASP/WAVE2. *J. Cell. Sci.* 114, 4533–4542 Medline

27. Ramalingam, N., Zhao, H. X., Breitsprecher, D., Lappalainen, P., Faix, J., and Schleicher, M. (2010) Phospholipids regulate localization and activity of mDia1 formin. *Eur. J. Cell Biol.* 89, 723–732 CrossRef Medline

28. Gorelik, R., Yang, C., Kameswaran, V., Dominguez, R., and Svitkina, T. (2011) Mechanisms of plasma membrane targeting of formin mDia2 through its amino-terminal domains. *Mol. Biol. Cell* 22, 189–201 CrossRef Medline

29. Veatch, S. L. and Keller, S. L. (2005) Miscibility phase diagrams of giant vesicles containing sphingomyelin. *Phys. Rev. Lett.* 94, 148101 CrossRef Medline

30. Veatch, S. L. and Keller, S. L. (2003) Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol. *Biophys. J.* 85, 3074–3083 CrossRef Medline

31. Rusinova, R., Hobart, E. A., Koeppe, R. E., 2nd., and Andersen, O. S. (2013) Phosphoinositides alter lipid bilayer properties. *J. Gen. Physiol.* 141, 673–690 CrossRef Medline

32. Levental, I., and Janney, P. A., and Cébers, A. (2008) Electrostatic contribution to the surface pressure of charged monolayers containing polyphosphoinositides. *Bioophys. J.* 95, 1199–1205 CrossRef Medline

33. Wang, Y. H., Collins, A., Guo, L., Smith-Dupont, K. B., Gai, F., Svitkina, T., and Janney, P. A. (2012) Divalent cation-induced cluster formation by polyphosphoinositides in model membranes. *J. Am. Chem. Soc.* 134, 3387–3395 CrossRef Medline

34. Bilkova, E., Pleskot, R., Rissanen, S., Sun, S., Czogalla, A., Cwiklik, L., Rög, T., Vattulainen, I., Cremer, P. S., Jungwirth, P., and Coskun, Ü. (2017) Calcium directly regulates phosphatidylinositol 4,5-bisphosphate headgroup conformation and recognition. *J. Am. Chem. Soc.* 139, 4019–4024 CrossRef Medline

35. Wong, Y. H., Slochover, D. R., and Janney, P. A. (2014) Counterion-mediated cluster formation by polyphosphoinositides. *Chem. Phys. Lipids* 182, 38–51 CrossRef Medline

36. Ridley, A. J. (2015) Rho GTPase signalling in cell migration. *Curr. Opin. Cell Biol.* 36, 103–112 CrossRef Medline

37. Senju, Y., Kalimeri, M., Koscela, E. V., Somerharju, P., Zhao, H., Vattulainen, I., and Lappalainen, P. (2017) Mechanistic principles underlying regulation of the actin cytoskeleton by phosphoinositides. *Proc. Natl. Acad. Sci. U.S.A.* 114, E8977–E8986 CrossRef Medline

38. Alvararawh, M., and Weresczynski, J. (2017) Investigation of the effect of bilayer composition on PKCa-C2 domain docking using molecular dynamics simulations. *J. Phys. Chem. B* 121, 78–88 CrossRef Medline

39. Bucki, R., Pastore, J. J., Randhawa, P., Vegers, R., Weiner, D. I., and Janney, P. A. (2004) Antibacterial activities of rhodamine B-conjugated gelosin-derived peptides compared with those of the antimicrobial peptides cathelicidin LL37, magainin II, and melittin. *Antimicrob. Agents Chemother.* 48, 1526–1533 CrossRef Medline

40. Wu, M., Huang, B., Graham, M., Raimondi, A., Heuser, J. E., Zhuang, X., and De Camilli, P. (2010) Coupling between clathrin-dependent endocytic budding and F-BAR-dependent tubulation in a cell-free system. *Nat. Cell Biol.* 12, 902–908 CrossRef Medline

41. Huang, M., Pring, M., Yang, C., Taoka, M., and Zigmond, S. H. (2005) Presence of a novel inhibitor of capping protein in neutrophil extract. *Cell Motil. Cytoskeleton* 62, 232–243 CrossRef Medline

42. Zigmond, S. H., Joyce, M., Yang, C., Brown, K., Huang, M., and Pring, M. (1998) Mechanism of Cdc42-induced actin polymerization in neutrophil extracts. *J. Cell Biol.* 142, 1001–1012 CrossRef Medline

43. Kim, D. E., Chivian, D., and Baker, D. (2004) Protein structure prediction and analysis using the Robetta server. *Nucleic Acids Res.* 32, W526–W531 CrossRef Medline

44. Eswar, N., Webb, B., Marti-Renom, M. A., Radhakrishnan, S. M., Eramian, D., Shen, M. Y., Pieper, U., and Sali, A. (2006) Comparative protein structure modeling using Modeller. *Curr. Protoc. Bioinformatics* 2006 Chapter 5, Unit 5.6 CrossRef Medline

45. Sievers, F., and Higgins, D. G. (2018) Clustal Omega for making accurate alignments of many protein sequences. *Protein Sci.* 27, 135–145 CrossRef Medline

46. Van Der Spoel, D., Lindahl, E., Hess, B., Groenhof, G., Mark, A. E., and Berendsen, H. J. (2005) Gromacs: fast, flexible, and free. *J. Comput. Chem.* 26, 1701–1718 CrossRef Medline

47. Stocker, U., and van Gunsteren, W. F. (2000) Molecular dynamics simulation of hen egg white lysozyme: a test of the GROMOS96 force field against nuclear magnetic resonance data. *Proteins* 40, 145–153 CrossRef Medline

48. Laskowski, R. A., Rullmann, J. A., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996) AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* 8, 477–486 Medline

49. Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Gallo Cassarino, T., Bertoni, M., Bordoli, L., and Schwede, T. (2014) Swiss-Model: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res.* 42, W252–W258 CrossRef Medline

50. DeLano, W. L. (2002) The PyMOL Molecular Graphics System. DeLano Scientific LLC, San Carlos, CA

51. Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: visual molecular dynamics. *J. Mol. Graph.* 14, 33–38, 27–8 CrossRef Medline

52. Bradley, R. P., and Radhakrishnan, R. (2016) Curvature-undulation coupling as a basis for curvature sensing and generation in bilayer membranes. *Proc. Natl. Acad. Sci. U.S.A.* 113, ES117–ES124 CrossRef Medline

53. Lee, S., Tran, A., Allsopp, M., Lim, J. B., Hénin, J., and Klauda, J. B. (2014) Charmm36 united atom chain model for lipids and surfactants. *J. Phys. Chem. B* 118, 547–556 CrossRef Medline

54. Ramakrishnan, N., Sunil Kumar, P. B., and Radhakrishnan, R. (2014) Mesoscale computational studies of membrane bilayer remodeling by curvature-underscoding proteins. *Phys. Rep.* 543, 1–60 CrossRef Medline

55. Dognert, M., and Leibler, S. (1993) Physical aspects of the growth and regulation of microtubule structures. *Phys. Rev. Lett.* 70, 1347–1350 CrossRef Medline