Phosphatidylinositol 4-phosphate (PtdIns4P) is essential for proper Cav clustering, caveola formation, and caveola dynamics and that membrane scrambling can perturb caveolar stability.

Caveolae ("little-caves") are bulb-shaped invaginations of the plasma membrane (PM) enriched in cholesterol and sphingolipids, with a diameter of 50–80 nm (1). A variety of physiological roles has been attributed to caveolae, including as endocytic carriers, mechanosensors, regulators of membrane stress, and as regulators of lipid transport (2, 3). The primary protein component of caveolae is the integral membrane protein caveolin encoded by three homologous genes in mammalian cells: Cav1, Cav2, and Cav3. Caveolin proteins are co-translationally inserted into the endoplasmic reticulum (ER) (4) and are delivered to the PM via the secretory pathway (5). Newly synthesized Cav1 molecules self-associate to form oligomers of 12 or 14 monomers (6, 7). These oligomers then traffic from the Golgi apparatus to the PM via a vesicular carrier containing syntaxin 6, the ganglioside GM1, and glycosylphophatidylinositol-linked proteins (8). Once the Cav1 oligomers reach the PM, they can assemble to form caveolae, higher order structures containing ~144 molecules of Cav1 protein (9). This basic structure has been referred to as a quantal unit (9). However, sub-quantal oligomeric structures of Cav1 have been described in the PM (9), as well as caveolar clusters formed by multiple caveolae in close, diffraction-limited proximity (10) or as larger rosette structures (11).

From a structural standpoint, Cav1 contains a hairpin loop structure, three palmitoylation sites, and a scaffolding domain.
that facilitates interaction with the PM (12). The scaffolding domain of Cav1 (amino acids 82–101) contains three cationic residues that bind to the negatively charged headgroups of PtdSer and PtdIns(4,5)P₂ via electrostatic interactions (13, 14). These in vitro studies revealed that PtdIns(4,5)P₂ is the preferred ligand on a per mole basis. However, when liposomes contained physiologically appropriate amounts of PtdIns(4,5)P₂ (1%) or PtdSer (17%), the PtdSer-containing liposomes were the preferred substrate (13). In the cellular context at first glance, the strength of the interaction between three cationic amino acids and the anionic phospholipid would rather modest. However, as caveolae have been estimated to contain ≈144 Cav1 molecules (9, 15), the number of basic residues per caveola is amplified to ≈432. Thus, the potential strength of the electrostatic interaction is considerable.

Although Cav1 is essential for the formation of caveolae, additional data have demonstrated the requirement for peripheral membrane proteins termed cavinols. The cavin family consists of four members, cav1 to cav4, that are required to stabilize the Cav1 proteins and to shape caveolae (16–19). The cavin family also have the ability to bind to PtdSer (16, 17, 20). Furthermore, a recent study demonstrated that there are 50 cavin1 molecules per caveola (15, 21). Together these observations suggest that PtdSer may play a critical role in the formation and stabilization of caveolae. Consistent with this notion, electron microscopic (EM) studies revealed an enrichment of a PtdSer-binding probe in caveolae (22, 23).

Despite these inferences, the precise role of PtdSer and PtdIns(4,5)P₂ in the assembly and stability of caveola has not been analyzed directly. In this study, we used several approaches to alter PtdSer and phosphoinositide levels of the inner leaflet of the PM, and we assessed the consequences of these manipulations on the caveolar number, size, and dynamics.

**Results**

**Characterization of Cav1-GFP membrane distribution**

To investigate the role of PtdSer and PtdIns(4,5)P₂ in caveola formation, we established a HeLa cell line stably expressing Cav1-GFP (HeLa/Cav1-GFP), which was imaged using a total internal reflection fluorescence (TIRF) microscope (Fig. 1A). Because the features resolved by light (fluorescence) microscopy cannot distinguish true caveolae from sub- or supra-caveolar clusters of Cav1-GFP, we will refer to all distinct foci of GFP fluorescence as “puncta” and to structures identified by the detection algorithms as “features.” Accordingly, Western blotting using an anti-caveolin antibody suggested that in the stable HeLa/Cav1-GFP cells, the ectopically expressed Cav1-GFP contributes only modestly to the total Cav1 pool. Moreover, the immunoblot analysis shows that the Cav1-GFP fusion appears to be quite stable, as no significant breakdown products or free GFP were detected using an anti-GFP antibody (Fig. 1B).

We observed a heterogeneous size and intensity distribution of Cav1-GFP foci that is consistent with multiple caveolin-enriched structures, such as caveolae, sub-caveolar oligomers, and rosettes (Fig. 1A, inset). To quantify this distribution, we utilized an automated spot detection algorithm that uses wave-let transforms to extract image features at multiple length scales (24, 25). As seen in Fig. 1C, the algorithm is robust at identifying Cav1-GFP puncta of varying sizes and intensities. For each detected feature, we computed an integrated intensity, defined as the product of its area and its average intensity. Assuming random incorporation of Cav1-GFP into endogenous structures, this integrated intensity is proportional to the total caveolin content of a feature. The distribution of integrated intensities spans approximately 2 orders of magnitude, reflecting the heterogeneity of the detected features (Fig. 1D).

To confirm that the ectopically expressed Cav1-GFP was incorporated into caveolae and did not perturb their formation, HeLa/Cav1-GFP cells were compared with untransfected HeLa cells immunostained with an anti-caveolin antibody (Fig. 1E). The distribution of the Cav1-GFP is virtually indistinguishable from that of immunostained endogenous Cav1 (Fig. 1F), demonstrating that the Cav1-GFP cell line is an excellent proxy to study the distribution of endogenous Cav1 in the parental cells.

**Diffusional analysis of Cav1-GFP features in untreated cells**

Previous studies have shown that caveolae are largely immobile in the plane of the membrane in unstimulated cells (5, 9, 26, 27). To confirm that our HeLa/Cav1-GFP cells behaved similarly, we used rapid time-lapse microscopy (25 acquisitions/s for a total of 20 s) and subsequently analyzed the mobility and trajectory of detected features using single-particle tracking (SPT) analysis (Fig. 1, G and H) (28). In this analysis, the type of motion and the diffusion coefficient are determined using a moment-scaling spectrum via a MATLAB algorithm (Fig. 1, I and J) (29). SPT analysis showed that the majority of the identified Cav1-GFP features displayed “sub-diffusive” motion (81.4% of 5046 particles), whereas “free” motion included a small portion of the Cav1-GFP structures during the 20 s of acquisition (Fig. 1I), consistent with previous reports (5). The sub-diffusive Cav1-GFP features also had reduced diffusion coefficients compared with the free Cav1-GFP features, with a median diffusion coefficient of $6.7 \times 10^{-3} \, \mu m^2/s$ compared with $1.7 \times 10^{-2} \, \mu m^2/s$ (Fig. 1J).

In contrast, caveolae are known to bud on and off the PM. Because SPT determinations are discontinuous, budding and reinserion into the membrane can be interpreted as increased lateral diffusion. Dynamin activity is associated with caveolae pinching off the PM (26, 27, 30, 31). To determine the impact of budding off and on the plasma membrane, we sought to inhibit dynamin. The treatment of cells with Dynogo-4aTm, a potent inhibitor of dynamin (32), illustrated that the fraction of tracks classified as sub-diffusive and free motions in cells treated with Dynogo-4aTm were very close to that of untreated, with the median diffusion coefficients of $3.0 \times 10^{-3}$ and $1.2 \times 10^{-2} \, \mu m^2/s$, respectively. This indicates that dynamin had minimal impact on the mobility of caveolae with this analysis (Fig. 1, I and J).

As a final step in quantitatively characterizing Cav1 dynamics, we down-regulated cav1, a crucial regulator of caveolar formation (16, 33). As confirmed in Fig. 1K, we could effectively deplete cav1 using siRNA. Depletion of cav1 caused a noticeable decrease in the number of Cav1 puncta (Fig. 1L). This was accompanied by a loss of the integrated spot intensity
Figure 1. Characterization of Cav1-GFP expressing HeLa cell. A, TIRF image of the HeLa cell stably expressing Cav1-GFP (HeLa/Cav1-GFP). Insets: magnifications of the indicated area by a dashed square. Scale bar, 10 µm unless stated. B, Western blot analysis of HeLa and HeLa/Cav1-GFP whole-cell lysates. Anti-caveolin antibody detected both the endogenous caveolin (22 kDa) and Cav1-GFP fusion protein (49 kDa). C, detection of caveolin in TIRF images using Spot Detector function in Icy. The raw TIRF image (left) and the detected caveolae by spot detector (center) were shown to be identical in the overlay (right). D, relative and the CFD of the integrated spot intensities in detected features of HeLa/Cav1-GFP, n = 36 cells. The shaded region in a 95% confidence band around each CFD, and the circle in the middle is the median. The same calculation for 95% confidence was applied throughout all the analyses. E, immunostaining of HeLa cells probed with the anti-caveolin antibody and imaged using TIRF. F, CFD of the integrated intensity of Cav1-GFP puncta in HeLa/Cav1-GFP cells and of endogenous Cav1 immunostained in untransfected HeLa cells, n = 27 cells. G, inverted TIRF image of HeLa/Cav1-GFP recorded for 20 s at a rate of 40 frames/s. H, representative image of the tracking for HeLa/Cav1-GFP with the tracks color-coded as follows: blue, sub-diffusive-Brownian trajectories (shown as sub-diffusive); cyan, pure-Brownian trajectories (displayed as free); red, linear trajectories (shown as directed); and yellow, unclassified trajectories (shown as unclassified, i.e. trajectories that were isotropic but too short for analysis). I, classification of Cav1-GFP features in HeLa/Cav1-GFP cells untreated (control) and treated with the dynamin inhibitor, Dyngo-4aTM (Dyngo). Cells were incubated with 30 µM Dyngo-4aTM for 30 min prior to observation. J, distribution of estimated diffusion coefficients for tracks classified as sub-diffusive and free using the MSS analysis. All above experiments were performed on 3 separate days. K, Western blot analysis demonstrating the levels of cavin1 in HeLa/Cav1-GFP cells treated with non-targeting or cavin1-targeting siRNA (top). L, TIRF images of HeLa/Cav1-GFP transfected with cavin1-targeting siRNA. Inset, magnifications of the indicated area by a dashed square. M, CFD of the product of spot size and intensity on HeLa/Cav1-GFP transfected with control-siRNA and cavin1-siRNA, n = 16 cells. N, ratio of diffuse to punctate fluorescence for HeLa/Cav1-GFP transfected with non-targeting or cavin1-targeting siRNA, n = 12 cells, mean ± S.E., *p < 0.05.
of Cav1-GFP structures as assessed by TIRF imaging and using our quantitative image analyses (Fig. 1M). Under circumstances that cause disassembly of caveolae, such as loss of cavin1 or hypotonic stress, the Cav1-GFP signal should become more diffuse in the plane of the membrane. To this end, we developed an image-processing scheme to measure the relative distribution of Cav1-GFP between the punctate features and the rest of the membrane (supplemental Fig. S1). Using this analysis, we find that in cells lacking cavin1 there is an ≈2-fold increase in the ratio of diffuse GFP to punctate GFP (Fig. 1N). Based on previous studies (16, 34) showing a loss of caveolae upon cavin1 down-regulation, we suspect that the remaining features detected by the algorithm are small clusters or oligomers of Cav1-GFP.

**PtdSer is required for the formation and stability of caveolae**

PtdSer is enriched in the PM and comprises 15–20 mol % of the inner leaflet phospholipid pool (35). To visualize the subcellular distribution of PtdSer on the cytosolic leaflet of organelles, we use the discoidin C2 domain of lactadherin (Lact-C2) fused to either GFP or mCherry (36). In this regard, we hypothesized that gross overexpression of a construct containing two Lact-C2 domains might be able to bind and sequester a significant fraction of the available PtdSer (37) in the PM and thereby serve as a tool to examine PtdSer-dependent pathways (Fig. 2A). To examine this possibility, we transiently expressed mCherry-2×Lact-C2 in HeLa cells and found that it localized to the same compartments as the monomeric Lact-C2, without obvious alterations to the cell structure (data not shown). We next confirmed that the expression of the probe did not significantly alter the presence and distribution of PtdIns(4,5)P₂ as monitored by the PH-PLCδ sensor (Fig. 2B). We conclude that any alterations in caveolar structure and function associated with the overexpression of the 2×Lact-C2 would be due to PtdSer sequestration and not significant alterations in PtdIns(4,5)P₂.

The overexpression of tandem Lact-C2 decreased the abundance of Cav1-GFP puncta visualized by TIRF microscopy (Fig. 2C). Additionally, the remaining puncta were smaller and had a reduction in the integrated fluorescence intensity (Fig. 2D), suggesting that they were clusters of Cav1-GFP. Because of the nature of these experiments, we were unable to determine whether the loss of the puncta was due to the prevention of assembly or enhanced disassembly of the structures. Superficially, the majority of the GFP signal is associated with puncta. However, analyzing the diffuse to puncta ratio revealed that a significant fraction of the GFP signal in the tandem Lact-C2-expressing cells is diffuse, with only ≈15% of the signal associated with features as characterized by the algorithm (Fig. 2E). We interpret these results to mean that a substantial amount of PtdSer is required for proper caveola formation.

As cholesterol is required for the formation of caveolae, one explanation for our results is that the overexpression of the tandem Lact-C2 depletes cholesterol from the plasma membrane. To examine this possibility, HeLa cells were transiently transfected with plasmids encoding soluble GFP, GFP-Lact-C2, or GFP-2×Lact-C2, fixed and stained with filipin to visualize the cholesterol distribution. As shown in Fig. 2F, regardless of the GFP protein expressed, the filipin readily stains the plasma membrane and a portion of the endosomes. Thus, it is unlikely that the absence of caveolae seen in the 2×Lact-C2-expressing cells is due to a depletion of plasmalemmal cholesterol. However, we cannot completely rule out a disruption in the nanoscale organization of cholesterol.
Sequestering plasmalemmal PtdSer interferes with caveolar association of cavin

Both Cav and cavin proteins bind to PtdSer in vitro, and this binding is postulated to be necessary for the function and assembly of caveolae in vivo (13, 14, 17, 20). To complement the experiments investigating the localization and dynamics of Cav1-GFP, we generated a cavin1-GFP stably expressing HeLa cells (HeLa/cavin1-GFP) to monitor cavin1 localization following perturbation of PtdSer. Again, Western blotting using an anti-cavin1 antibody indicated that the cavin1-GFP fusion protein accounted for a small fraction of the total cavin1 in HeLa/cavin1-GFP cells (Fig. 3A). Consistent with previous findings, HeLa/cavin1-GFP formed punctate structures similar to HeLa/Cav1-GFP as reported previously (16). Next, these cells were transiently transfected with mCherry or mCherry-2×Lact-C2 expressing cells compared with that of untransfected cells. n ≥ 18 cells, mean ± S.D. D, comparison of the total cellular cavin1-GFP content in mCherry and mCherry-2×Lact-C2 expressing cells. Z-stacks were used to measure the total cavin1-GFP intensity in the entire cell and divided by pixel area, which was then contrasted by that of untransfected cells. n ≥ 15 cells, mean ± S.D. n.s., not significant, **, p < 0.01; ****, p < 0.001. E, cavel1 abundance was quantified in BHK and HeLa cells transiently transfected with fluorescent probes tagged with or without 2×Lact-C2. F, trajectories of cavelin features in HeLa/Cav1-GFP cells overexpressing the 2×Lact-C2. G, classification of 4815 Cav1-GFP features in HeLa/Cav1-GFP cells overexpressing the 2×Lact-C2. H, relative frequency of the diffusion coefficients for the “sub-diffusive” and “free” motion of Cav1-GFP.

Figure 3. Sequestering phosphatidylserine depletes cavin1 levels and caveolae. A, Western blot analysis with anti-cavin1 antibody depicting the levels of cavin1 (44 kDa) and cavin1-GFP (71 kDa) in HeLa and HeLa/cavin1-GFP cells. B, confocal images of the bottom of the cells with cavin1-GFP stably expressing HeLa cells (HeLa/cavin1-GFP), which were transiently transfected with mCherry (top) or mCherry-2×Lact-C2 (bottom). Scale bar, 10 μm unless indicated. C, normalized integrated cavin1 spot intensity from B. The sum of the product of spot area and intensity for each cavin1 punctum at the cell base slice in mCherry or mCherry-2×Lact-C2-expressing cells were compared with that of untransfected cells. n ≥ 18 cells, mean ± S.D. D, comparison of the total cellular cavin1-GFP content in mCherry and mCherry-2×Lact-C2 expressing cells. Z-stacks were used to measure the total cavin1-GFP intensity in the entire cell and divided by pixel area, which was then contrasted by that of untransfected cells. n ≥ 15 cells, mean ± S.D. n.s., not significant, **, p < 0.01; ****, p < 0.001. E, cavel1 abundance was quantified in BHK and HeLa cells transiently transfected with fluorescent probes tagged with or without 2×Lact-C2. F, trajectories of cavelin features in HeLa/Cav1-GFP cells overexpressing the 2×Lact-C2. G, classification of 4815 Cav1-GFP features in HeLa/Cav1-GFP cells overexpressing the 2×Lact-C2. H, relative frequency of the diffusion coefficients for the “sub-diffusive” and “free” motion of Cav1-GFP.

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Despite the significant reduction in Cav1-GFP puncta in cells overexpressing the tandem Lact-C2, Cav1-GFP features were still detectable through TIRF. To verify that sequestration of PtdSer by the overexpression of tandem Lact-C2 precipitated a loss of bona fide caveolae, we used electron microscopy (EM). As illustrated in Fig. 3E, a decline in the density of caveolae was observed in baby hamster kidney cells (BHK) and HeLa cells upon overexpression of tandem Lact-C2. The remaining Cav1-GFP features demonstrated an increase in free motion and diffusion, with an attendant decrease in the sub-diffusive components (81.0 to 60.5%) (Fig. 3, F–H). Thus, sequestration of plasmalemmal PtdSer significantly inhibited the formation of caveolar puncta, and the remaining structures (Cav1-GFP oligomers or caveolae) were on average more mobile and less confined.
**Chronic reduction of PtdSer results in a loss of caveolae**

In mammalian cells, PtdSer is synthesized in the ER by two separate enzymes, PtdSer synthase (PSS) 1 and 2. PSS1 catalyzes PtdSer biosynthesis from phosphatidylcholine, and PSS2 uses phosphatidylethanolamine as a substrate (PtdEtn) (35). Mice deficient for both PSS1 and PSS2 are not viable (39). However, previous studies identified and isolated a mutant Chinese hamster ovary (CHO) cell line, termed PSB-2, with greatly reduced PtdSer synthase activity, resulting in an ~80% reduction in PtdSer (40). The sustained reduction of PtdSer causes phenotypic changes in the cell with the most notable being elongation (Fig. 4A). Conveniently, the levels of PtdSer can be largely restored in these cells by supplementing the medium with PtdSer (40), which is accompanied by reacquisition of a more rounded phenotype, similar to the parental CHO cells (Fig. 4A). To confirm the requirement for PtdSer in the formation of caveolae, we compared the number of caveolae in PSB-2 and parental CHO cells by EM (Fig. 4B). EM examination of the PSB-2 cells revealed that these cells had a substantially reduced caveolae density: from 14.1 per cell profile in CHO cells to 2.9 per cell profile in the PSB-2 cells (Fig. 4C). This defect could be rescued by supplementation of the growth medium with PtdSer, restoring the number of caveolae to 9.7 per cell profile (Fig. 4, B and C).

Next, we examined the distribution of cavin1 in PSB-2 cells and the contribution for the caveolae formation. CHO cells and PSB-2 cells with or without PtdSer supplementation were transiently transfected with a plasmid encoding cavin1-GFP. After 18 h of transfection, the cells were fixed, permeabilized, and immunostained for endogenous caveolin. Consistent with previous findings, cavin1-GFP and immunostained caveolin were both punctate in appearance and displayed extensive overlap in the parental CHO cells. Conversely, in PSB-2 cells, the cavin1-GFP was largely diffuse in nature, although caveolin remained punctate (Fig. 4, D and E). Intriguingly, and as anticipated, the cavin1 in PtdSer-supplemented PSB-2 cells relocalized with the caveolin puncta. Together, the results suggest that the restoration of PtdSer levels promotes not only the re-association of caveolin and cavin1 but more importantly the reassembly of caveolae.

The PSB-2 cells have not been extensively used for cellular experiments. One possibility is that the reduction in PtdSer could result in a reduction of PtdIns(4,5)P₂. To compare the distribution of PtdIns(4,5)P₂ in CHO and PSB-2 cells, we used the GFP-PH-PLCβ8 probe. As depicted the Fig. 4F, the probe decorates the plasma membrane in both the parental CHO cells and the PtdSer-depleted PSB-2 cells. Additionally, staining of the actin cytoskeleton with rhodamine-phalloidin revealed the presence of polymerized actin (not depicted) thereby confirming the presence of plasmalemmal PtdIns(4,5)P₂.

**Cav1-GFP features are more dynamic in PtdSer-depleted cells**

To determine the lateral mobility of Cav1-GFP in PSB-2 cells, we generated CHO and PSB-2 cell lines stably expressing the Cav1-GFP construct: CHO/Cav1-GFP and PSB-2/Cav1-GFP. Both cell lines showed similar levels of expression of Cav1 and Cav1-GFP by Western blotting (Fig. 5A). Again, to confirm that the generated stable transfectants behaved as parental cells, we examined the number and appearance of Cav1-GFP puncta expressed in CHO/Cav1-GFP and PSB-2/Cav1-GFP and compared it with the immunostaining caveolin I, the parental cells. As depicted in Fig. 5, B and C, the stable expression of low levels of Cav1-GFP did not impact the appearance of caveolin. We next sought to characterize the fate of Cav1-GFP in PSB-2 cells by using TIRF microscopy. The analysis revealed a reduction in the integrated spot intensity (Fig. 5D). Additionally, we find a trend toward an increase in the fraction of diffuse Cav1-GFP (Fig. 5E). As before, we analyzed the mobility of the remaining Cav1-GFP puncta in PSB-2 cells using SPT. The identified features in the PSB-2/Cav1-GFP cells show less sub-diffusive motion 65.5% compared with 80.1% in CHO/Cav1-GFP (Fig. 5, F–H) and display enhanced rates of sub-diffusion compared with the parental CHO cells (7.8 × 10⁻³ μm²/s versus 6.1 × 10⁻³ μm²/s, respectively) (Fig. 5I). Although the PSB2/Cav1-GFP cells showed more free motion of Cav1 than the CHO/Cav1-GFP, this does not appear to be a result of budding off and on the PM. As shown in Fig. 5H, inhibition of dynamin did not alter the classification of the Cav1-GFP tracks. This dynamin-independent motion seems logical because few bona fide caveolae are seen in PSB-2 cells by EM (Fig. 4B). The results suggest that Cav1 clusters or oligomers may have free, long-range motion that may, in fact, help with the reassembly of caveolae after disassembly caused by stress (i.e. membrane stretching).

**Phosphoinositide depletion increases the fraction of freely diffusible Cav1-GFP features**

The acidic nature of PtdIns(4,5)P₂, PtdIns4P, phosphatidylinositol, and PtdSer combines to generate the negatively charged surface of the inner leaflet of the PM (41). The relative contribution of each of these lipids to the surface charge is dictated by both their relative abundance and their valency (41, 42). Thus, although the abundance of PtdIns4P and PtdIns(4,5)P₂ is low, they are major contributors to the surface charge by virtue of their relatively high valence, and we sought alternative ways to alter the levels of PtdIns(4,5)P₂ and PtdIns4P acutely to evaluate their roles in caveola formation.

Pseudojanin (PJ) is a synthetic construct encoding a rapamycin-based heterodimerization domain (FKBP) and two phosphatase domains as follows: one isolated from INPP5E, a PtdIns(4,5)P₂ 5-phosphatase, and a second one from Sac, a 4-phosphatase (43). Recruitment of PJ to the PM by co-expression of the Lyn11-FRB targeting protein followed by addition of rapamycin simultaneously degrades both PtdIns4P and PtdIns(4,5)P₂ (43, 44). As a control for these experiments, a PJ-dead construct was developed in which both phosphatase domains are inactivated (43). Additionally, to better elucidate the specific roles of PtdIns4P and PtdIns(4,5)P₂, a PJ-INPP5E construct, which contains an inactivated Sac1 domain (and therefore serves only as a 5-phosphatase), and a PJ-Sac construct, which contains an inactivated INPP5E domain (and functions only as a 4-phosphatase), have also been engineered (Fig. 6A and supplemental Fig. 2A) (43). When recruited to the membrane, PJ was able to dephosphorylate PtdIns(4,5)P₂ and PtdIns4P, causing a sizable decrease in membrane surface charge (Fig. 6A and supplemental Fig. 2A). Deple-
Figure 4. Caveolae formation is impaired in PtdSer-depleted cells. A, differential interference contrast (DIC) images of CHO and PSB-2 cells. PSB-2 cells were incubated in delipidated medium supplemented with 30 μM PtdSer (middle), 30 μM PtdEtn (right), or without supplementation (left). Scale bar, 40 μm. B, representative electron micrographs of CHO and PSB-2 cells with and without PtdSer supplementation. The arrowheads indicate caveolae or cavicles, and the asterisks indicate clathrin-coated pits. Scale bar, 500 nm. C, quantitation of caveolae per cell slice examined by electron microscopy. *, p < 0.05. D, cavin1-GFP transiently expressing CHO and PSB-2 cells with or without PtdSer supplementation were immunostained for caveolin. Scale bar, 10 μm. Insets, the magnifications of the indicated area shown by a square. E, Mander’s correlation coefficient calculated caveolin signal co-localizing with the cavin1 signal, n ≥ 20 cells, mean ± S.D. ****, p < 0.0001. F, confocal images of CHO and PSB-2 cells transiently transfected with the mCherry-PH-PLCδ. Scale bar, 10 μm.
tion of the phosphoinositides did not alter the transbilayer distribution of PtdSer, as monitored by annexin-V staining (supplemental Fig. 2A). Recruitment of the construct with only an active INPP5E domain led to the rapid depletion of available PtdIns(4,5)P2, as monitored by the GFP-PH-PLC6/H9254. Similarly, the targeting of the Sac1 domain caused a rapid decrease in the available PtdIns4P, as monitored with GFP-2PH-Osh2 (45). The judicious use of these constructs, therefore, allowed us to degrade PtdIns4P and/or PtdIns(4,5)P2 acutely in a controllable fashion.

To examine the specific roles of PtdIns4P and PtdIns(4,5)P2 in the formation of caveolae, HeLa/Cav1-GFP cells were transiently transfected with PJ and Lyn11-FRB. The following day, the cells were visualized using TIRF microscopy either before or after the addition of rapamycin. The depletion of both PtdIns4P and PtdIns(4,5)P2 caused a rapid decrease in the number of Cav1-GFP puncta and a slight increase in the ratio of diffuse to puncta (Fig. 6, B and C). However, the remaining structures were unchanged as monitored by the integrated intensity of the Cav1-GFP foci (Fig. 6, B and D). In cells expressing PJ and PJ-Dead, both demonstrated a small shift to the left in the integrated spot intensity (Fig. 6D) that could be the result of photobleaching in these time-lapse video microscopy experiments. However, the depletion of the phosphoinositides resulted in a decrease in the percentage of sub-diffusive Cav1-GFP puncta from 83.2 to 57.8%, and a corresponding increase in the number of freely diffusible puncta from 8.6 to 35.0% (Fig. 6, E and F).

Through the recruitment of the PJ-INPP5E and the PJ-sac-Dead constructs, we examined the impact of acutely and separately decreasing PtdIns(4,5)P2 and PtdIns4P, respectively, on the stability and (im)mobility of caveolae. Recruitment of the specific phosphatases caused essentially no disassembly of caveolae (Fig. 6C). However, such separate recruitment led to an increase in the population of freely diffusible Cav1-GFP features, with an accompanying decrease in the sub-
diffusive component (Fig. 6F). The reduction in the fraction of sub-diffusive features and the rise in freely mobile features correlate with the charge density of the individual phospholipids, consistent with PtdIns4P and PtdIns(4,5)P₂ interactions being important for the confinement and immobilization of caveolae/Cav1-GFP puncta.

Membrane lipid scrambling alters Cav1-GFP features

The results suggest that caveolae are very sensitive to alterations in PtdSer and to a lesser extent phosphoinositides. One of the primary functions of PtdSer is to promote blood coagulation by platelets and to act as an “eat-me” signal on apoptotic cells. In both these situations and others, PtdSer is scrambled in...
the plasma membrane, which will effectively reduce the concentration of PtdSer by \( \approx 50\% \). Thus, it is conceivable that plasma membrane lipid scrambling will impact the stability of caveolae. To test this hypothesis, we made use of dibucaine, an analgesic, to scramble the lipids across the PM (46, 47). To validate the effects of dibucaine, we added Alexa-555-conjugated annexin V, which detects exofacial PtdSer (Fig. 7A). Treatment of HeLa/Cav1-GFP cells with dibucaine decreased the abundance of Cav1-GFP puncta (Fig. 7B). The puncta that remained at the PM were smaller and had a reduced integrated fluorescence intensity (Fig. 7C), meaning disassembled. Concomitant with the decrease of punctate spots, an increase in diffuse GFP signal was observed in the TIRF field in dibucaine-treated cells (Fig. 7D).

Next, we determined the increase in mobility of the Cav1-GFP features that persisted after dibucaine treatment, using TIRF microscopy and SPT. The fraction of features showing sub-diffusive Brownian motion decreased from 82.1% in control cells (Figs. 1I and 7H) to 40.6% after dibucaine treatment (Fig. 7, E and H). Conversely, Cav1-GFP foci displaying free motion increased from 13.3% in controls to 45.1% after dibucaine (Fig. 7, E and H). Furthermore, after treatment with dibucaine, the diffusion coefficient raised to a median 17.8 \( \times 10^{-3} \) \( \mu \text{m}^2/\text{s} \) for the tracks classified as sub-diffusive and to 18.1 \( \times 10^{-2} \) \( \mu \text{m}^2/\text{s} \) for freely diffusing features. The distribution of diffusion coefficients for both modes of motion shifted toward higher values in response to dibucaine treatment (Fig. 7, I and J). Thus, scrambling PtdSer

Figure 7. Dibucaine-induced scrambling alters Cav1 features. A, HeLa cells were treated with 1 mM dibucaine for 10 min resulting in PtdSer scrambling as shown by annexin-V staining. Scale bar, 10 \( \mu \text{m} \) unless indicated. B, TIRF images of HeLa/Cav1-GFP before and after dibucaine treatment. The dashed line in white indicates the cell margin. Insets, magnifications of the indicated area by a square in blue. C, CDF of integrated spot intensities in HeLa/Cav1-GFP before and after addition of dibucaine. Identical cells before and after treatment were selected for the analysis, \( n = 18 \) cells. D, ratio of diffuse to punctate fluorescence, \( n = 16 \) cells, mean \( \pm \) S.E. E–G, representative trajectories of Cav1-GFP features in cells treated with only dibucaine (Dib) (E), only Dyngo-4aTM (Dyg) (F), or both (G). Cells were incubated with 30 \( \mu \text{M} \) Dyngo-4aTM before dibucaine treatment. Scale bar, 2 \( \mu \text{m} \). H, classification of Cav1-GFP features in HeLa/Cav1-GFP cells from E to G. Please note: As a reference we have included the control experiments from Fig. 1. I and J, relative frequencies of the diffusion coefficients for the “sub-diffusive” movement, and J is “free” motion of caveolin in HeLa/Cav1-GFP. *, \( p < 0.05 \).
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from the cytosolic leaflet increased the lateral motion of caveolin.

Sequestration and depletion of PtdSer led to increased Cav1-GFP feature motion (Figs. 3, H and I, and 5, H and J). To distinguish between budding and bona fide lateral diffusion, we again used Dynago-4aTM to distinguish internalization from the planar mobility of the remaining Cav1-GFP features (Fig. 7, F–H); please note for comparison we have reposted data from Fig. 1, I (frequency of motion types) and J (frequency of diffusion coefficients). The fraction of tracks classified as sub-diffusive in cells treated with Dynogo-4aTM, dibucaine, or both averaged 82.1, 40.6, and 71.4%, with median diffusion coefficients of 3.4 \times 10^{-3}, 17.8 \times 10^{-3}, and 7.0 \times 10^{-3} \mu m^2/s, respectively (Fig. 7, H and I). Conversely, 13.3, 45.1, and 21.3% of the tracks exhibited free motion, with median diffusion coefficients of 1.2 \times 10^{-2}, 18.1 \times 10^{-2}, and 5.1 \times 10^{-2} \mu m^2/s, respectively (Fig. 7, H and J). These results indicate that a significant proportion of the Cav1-GFP features display enhanced mobility following dibucaine treatment and that the mobility is due to both the stimulation of a dynamin-dependent internalization pathway and an increase in lateral diffusion. These findings on PtdSer scrambling together with the data on PtdSer sequestration and degradation support an important role of this lipid in caveola formation. However, it should be noted that phospholipid scrambling also scrambles other lipids, including PtdEtn. Whether PtdEtn is required for caveola assembly or function is currently unknown.

Transient increase in membrane fluidity does not alter caveolae

In addition to being a potent inducer of membrane scrambling, dibucaine can also fluidize membranes (48, 49). This raises the possibility that the substantial alterations in caveolar behavior and abundance could be due to a combination of scrambling and increasing membrane fluidity. Typically, mammalian cells maintain membrane fluidity within a narrow range by a process known as homeoviscous adaptation (50). However, this adaptation to temperature changes or fatty acid supplementation occurs over the course of hours and requires extensive remodeling of the lipidome. Fortunately, it has been reported that valproic acid, a branched chain weak organic acid, can rapidly increase membrane fluidity within minutes of addition to cells (51). To monitor the changes in membrane fluidity, we used polarized TIRF microscopy to access the degree of orientational order of the lipophilic membrane dye 1,1-decyl-3,3,3'-tetrachlorinatedocarboxycyanine (DiI) to determine the order parameter \(P_2\), as described previously (52, 53). As depicted in Fig. 8A, cells were imaged at 0° (p-polarization) and 90° (s-polarization) and processed to generate the single pixel level \(P_2\) image. In control cells, the orientation of the absorption transition moment of the DiI is largely parallel to the membrane surface resulting in negative \(P_2\) values (Fig. 8B) (53, 54). Having determined the average value in control cells, we next confirmed that dibucaine and valproate increased the fluidity of the cells. Again, the DiI-containing cells were captured using the polarized TIRF microscopy before and after incubation with the drugs for 10 min. Following the incubation with dibucaine or valproate, the calculated \(P_2\) value remains negative, but the magnitude is less than that obtained in control cells. This indicates that the orientation of the absorption transition moment has changed as a result of increasing membrane fluidity. Collectively, the images and histograms demonstrate that treatment with either 1 mM dibucaine or 40 mM valproate for 10 min increased membrane fluidity (Fig. 8, C–F).

Next, we examined the impact of the addition of valproate on the Cav1-GFP features using TIRF and signal particle tracking. In contrast to the addition of dibucaine (Fig. 7B), the addition of valproate had little impact on the number or intensity of the Cav1-GFP features (Fig. 8G). Likewise, the addition of valproate did not alter the diffusive properties of Cav1-GFP features (Fig. 8, H and J) compared with the cells treated with dibucaine (Fig. 7, E, G, and H). Collectively, these results suggest that transient increases in membrane fluidity do not greatly impact the stability of non-diffusive nature of caveola.

Discussion

Phosphatidylserine is required to support the formation of caveolae

In this paper, we have examined the role of PtdSer and PtdIns(4,5)P2 in the formation and assembly of caveolae. PtdSer is abundant in the inner leaflet of PM and enriched in caveola (23), and now using a variety of techniques, we have shown that PtdSer is required for their assembly. Cells with reduced availability of PtdSer, via the overexpression of the tandem Lact-C2, showed a reduction in the number of caveolae observed by EM as well as a decrease in the intensity of Cav1 puncta detected by TIRF microscopy. Intriguingly, the silencing of cavin1 results in a similar pattern of Cav1 redistribution in the PM. This result is consistent with cavin1 being the PtdSer-sensitive component needed for proper function or stabilization. The polybasic sites of cavin1, HR1 and HR2 domains, were proposed to bind anionic phospholipids, including PtdSer through charge-based interactions (16, 17, 20, 55). In support of this notion, we find that chronic depletion of the majority (~80%) of PtdSer limits the ability of cavin1 to associate with Cav1 and the PM. Additionally, sequestering the plasmalemmal PtdSer using transient overexpression of the tandem Lact-C2 also displaced cavin1 from the PM and ultimately caused the disappearance of GFP-cavin1. These results are consistent with a previous study that demonstrated that upon release from the PM, cavin1 can be ubiquitinated on its HR1 domain leading to rapid proteasomal degradation (38). Together, our results show that PtdSer is required for Cav1 and cavin1 to form stable complexes in vivo, thereby explaining the enrichment of PtdSer in caveola (23, 56).

Non-caveolar Cav1-GFP clusters are mobile

Sequestering or limiting PtdSer prevents the assembly of caveola but does not cause the loss of Cav1. Instead, PtdSer limitation generates Cav1-GFP clusters capable of diffusing in the plasma membrane. Although PtdSer-limiting manipulations are somewhat extreme, other physiological conditions do occur that result in the presence of non-caveolar Cav1. Cav1 is delivered to the PM via vesicular transport with Cav1 organized into oligomers of 12–14 monomers (6). Upon delivery to the plasma membrane, individual clusters will have to diffuse and collide with other clusters to form the larger caveola.
Growing evidence suggests that non-caveolar Cav1 may also have biological significance. For instance, select prostate cancers contain non-caveolar Cav1 due to the absence of cavin1 (16, 57). Non-caveolar Cav1 has been described to impact a variety of traits necessary for the progression of cancer. In prostate cancer cells, Cav1 influences JAK/STAT and Src signaling with high levels of Cav1 correlating with worse outcomes (58). Importantly, in these cells exposure to radiation or the chemotherapeutic agents, gemcitabine and 5-fluorouracil, results in enhanced levels of Cav1 (58). This up-regulation could be quite problematic as Cav1 is thought to promote the dissemination of cancer via metastatic migration through the lymphatic system and to protect cells from apoptosis (59). Although a previous study has demonstrated that caveolae can provide resistance to apoptosis in androgen-independent prostate cancer cells (60), the biological signals associated with non-caveolar Cav1 are incompletely understood. In this regard, non-caveolar Cav1 has been shown to influence the expression of important mediators of metastasis and lymphangiogenesis such as matrix metalloprotease 9 and vascular endothelial growth factor, respectively.

**Figure 8. Rapid increase in membrane fluidity does not alter caveolae.** A, HeLa cells containing the fluorescence probe DiI was excited by parallel (s) and perpendicular (p) polarized light, relative to the substrate surface using polarized TIRF microscopy. The inset and B correspond to the order parameter, \(<P_p^2>\). Cells loaded with DiI and treated with dibucaine (C and D) or valproate (E and F) were also imaged, and their relative order parameter was calculated. Scale bar, 3 mm.
G, TIRF images of HeLa/Cav1-GFP following a 10-min incubation with valproate. H, representative trajectories of Cav1-GFP features in cells treated with valproate; I, the classification of the tracks.
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(59, 61). Thus, it is tempting to speculate that improved understanding of the biophysical properties of non-caveolar Cav1 will help understand its role in cancer metastasis.

Anionic lipids in caveola formation and immobilization in the PM

Examination of the role of the plasmalemmal phosphoinositides PtdIns4P and PtdIns(4,5)P2 in caveola formation required more acute depletion of these lipids. Thus, we used the controllable enzymatic degradation of the phosphoinositides rather than the inhibition of their synthesis. The acute depletion of PtdIns(4,5)P2 had minimal impact on the total number of observable caveolae. Because of the robustness of this detection algorithm to identify puncta in the TIRF images, depletion of phosphoinositides had minimal impact on the number of puncta. However, integrated spot intensity for the population of foci was only minimally decreased after the exhaustion of PtdIns4P and PtdIns(4,5)P2 and to a slightly lesser extent PtdIns(4,5)P2 alone. Consistent with this finding, rapid depletion of phosphoinositides cause only a minor increase in the fraction of diffuse Cav1-GFP. The depletion of PtdIns(4,5)P2 and PtdIns4P did lead to changes in the percent of sub-diffusive versus freely diffusible caveolae. It is possible that this observation is related more to the impact of phosphoinositide depletion of actin dynamics. Furthermore, although the requirement for PtdIns(4,5)P2 in caveola formation is less obvious than PtdIns4P and PtdIns(4,5)P2 and to a slightly lesser extent PtdIns(4,5)P2 alone, consistent with this finding, rapid depletion of phosphoinositides cause only a minor increase in the fraction of diffuse Cav1-GFP. The depletion of PtdIns(4,5)P2 and PtdIns4P did lead to changes in the percent of sub-diffusive versus freely diffusible caveolae. It is possible that this observation is related more to the impact of phosphoinositide depletion of actin dynamics. Furthermore, although the requirement for PtdIns(4,5)P2 in caveola formation is less obvious than PtdIns4P and the caveolar pool of PtdIns(4,5)P2 is not readily exchangeable with the rest of the plasma membrane.

In summary, through the use of a variety of techniques to manipulate the levels or availability of plasmalemmal PtdSer, we have dissected the role for the lipid in supporting the formation of caveola and Cav1-cavin1 association. PtdSer appears to be essential to support caveola formation, although Cav1 molecules still cluster when it is greatly diminished.

Experimental procedures

Reagents and solutions

Rabbit polyclonal antibodies to caveolin (catalog no. 610060) were purchased from BD Biosciences, and rabbit polyclonal antibodies to cavin1 (ab48824) were from Abcam. Goat polyclonal anti-GFP antibodies were purchased from Rockland Inc. (catalog no. 600-101-215). Mouse monoclonal anti-GAPDH antibodies were obtained from Millipore (MAB374). Cy3-conjugated IgG for a secondary antibody was obtained from Jackson ImmunoResearch Laboratories. Enhanced chemiluminescence reagents were obtained from GE Healthcare, and BCA protein assay reagent was from Pierce. Fluorescently conjugated annexin-V and puromycin were purchased from Thermo Fisher Scientific. Dyngo-4a™ was also acquired from Abcam. Oligonucleotides for PCR and siRNA-mediated gene silencing were purchased from Integrated DNA Technologies and Dharmacon, respectively. All other reagents were from Sigma unless stated.

Plasmids

Construction of the plasmids encoding Lact-C2 (36), PH-PLCδ (62), and EGFP-PTRF (cavin1) (16) has been described previously.
siRNA (final 20 nM) mixed with 8 µl of HiPerFect was incubated at room temperature for 15 min. Cells expressing PJ and its related constructs, PJ-JNPP5E, PJ-sac, and PJ-dead, were treated for 5 min with 1 µM rapamycin before analysis. Unless otherwise indicated, all treatments and assays were at 37 °C.

**Western blotting**

Cells were lysed in RIPA buffer, 150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 µM leupeptin, and 20 mM Tris, pH 7.4, supplemented with protease inhibitor, Complete (Roche Applied Science). Protein was quantified with the BCA Protein Assay Reagent Kit, using bovine serum albumin as a standard. The lysate was denatured with Laemmli Buffer and separated by electrophoresis on a 10% polyacrylamide gel. Proteins were transferred to a PVDF membrane and the blots probed with the anti-caveolin, anti-GFP, anti-GAPDH, or anti-cavin1 antibodies as indicated. The proteins were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL system (GE Healthcare).

**Immunostaining**

Cells were fixed with 4% paraformaldehyde in PBS, permeabilized–blocked with 0.1% Triton X-100 and 5% skim milk in PBS, and incubated with the anti-caveolin antibody diluted 1:2000 in 5% skim milk containing PBS, for 60 min. After extensive rinsing with PBS, cells were incubated for 1 h with Cy3-coupled donkey anti-rabbit antibodies in PBS containing 5% skim milk.

**Microscopy**

In the acquisition of all images or movies, coverslips with cells were transferred to an 18-mm magnetic chamber (Chamlide CMB, Live Cell Instrument); medium was replaced with a synthetic medium as indicated (see under “Reagents and solutions”), and the chamber was placed on a microscope stage maintained at 37 °C. For acquiring differential interference contrast microscopy, the cells were imaged using a Leica DM IRB microscope, and images were captured by a cooled charge-coupled device (CCD) camera (Cascade II; Photometrics) using MetaMorph software (MDS Analytical Technologies). Fluorescence images were acquired by spinning-disc confocal systems (MetaMorph software (MDS Analytical Technologies). Fluorescence microscopy was carried out on a custom-built combinatorial microscopy system. The rig is built around an Olympus IX70 inverted microscope with four laser lines and two Evolve 512 EM-CCD cameras (Photometrics, Tucson). The system is capable of performing polarized and traditional TIRF microscopy, atomic force microscopy, confocal microscopy, and direct stochastic optical reconstruction microscopy. The images were captured with a ×60 1.45 NA oil-immersion TIRF objective (Olympus Canada). The fluorescence probe DilC18(3) was excited by parallel ($\parallel$) and perpendicular ($\perp$) polarized light, relative to the substrate surface, through a half-wave liquid crystal variable retarder LCC25 1111A (Thorlabs, Newton, NJ). Both the EMCCD cameras and liquid crystal variable retarder were controlled by Micro-Manager. Cells were imaged before and after a 10-min treatment with dibucaine or valproate, respectively. The ($P_x$) values were calculated using an ImageJ macro that considers the evanescent electric field vector amplitude, excitation incident angle, and fluorescence detected dichroic rotation, see Ref. 53 for further details.

**Electron microscopy**

Cells were rinsed briefly in 100 mM cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, PA), and then fixed for 1 h in primary fixative 100 mM cacodylate buffer, pH 7.4, containing 1 mg/ml ruthenium red (Calbiochem) and 2.5% glutaraldehyde at room temperature. Next, cells were washed with 100 mM cacodylate buffer and stained with 100 mM cacodylate buffer, pH 7.4, containing 1% osmium tetroxide and 1 mg/ml ruthenium red for 3 h at room temperature. After that, cells were washed with 100 mM cacodylate buffer, pH 7.4, and rinsed with double-distilled water. Cells were observed in a transmission electron microscope (JEOL 1011; JEOL Ltd.), and electron micrographs were taken with a digital camera (Morada; Olympus) using AnalySIS software (Olympus).

**Post-acquisition analysis**

Quantifying punctate versus diffuse fluorescence—To restrict our analysis to a region of interest, we first constructed a membrane mask, algorithm for DualView images (Cav1-GFP + membrane label). For these images, we first split the image into the two channels (supplemental Fig. 1A), averaged the membrane channel images, and performed the following operations on the averaged image: 1) threshold to a binary image; 2) apply morphological closing with a disk-structuring element; 3) fill any holes to create a membrane mask (supplemental Fig. 1B). The membrane mask was combined with the spot detection output to generate a mask for punctate Cav1-GFP (supplemental Fig. 1C), and with the inverse of the spot detector output to generate a mask for diffuse Cav1-GFP (supplemental Fig. 1D). Applying these masks to the Cav1-GFP generated corresponding images of the membrane distribution of Cav1-GFP in either
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development and function.

The motion of individual particles was defined by SPT. For analysis, raw images were cropped in ImageJ (National Institutes of Health, Bethesda) (Fig. 1G), and tracking was performed with MATLAB (MathWorks, Natick, MA), detecting local intensity maxima and then fitting two-dimensional Gaussian kernels approximating the point-spread function of the microscope as described previously (28). The detected particles throughout a time-lapse image sequence were first linked between consecutive frames, and the resulting track segments were then linked to generate complete trajectories by closing gaps and capturing merging and splitting events (Fig. 1H).

Diffusion analysis—Motion types and diffusion coefficients were also derived with MATLAB using a moment scaling spectrum (MSS) analysis (64). The motion type (sub-diffusive, free, or directed) was determined by the value of the slope of the MSS (29).

Author contributions—G. D. F. conceived the study, coordinated the experiments, and wrote the paper. T. H., Y. Y., C. F., and A. W. performed experiments and analyzed data. R. D. generated Matlab code, analyzed data, and generated figures. J. G. K. provided new materials used in the manuscript. S. G., C. M. Y., and R. G. P. helped to edit the paper and provided input into the experiments. All authors reviewed the results and approved the final version of the manuscript.

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