A synergy between mechanosensitive calcium- and membrane-binding mediates tension-sensing by C2-like domains

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When nuclear membranes are stretched, the peripheral membrane enzyme cytosolic phospholipase A2 (cPLA2) binds via its calcium-dependent C2 domain (cPLA2-C2) and initiates bioactive lipid signaling and tissue inflammation. More than 150 C2-like domains are encoded in vertebrate genomes. How many of them are mechanosensors and quantitative relationships between tension and membrane recruitment remain unexplored, leaving a knowledge gap in the mechanotransduction field. In this study, we imaged the mechanosensitive adsorption of cPLA2 and its C2 domain to nuclear membranes and artificial lipid bilayers, comparing it to related C2-like motifs. Stretch increased the Ca2+-sensitivity of all tested domains, promoting half-maximal binding of cPLA2 at cytoplasmic resting-Ca2+ concentrations. cPLA2-C2 bound up to 50 times tighter to stretched than to unstretched membranes. Our data suggest that a synergy of mechanosensitive Ca2+ interactions and deep, hydrophobic membrane insertion enables cPLA2-C2 to detect stretched membranes with antibody-like affinity, providing a quantitative basis for understanding mechanotransduction by C2-like domains.

Significance

Eicosanoids are powerful bioactive lipids generated by the enzymatic oxidation of the polyunsaturated fatty acid arachidonic acid (AA). Among other functions (1), eicosanoids participate in mechanotransduction, for example, during bone load sensing in mammals and osmotic wound detection in zebrafish (2, 3). The rate-limiting step of eicosanoid synthesis is the cleavage of AA from nuclear and endoplasmic reticulum (ER) membranes by the inflammatory lipid hydrolase cPLA2. Recently, this enzyme emerged as a nuclear membrane (NM) mechanosensor that detects cell swelling and confinement (2, 4–6). Together with Ca2+, NM-stretch promotes the bilayer adsorption of cPLA2 via its C2-domain, which allows the enzyme to hydrolyze AA from phospholipids. Lipoxigenases (ALOX5, ALOX12, etc.), cyclooxygenases (PTGS1 and PTGS2), and other enzymes convert AA into the eicosanoids, which include the leukotrienes, prostaglandins, and other bioactive lipids. There are more than 150 vertebrate proteins with C2-like domains, which include both classic C2 domains, such as those in protein kinase C (PKC) and cPLA2, as well as the structurally related PLAT (Polycystin-1, Lipoxigenase, Alpha-Toxin) domain as in ALOX5 and ALOX12. Classic C2 domains form a β-sandwich composed of eight β-strands, whereas PLAT domains form a β-sandwich composed of two β-sheets and four β-strands. The mechanosensitivity of these Ca2+-dependent membrane-interaction motifs has not been systematically investigated. Therefore, it is currently difficult to predict which C2-like domains act as membrane tension sensors and what the distinguishing features of mechanosensitive C2-like domains are. To gain quantitative insights into this, we decided to focus on a representative group of two classical C2-domains and two PLAT domains, namely, drcPLA2-C2 and hs cPLA2-C2 (Danio rerio and Homo sapiens), hs PKCγ-C2 (H. sapiens), hr Alox5a-PLAT (D. rerio), and dr Alox12-PLAT (D. rerio).

cPLA2-C2’s calcium-binding loops (CBRs) contain Ca2+-binding residues and two clusters of exposed hydrophobic amino acids that promote membrane-binding and penetration. The Ca2+ ions neutralize negative surface charges of lipid headgroups, whereas CBR1 and CBR3 deeply insert into the lipid bilayer and interact with lipid hydrocarbons (7–11). Like cPLA2-C2, ALOX5-PLAT binds to zwitterionic membranes via hydrophobic membrane insertion and electrostatic interactions (11, 12), whereas the binding mechanism of ALOX12-PLAT is less clear. PKCγ-C2 binds phosphatidylserine (PS)-rich membranes primarily via electrostatic interactions (13). Electron paramagnetic resonance and molecular dynamics studies of the related PKCζ-C2 domain underline that compared to cPLA2-C2, PKCs interact more shallowly with membranes (7, 14, 15).

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Here, we purified the fluorescent protein fusions of these domains from bacteria and analyzed their adsorption to stretched and unstretched artificial bilayers at different Ca\(^{2+}\)-and C2-like domain concentrations. As an artificial membrane system, we chose giant unilamellar vesicles (GUVs) generated by a nylon net hydration technique. GUV bilayers are basically flat and thus represent much better the topology of large cell organelles (e.g., the nucleus) than the highly curved, small unilamellar vesicles (SU\(\text{Vs}, <100\) nm) often used for spectroscopic measurements of membrane–protein interactions. Osmotic swelling enabled the confocal imaging of stretch-dependent domain adsorption to many GUVs in parallel (i.e., under the same conditions), which is hard to achieve via one-by-one stretching methods (e.g., micropipette). The resultant robustness of our assay allowed us to define distinguishing features of mechanosensitive C2-like domains.

**Results**

**Membrane Tension Increases the Ca\(^{2+}\) Sensitivity of C2-Like Domains.** GUVs were produced either by drying lipid films on nylon nets and hydrating them with a 0.5 M sucrose solution (SI Appendix, Fig. S1A) or by standard electroformation (16). To this end, we used binary lipid mixtures of zwitterionic dipalmitoyl-phosphatidylcholine (DPPC) with or without 16% or 26% brain-derived PS or mixtures of dioleoyl-phosphatidylcholine (DOPC) with or without 18% conical dioleoyl glycerol (DOG) to vary bilayer-charge and -packing density through the inclusion of anionic and differently shaped lipids. Our nylon net method markedly increased the homogeneity and yield of unilamellar vesicles (median radius \(~7\) \(\mu\)m) compared to a standard gentle hydration protocol (SI Appendix, Fig. S1 B and C). We suppose that the advantageous wetting properties of nylon nets support a particularly thin lipid film (i.e., low thickness) thereby increasing the likelihood of unilamellar structures during hydration.

To osmotically stretch multiple GUVs at the same time, we exposed them to a hypotonic buffer, and measured domain adsorption by confocal fluorescence microscopy followed by automated image recognition and processing (Methods). After all visible surface invaginations had disappeared and the GUVs had reached maximal circularity, we observed domain-binding (Fig. L4 and Movie S1). As expected, mutation of Ca\(^{2+}\)-binding residues completely abrogated the membrane interactions of the cPla2-C2-eGFP fusion protein (SI Appendix, Fig. S2 C and D).

Eventually, the GUVs ruptured, which caused a sudden decrease in light refraction due to sucrose leakage, a decrease in vesicle diameter, and a loss of domain fluorescence from the GUV rim (Fig. 1 B and C, SI Appendix, Figs. S3 and S4A, and Movies S2, S3, and S4). To confirm the direct correlation of osmotic swelling and membrane tension in our GUV assay, we used the fluorescent lipid tension reporter Flipper-TR (17) that measures membrane tension by changes in fluorescence lifetime. GUVs in hypotonic buffer exhibited increased Flipper-TR fluorescence lifetimes compared to isotonic buffer, consistent with their expected, higher-membrane tension (SI Appendix, Fig. S4B). GUV rupture led to a sudden drop of Flipper-TR fluorescence lifetime indicative of membrane relaxation (SI Appendix, Fig. S4C).

Without any GUV stretch (\(\Delta\text{MOSM} = 0\) mOsm), Ca\(^{2+}\)-dependent domain adsorption was often too low for reliable curve fitting. As a result, we quantitatively compared the [Ca\(^{2+}\)]-versus-adsorption isotherms of weakly (\(\Delta\text{MOSM} = 100\) mOsm; Fig. 1D and SI Appendix, Fig. S5, dashed lines) and strongly stretched GUVs (\(\Delta\text{MOSM} = 240\) mOsm; Fig. 1D and SI Appendix, Fig. S5, solid lines) by fitting a Hill equation. GUV tension lowered the Ca\(^{2+}\) concentration required for half-maximal domain-binding ([(Ca\(^{2+}\)]\(_{50}\)). We conclude that mechanosensitive Ca\(^{2+}\) interactions are a common feature of C2-like domains. In part, they may result from the coupled-binding equilibria of domain-Ca\(^{2+}\)- and domain-membrane interactions. Namely, the increase in membrane affinity (that occurs when stretching lowers the thermodynamic cost of membrane insertion) should trigger a simultaneous increase in the Ca\(^{2+}\) affinity of the domain–membrane complex. In addition, membrane stretch may facilitate Ca\(^{2+}\) adsorption to lipid bilayers (18), as previously observed for membrane curvature (19).

**Tension Increases the Membrane Affinity of Some C2-Like Motifs.** To determine the effect of stretch on the maximal adsorption and apparent membrane affinity of C2-like domains, we varied domain concentration at low (i.e., [Ca\(^{2+}\)] = 20 \(\mu\)M; Fig. 1 E and F, open bars; SI Appendix, Fig. S6) or high-Ca\(^{2+}\) concentrations (i.e., [Ca\(^{2+}\)] = 200 to 500 \(\mu\)M; Fig. 1 E and F; gray bars; SI Appendix, Fig. S6) in the absence (\(\Delta\text{MOSM} = 0\) mOsm, Fig. 1 E and F and SI Appendix, Fig. S6, dashed lines) or presence (\(\Delta\text{MOSM} = 240\) mOsm, Fig. 1 E and F and SI Appendix, Fig. S6, solid lines) of membrane stretch. These two [Ca\(^{2+}\)] regimes were picked to roughly match the [Ca\(^{2+}\)]\(_{50}\) of the domains. For a better quantitative comparison, we fitted the adsorption versus [Domain] curves with a Langmuir equation. Osmotic stretch and conical lipids increased the membrane adsorption capacity \(B_{\text{max}}\) for all tested C2-like domains (SI Appendix, Fig. S6B, Upper), except for hs cPLA\(_{2}\)-C2 in which the uncertainty of \(B_{\text{max}}\) under isotonic, unstretched conditions was too large to allow this conclusion. C2-like domains partially insert into the membrane, regardless of whether they hydrophobically interact with its acyl-chain core, such as cPLA\(_{2}\)-C2 or ALOX5-PLAT, or merely shallowly attach via Ca\(^{2+}\) and electrostatics, such as PKC\(_{\gamma}\)-C2. Pulling lipid headgroups apart by stretch makes space for partial insertion, which increases the number of potential adsorption sites and with it, \(B_{\text{max}}\). Accordingly, incorporation of lipids to loosen lipid packing because of their small headgroups or kinked fatty acid side chains (DOG or DOPC) raised \(B_{\text{max}}\) even in the absence of membrane tension (SI Appendix, Fig. S6E; compare adsorption at \(\Delta\text{MOSM} = 0\) mOsm between DPPC, DOPC, and DOPC/DOPC vesicles).

Interestingly, stretch differentially affected the apparent domain affinity (K\(_{d}'\)) to these adsorption sites: With Ca\(^{2+}\) provided in excess (200 to 500 \(\mu\)M), stretch tightened membrane-binding only for cPla2-C2 and Alox5a-PLAT, whereas it did not affect or even weakened binding for Alox12-PLAT and PKC\(_{\gamma}\)-C2, respectively (Fig. 1F). Importantly, this selective mechanosensitive effect correlates with the ability of these domains to hydrophobically interact with the membrane acyl-core: cPLA\(_{2}\)-C2, whose bilayer interactions are dominated by hydrophobic insertion into the membrane, tightened its membrane-binding most drastically upon stretch. In line with the conservation of critical hydrophobic residues in the CBRs of \(d\) and hs cPLA\(_{2}\)-C2 (SI Appendix, Fig. S2 A and B), the membrane affinities of both domains were very sensitive to membrane stretch (SI Appendix, Fig. S6B, Bottom). By contrast, PKC\(_{\gamma}\)-C2, whose membrane association is dominated by electrostatics, showed the opposite effect. A “C2-unlike” PH domain (AKT1) showed no considerable mechanosensitivity to osmotic membrane stretch or rupture (SI Appendix, Fig. S7). Hence, stretch-sensing does not seem to be a universal feature of C2-like and other membrane-binding motifs.

The stretch-induced affinity changes depended on lipid composition: On DPPC/PS and DOPC GUVs, stretch strengthened the membrane interactions of cPla2-C2 by up to 50 times. For DOPC/DOG and DPPC GUVs, much-lower affinity changes were observed (approximately two to four times). To directly test the role of hydrophobic interactions for cPla2-C2’s mechanosensitivity, we mutated two pairs of conserved, hydrophobic
Fig. 1. Probing the mechanosensitivity of C2-like domains with GUVs. (A) Representative time-lapse montage of drcPla2-C2-eGFP adsorption to GUVs after transferring GUVs (DPPC+16% Brain PS) from hypertonic (ΔM<sub>Osm</sub> = 400 mOsm) to hypotonic (ΔM<sub>Osm</sub> = 240 mOsm) solution. (Movie S1) (B) Representative time-lapse montage of drcPla2-C2-eGFP desorption from a ruptured GUV (DPPC+16% Brain PS). TMR PC, TopFluor TMR labeled phosphatidylcholine. (Scale bars, 10 μm.) (Movie S2). (C) Quantification of the experiment depicted in B. (D) Ca<sup>2+</sup> concentrations required for half-maximal equilibrium binding of the indicated domains to GUVs exposed to low (dashed lines) or high (solid lines) osmotic stretch (SI Appendix, Fig. S5). (E and F) Apparent membrane-binding affinity (K<sub>d'</sub>) on unstretched (dashed lines) and stretched (solid lines) GUVs at low [Ca<sup>2+</sup>] (20 μM) (E) or high [Ca<sup>2+</sup>] (200 to 500 μM) (F) (SI Appendix, Fig. S6). Error bars, 95% CI of Hill or Langmuir equation-fitting through isotherm data consisting of n > 300 individual GUV measurements across nine different Ca<sup>2+</sup> (Hill fit) or domain concentrations (Langmuir fit). GUV lipid composition: 74% DPPC + 26% Brain PS for PKCγ-C2 and 84% DPPC + 16% Brain PS for all other domains. For sample sizes, refer to Dataset S2A.
residues known to participate in membrane penetration. Indeed, both L32A/L33A and Y90A/V91A double mutants showed severely reduced stretch-sensing, whereas the corresponding conservative L32F/L33F and Y90F/V91F double mutants did not (Fig. 1E and SI Appendix, Fig. S6).

Tension and Curvature Sensors Recognize the Same Membrane Sites. The concept that hydrophobic protein insertion measures internal membrane stresses has been mostly studied in the context of membrane curvature-sensing by amphiphatic lipid-packing sensor (ALPS) motifs (20–24). ARFGAP1, a regulator of vesicle-trafficking, contains tandem ALPS motifs (ARFGAP1-ALPS, SI Appendix, Fig. S1D). If curvature- and stretch-sensing occurs through the same structural membrane intermediates, ARFGAP1-ALPS should be a tension sensor and compete with cPLA2-C2 for the same adsorption sites. Indeed, ARFGAP1-ALPS showed preferential adsorption to stretched and desorption from ruptured GUVs (SI Appendix, Fig. S8 and Movie S5). Furthermore, prebound cPLA2-C2 blocked ARFGAP1-ALPS adsorption to GUVs (Fig. 2A and Movie S6). Prebound ARFGAP1-ALPS was displaced from stretched GUVs only in the presence but not absence of cPLA2-C2 (Fig. 2B and SI Appendix, Fig. S8C and Movies S7 and S8). When prebound cPLA2-C2 desorbed due to Ca²⁺ removal, ARFGAP1-ALPS started to bind (Fig. 2C and Movie S9). Altogether, these data argue that stretch and curvature sensors compete for the same adsorption sites. Indeed, cPLA2-C2 displaces the curvature sensor ARFGAP1-ALPS from stretched GUVs (Fig. 2A).

NM-Tension Promotes cPLA₂ Adsorption at Resting [Ca²⁺]. To test whether the insights from our reconstitution experiments hold up in a more physiologically complex situation, we sought to determine the binding kinetics of full-length cPLA₂ to stretched and unstretched, intact nuclei. Whereas it is hard to vary cellular protein concentrations precisely enough to generate reliable Langmuir isotherms, adjusting Ca²⁺ concentrations together with NM-stretch is feasible. To this end, we took advantage of a previously established experimental setup (4): Using low-dose digitonin, we permeabilized the plasma membrane of A549 and HeLa cells (4) expressing fluorescent zebrafish cPLA₂ (cPLA₂-mKate2), leaving the cell nuclei visibly intact. [Ca²⁺] and NM-stretch were adjusted by varying the concentrations of calcium chloride and high–molecular weight polyvinylpyrrolidone (PVP) in the cell lysis buffer (Fig. 3A). Lowering extranuclear [PVP] caused colloid osmotic swelling of the nucleus and cPLA₂ adsorption to the inner NM as a function of osmotic stretch (Fig. 3B). Intriguingly, membrane tension diminished the Ca²⁺ concentration required for half-maximal adsorption from [Ca²⁺]₁/₂ ≈1.6 μM (low NM-stretch) to [Ca²⁺]₁/₂ ≈60 nM (high NM-stretch) (Fig. 3C). In HeLa cells, Ca²⁺-sensitivity increased from [Ca²⁺]₁/₂ ≈250 nM (low NM-stretch) to ≈90 nM (high NM-stretch) (SI Appendix, Fig. S9). The different baseline-binding of cPLA₂ to the osmotically unstretched inner NM of A549 and HeLa cells might reflect different conical lipid concentrations or different baseline NM-tension in these cells. Considering the different assay conditions, these nanomolar [Ca²⁺]₁/₂ values agree remarkably well with the available literature: The [Ca²⁺]₁/₂ required for half-maximal activity of Ca²⁺-dependent PLA₂ (i.e., likely cPLA₂) in the sonicated particulate fraction of homogenized macrophages ranges around 100 to 200 nM (27). This is not too far off from the 60 to 90 nM measured by us for cPLA₂ adsorption to stretched NMs. By contrast, the [Ca²⁺]₁/₂ values for the recruitment of recombinant cPLA₂-C2 to stretched GUVs ([Ca²⁺]₁/₂ ≈1.2 to 1.7 μM; Fig. 1D and Dataset S1) or unstretched SUVs ([Ca²⁺]₁/₂ =3.3 ± 0.4 μM) (28) are substantially higher.

**Fig. 2.** cPLA₂-C2 displaces the curvature sensor ARFGAP1-ALPS from stretched GUVs. (A, Top) Representative time-lapse montage of domain–membrane interactions after transferring GUVs with prebound cPLA₂-C2-eGFP to hypotonic solution ([ΔmOsm] = 240 mOsm) supplemented with hsARFGAP1-ALPS-mCherry (Movie S5). (Bottom) Quantification of normalized mean GUV rim fluorescence of ARFGAP1-ALPS-mCherry (n = 55 GUVs) and cPLA₂-C2-eGFP (n = 29 GUVs). [Ca²⁺] = 20 μM. (B, Top) Representative time-lapse montage of domain–membrane interactions after transferring GUVs with prebound ARFGAP1-ALPS-mCherry to hypotonic solution ([ΔmOsm] = 240 mOsm) supplemented with cPLA₂-C2-eGFP. Note, ARFGAP1-ALPS-mCherry desorption was not observed in the absence of cPLA₂-C2-eGFP (SI Appendix, Fig. S8C and Movies S7 and S8). (Bottom) Quantification of mean normalized GUV rim fluorescence of ARFGAP1-ALPS-mCherry (n = 18 GUVs) and cPLA₂-C2-eGFP (n = 22 GUVs). [Ca²⁺] = 20 μM. (C, Top) Representative time-lapse montage of domain–membrane interactions after transferring GUVs with prebound cPLA₂-C2-eGFP to Ca²⁺-depleted solution ([ΔmOsm] = 240 mOsm) supplemented with ARFGAP1-ALPS-mCherry (Movie S9). (Bottom) Quantification of mean fluorescence of ARFGAP1-ALPS-mCherry (n = 14 GUVs) and cPLA₂-C2-eGFP (n = 22 GUVs) on GUV membranes. [Ca²⁺] = 20 to 0 μM. (All subfigures: Scale bars, 10 μm.) Shaded error, SD. GUV lipid composition: 100% DPPC. For sample sizes, refer to Dataset S2B.
higher. In part, these discrepancies might reflect the physicochemical differences between artificial membrane systems and intact NMs as discussed below. Besides, full-length cPLA$_2$ may adsorb tighter to bilayers than its isolated C2-domain owing to additional membrane contacts of its catalytic domain. Since cytoplasmic resting [Ca$^{2+}$] is estimated to be $\sim$100 nM, our intact nuclei experiments raise the possibility that very strong nuclear stretch, in principle, can replace signal-induced Ca$^{2+}$ transients as cPLA$_2$ activation signal. Under less-extreme conditions, cPLA$_2$ may act as a “coincidence detector” or “AND-gate” for NM-stretch and Ca$^{2+}$ transients. These experiments provide proof of principle that the mechanosensitive features of C2-like domains revealed in our GUV assay likely apply to tension-sensing of full-length proteins on intact cell membranes.

**Discussion**

Judging from our representative set of membrane-binding domains, we propose that cPLA$_2$-C2 owes its exceptional and conserved stretch-sensitivity to a synergy of at least two mechanosensitive features (SI Appendix, Fig. S10). First, stretch increases its Ca$^{2+}$ sensitivity and that of the other tested C2-like domains. Second, stretch permits the insertion of cPLA$_2$-C2 into the hydrophobic membrane core. This distinguishing feature may be restricted to a subgroup of C2-like domains with pronounced, hydrophobic interaction features (i.e., protruding hydrophobic loops or residues), which may have evolved to increase their dynamic mechanosensing range.

We suspect that any membrane-binding motif, naturally occurring or engineered, that exhibits the outlined synergy will be sensitive to membrane tension. Whether membrane insertion and Ca$^{2+}$-binding must occur within the same domain (as in C2-like domains) or whether interdomain cooperation of these features can also establish mechanosensitivity stands to be seen. Likewise, an intriguing question remains as to whether other chemical ligands in the membrane can substitute for Ca$^{2+}$ in this synergy.

Although our data suggest that cPLA$_2$, in principle, can bind to stretched membranes in the absence of Ca$^{2+}$ signals, it is unclear whether strong nuclear deformation ever occurs

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Fig. 3. Membrane tension amplifies cPla2 adsorption to intact NM in A549 cells. (A) Experimental scheme. Digitonin was used to permeabilize the plasma membrane. Extranuclear colloid osmotic pressure was adjusted with 360 kDa PVP to control nuclear swelling tension at defined Ca$^{2+}$ concentrations. (B) Top) Representative images of cPla2 located either in the nucleoplasm or bound to the inner NM of A549 cells. (Scale bars, 10 $\mu$m.) Bottom Quantification of cPla2-mKate2 membrane interaction. n > 200 cells per condition. Error bars, SD. [Ca$^{2+}$] = 200 nM. (C) Top) Representative images of cPla2 located either in the nucleoplasm or bound to the inner NM of A549 cells. (Scale bars, 10 $\mu$m.) Bottom) Ca$^{2+}$-isotherms for varying [PVP]. In the table, Hill-fitting of binding curves and binding parameters were determined by nonlinear least-square fitting of data. n > 4 0 cells per condition. Error bars, SD. For sample sizes, refer to Dataset S2C.
without cyto-/nucleoplasmic Ca²⁺ elevation. Cytoplasmic Ca²⁺ influx from the extracellular space or the ER is directly coupled to cell death through mechanosensitive channels like Piezo1 and others (5, 6, 29). In any case, NM-stretch will prime cPLA₂ for activation by enabling the enzyme to detect minuscule Ca²⁺ transients. Strong mechanosensitivity, as realized in cPLA₂-C2, is not intrinsic to all peripheral membrane-binding motifs, as suggested by our AKTI-PH, Alox12-PLAT, and PKCy-C2 results.

A popular artificial membrane model for measuring protein–bilayer interactions are highly curved liposomes (e.g., SUVs) generated by membrane sonication or comparable techniques (11, 27, 28). The total curvature of GUVs or nuclei is roughly two orders of magnitude lower than that of SUVs. In “flat” GUV membranes, lipid headgroups are more densely packed than in highly curved membranes of similar lipid composition. Given the larger voids between their headgroups, SUV membranes are intrinsically primed for adsorption (20, 22, 24, 25, 30, 31). GUV membranes, on the other hand, are basically flat and require stretch to achieve a similar priming. Consistent with this, [Ca²⁺]₁/₂ for cPLA₂-C2–binding to stretched GUVs (~1.7 μM) corresponds well to literature measurements on unstretched but curved SUVs (e.g., 3.3 μM) (28), whereas the Ca²⁺ sensitivity on unstretched GUVs is much lower ([Ca²⁺]₁/₂ ~18 to 36 μM). Likewise, the Ca²⁺ sensitivity of cPLA₂ interaction with stretched membranes ([Ca²⁺]₁/₂ ~60 to 90 nM) corresponds well to the Ca²⁺ sensitivity of PLAT activity ([Ca²⁺]₁/₂ ~100 to 200 nM) on sonicated macrophage membranes, which likely are highly curved (27). In the case of A549 cells, the Ca²⁺ sensitivity of cPLA₂ interaction with relaxed NM is much lower ([Ca²⁺]₁/₂ ~1.6 μM). Chemical variables, such as different protein-tagging strategies, recombinant protein quality, and differences in membrane composition may, of course, also contribute to the noted differences between our observation and literature measurements. Notwithstanding, the above relations and our ALPS experiments together argue that curvature and stretch promote domain adsorption through similar mechanisms (i.e., alternation of lipid-packing), albeit on very different length scales.

By providing a quantitative assay for protein interactions with flat membranes, our GUV method should help to further clarify how physical cues, such as stretch and curvature, regulate peripheral membrane proteins. At the same time, our data establish a quantitative baseline for understanding mechanotransduction through peripheral membrane proteins. We hope that our insights will facilitate the rational design of noninvasive probes that can measure membrane tension in intact cells and tissues.

**Methods**

**Lipids.** DPPC, L-α-phosphatidylserine (Brain PS), L-α-phosphatidylinositol-4-phosphate (Brain PI(4)P), DOPC, 1-2-dioleoyl-sn-glycerol (18:1 DOG), 1-oleyl-2-(6-(4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-1-bora-3a,4a-diaza-s-indacene-2-propionyloxy)amino)-hexanoyl)-sn-glycero-3-phosphocholine (TopFluor TMR PC), and 1,2-dioleoyl-sn-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (18:1 Liss Rhod PE) were purchased from Avanti Polar Lipids. All other chemicals were purchased from MilliporeSigma.

**Constitution of Expression Vector and Mutagenesis.** For bacterial expression, zebrinII cPLA₂-C2-eGFP-6HIS (zebrafish cPLA₂, C2 domain spanning amino acids 1 to 131), human cPLA₂-C2-eGFP-6HIS (human cPLA₂, C2 domain spanning amino acids 1 to 137), Alox12A-PLAT-6HIS (zebrafish Alox12A N-terminal PLAT domain spanning amino acids 1 to 118), Alox12B-PLAT-6HIS (zebrafish Alox12B N-terminal PLAT domain spanning amino acids 1 to 117), PKCy-C2-eGFP-6HIS (human PKCy-C2 domain spanning amino acids 158 to 289), and AKTI-PH-eGFP-6HIS (human AKTI-PH domain spanning amino acids 1 to 148) were amplified from complementary DNA (cDNA) and cloned into a modified pHAT2 bacterial expression vector backbone. ARFGAP1-ALPS-eGFP and ARF- GAP1-ALPS-mCherry (human ARFGAP1-ALPS domain covering amino acids 192 to 304) were cloned into a modified pGEX-6P-1 backbone, leading to N-terminal GST fusion with Human Rhinovirus-3C (HRV-3C) protease cleavage sites.

Double-α-amino acid mutations of the cPLA₂-C2-eGFP hydrophobic regions (L32A33A, L32FL33F, Y90A91A, and Y90F91F) and calcium-binding regions (D34RDB7R and D34KDB7K) were introduced using the QuickChange Lightening Site-Directed Mutagenesis Kit (Agilent Technologies) and the Phusion Site-Directed Mutagenesis Kit (Tbermo Fisher Scientific), respectively, with appropriate mutagenesis primers (Table 1) according to the manufacturer’s protocol. For mutagenesis of calcium-binding regions, mutations at residue 34 and residue 87 were sequentially introduced to the plasmid.

For mammalian expression, cPLA₂-mKate2-P2A-eGFP-Alox12 or cPLA₂-mKate2 were cloned into the pSBE-1/2-mMCS/Puro transposon plasmid as previously described (32). Within this plasmid, an expression cassette containing cPLA₂-mKate2-P2A-eGFP-Alox12 or cPLA₂-mKate2 is driven by a constitutive EF-1α promoter along with another expression cassette that encodes a puromycin selection marker under the control of synthetic RPB3A promoter. Both expression cassette flanks are blanked by inverted terminal repeats that contain transposase recognition sites and allow transposition into host genome when coexpressed with the hyperactive Sleeping Beauty transposase.

**Recombinant Protein Expression and Purification.** The expression and purification of C-terminally tagged C2 or PLAT domains was performed as previously described (4). In brief, the pHAT2 vectors containing the C-terminally tagged C2 or PLAT domains were transformed into Escherichia coli BL21 cells (New England Biolabs). Expression was induced by 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega) in 1 L Luria Bertani (LB) culture supplemented with Ampicillin (100 μg/mL) at optical density (OD₆₀₀ = 0.5 under vigorous shaking at 250 revolutions per minute (RPM) overnight at 18°C. Bacteria were centrifuged at 2,600 relative centrifugal force (RCF) for 30 min, pelleted, and frozen at –80°C. For purification, bacteria were resuspended in 25 mM Heps (pH = 7.4) and 0.1% Triton X-100 with protease inhibitors (Complete, ethylenediaminetetraacetic acid-free protease inhibitor mixture, Roche). Sonication was conducted on an ice/water mixture for 7 to 10 min with the following settings: 50% Amplitude, 1-s on/2-s off pulses (Thermo Fisher Scientific Sonic Dismembrator Model 500). After sonication and centrifugation at 17,000 RCF for 30 min at 4°C (Sorvall RC 6 PLUS, Rotor SS-34), the supernatants were collected and incubated with TALON metal affinity resin (Takara) for 3 h under rotation at 4°C. The affinity resin was rinsed three times with 25 mM Heps (pH = 7.4) and 200 mM potassium chloride (KCl) buffer. For protein elution, the TALON beads were incubated with 2 mL 100 mM imidazole (dissolved in 25 mM Heps and 200 mM KCl buffer) in disposable gravity filter devices. Protein concentration was determined using the Pierce bichoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). Protein purity was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Aliquots were snap frozen in liquid nitrogen and stored at –80°C.

Double-α amino acid mutations of C-terminally tagged ARFGAP1-ALPS and ARFGAP1-ALPS were introduced as previously described (30). The pGEX-6P-1 expression vector harboring tagged ARFGAP1-ALPS was transformed into Rosetta 2 pLysS cells (Novagen). Expression of ARFGAP1-ALPS was induced by 0.2 mM IPTG at 37°C under vigorous shaking (250 RPM) for 3 h in LB medium supplemented with Ampicillin (100 μg/mL) at OD₆₀₀ = 0.8. After expression, bacteria were resuspended, sonicated, and centrifuged. Supernatants were incubated with Glutathione Sepharose 4B gel beads (Cytiva) overnight at 4°C. The beads were washed three times in 25 mM Heps (pH = 7.4) and 200 mM KCl afterward. Beads with GST-tagged ARFGAP1-ALPS were incubated with HRV-3C protease (PreScission Protease, Cytiva) in 25 mM Heps (pH = 7.4) and 200 mM KCl overnight at 4°C to elute ARFGAP1-ALPS. Eluates were concentrated with Amicon Ultra-2 10K Centrifugal Filter Devices, MilliporeSigma. The total protein concentration was determined using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). Protein purity was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Aliquots were snap frozen in liquid nitrogen and stored at –80°C.

**GUV Preparation.** For GUVs composed of DPPC and PS (84% DPPC and 16% Brain PS; 83.5% DPPC, 16% Brain PS and 0.5% TopFluor TMR PC [mol/mol]; 84% DPPC and 16% Brain PS; and 83.5% DPPC, 16% Brain PS, and 0.5% 18:1 Liss Rhod PE), a nylon net hydration method was used. Briefly, 41 μm pore size nylon net filters (MilliporeSigma) were submerged in 25 mL of lipid mixture containing lipids (total amount: 160 μM) dissolved in 1:1 chloroform/methanol based on the ratios described above. Subsequently, the filter was dried in vacuum for 10 min. In total, 10 mL of warm 500 mM sucrose solution was
added and incubated with the nylon net filters in tightly closed, amber glass jars (Thermo Fisher Scientific). The jars were incubated for 4 to 6 h at 42 °C. The GUV suspension was diluted with 4 volumes of osmotically matched salt and sucrose solution (107.5 mM KCl, 1 mM MgCl₂, 10 mM Hepes, 5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), and 267 mM sucrose). Vesicles were pelleted at 50 KCF for 1 h and resuspended in the same salt and sucrose solution. For experiments that involved PKCγ-C2, a lipid mixture with increased brain P₅₇ concentration was used: 73.5% DPPC, 26% Brain P₅₇, and 0.5% TopFluo TMR PC dissolved in 1:1 chloroform/methanol (up to a maximum of 30% mol/mol Brain P₅₇ concentration above which natural swelling was inhibited). For comparison, gentle hydration by a standard protocol was conducted as described previously (34).

To generate GUVs with alternative lipid compositions (99.5% DOPC and 0.5% 18:1 Liss Rhod PE; 81.5% DOPC, 18% 18:1 DOG, and 0.5% 18:1 Liss Rhod PE; 89.5% DOPC, 10% 18:1 DOG, and 0.5% 18:1 Liss Rhod PE; 89.5% DOPC, 10% 18:1 DOG, and 0.5% 18:1 Liss Rhod PE; 99.5% DPPC and 0.5% 18:1 Liss Rhod PE; 99.5% DOPC, 10% 18:1 DOG, and 0.5% 18:1 Liss Rhod PE; 99.5% DOPC, 10% 18:1 DOG, and 0.5% 18:1 Liss Rhod PE), the electroformation technique was performed as previously described (16). Briefly, 0.11 μM of lipid mixture according to the above described ratios was spread on 30 to 60 μM indium tin oxide (ITO) coverslips (Structure Probe Inc. supplies) and dried under a N₂ stream. ITO coverslips were assembled into an electroformation chamber with 500 mM sucrose solution inside and incubated at 60 °C. The chambers were connected to a function generator (RIGOL DG1022). A sine wave with a frequency of 10.0 Hz and peak-to-peak voltage at 1.41 V was applied for 4 to 6 h followed by a square wave with a frequency of 4.5 Hz and peak-to-peak voltage at 2.12 V for 30 min. After electroformation, GUVs were extracted from the chamber using a gel loading tip. The chamber was washed twice with 500 mM glucose solution to collect residual GUVs. GUVs were used for experiments within 5 d after preparation.

### Recombinant Protein-Binding Experiments

GUV-binding buffers (Table 2) generally contained KCl, EGTA, Hepes, MgCl₂, and varying concentrations of CaCl₂ and sucrose at pH = 7.4 to 7.5. Free calcium concentration was calibrated by mixing EGTA and CaCl₂ as described previously (35).

| Mutagenesis primers | Primer sequence |
|---------------------|-----------------|
| Y90F V91F Forward   | ccaagattttcatcctgaaaaagtttgcctccatcagtgtccacc |
| Y90F V91F Reverse   | ggtgacagtgttgaagttaaatcrttttttttttggagactggccgacc |
| Y90F V91F Reverse   | gctgcccgttcagaaatccttctctctagt |
| Y90F V91F Reverse   | taaagttcgccgggtgctgctgtcatcctcctttctcattatctaat |
| Y90F V91F Reverse   | gattttttagcaccgcgacc |
| Y90F V91F Reverse   | cacaatttctcttcttgatc |
| Y90F V91F Reverse   | gactctaaaaatgcttcatcagtcatcagtccctgagttaggcacgacaccccggcttcctttagttac |
| Y90F V91F Reverse   | ccaatttctcttcttgatc |
| Y90F V91F Reverse   | gactctaaaaatgcttcatcagtcatcagtccctgagttaggcacgacaccccggcttcctttagttac |
| Y90F V91F Reverse   | acctctaaaaatgcttcatcagtcatcagtccctgagttaggcacgacaccccggcttcctttagttac |

### Table 1. Mutagenesis primers

Cells were coaxed with 20 mg/mL bovine serum albumin (BSA) solution for 1 h before the binding experiments to avoid GUV adhesion to the glass. For the experiment depicted in Fig. 1A, GUVs were first shrunk by preincubation in hypertonic solution (ΔMₜot = −400 Osm) for 1 h. Binding experiments were started by transferring these GUV suspensions (2 to 4 μL) to binding buffer supplemented with 1 μM eGFP-tagged recombinant protein in BSA-coated glass-bottom dishes. For the cPLA₂-C2 domains (wild-type and mutants), the free calcium concentration was set to 20 μM. For the C₂ and PLAT domains of Alox5a, Alox12, and PKCγ, free calcium concentration was maintained between 200 and 500 μM as indicated. For the AKT1-PH domain, free [Ca²⁺] was maintained at 500 μM. For ARFGAP1-ALPS, calcium was not included in the solution. Protein binding to GUV membranes was measured at room temperature using a Nikon Eclipse Ti inverted spinning-disk confocal microscope with a Plan Apo 100x/1.45 oil objective or a Plan Apo 40x/0.95 air objective and equipped with a Yokogawa CSXU-1 Sxinning Disk unit, an ANDOR iXon ULTRA 897BV EMCCD camera, 488-nm and 561-nm solid-state laser lines (Andor Revolution) and NIS-Elements Software (Nikon). GUV membrane fluorescence was excited at 561 nm and collected through a 620 bandpass emission filter (590 to 650 nm). eGFP fluorescence was excited at 488 nm and collected through a 525 bandpass emission filter (515 to 555 nm). All protein domains were imaged using identical microscope settings. GUVs were allowed to settle in solution for 1 to 2 mins before acquisition. Z-stacks (1.4- to 2-μm steps over 30 μm) were acquired every minute for 30 min.

**Competition Experiments.** Generally, GUVs with preadsorbed “protein A” were transferred to a buffer containing “protein B” (the competitor) but no “protein A” ± calcium. Specifically, 4 to 6 μL unlabeled GUVs (100% DPPC) were first preincubated with 1 μM fluorescent “protein A” (cPla2-C2-eGFP or ARFGAP1-ALPS-mCherry) in 200 μL hypotonic solution (ΔMₜot = 240 mOsm; 20 μM free Ca²⁺) for 1 h to allow for membrane adsorption. To initiate competition, ~195 μL of this solution was replaced by 200 μL hypotonic solution with 1 μM “protein B” (ARFGAP1-ALPS-mCherry or cPla2-C2-eGFP). Protein (de-)binding was captured for 1 h using confocal microscopy as described in *Recombinant Protein-Binding Experiments*. To study ARFGAP1-ALPS binding after cPla2-C2 membrane desorption, ~195 μL supernatant of the hypotonic cPla2-C2-eGFP (1 μM) was replaced by 200 μL hypotonic ARFGAP1-ALPS-mCherry (1 μM) solution without Ca²⁺. As control, we also monitored ARFGAP1-ALPS-mCherry desorption after transferring GUVs into hypotonic buffer without cPla2-C2-eGFP (ΔMₜot = 240 mOsm; 20 μM free Ca²⁺).

**GUV Membrane Rupture and Protein Desorption.** To image GUV rupture, concentrated GUV suspensions (4 to 6 μL) were first incubated for 1 h in hypotonic binding buffer (ΔMₜot = 240 mOsm) containing 1 μM of the indicated domain. Next, the GUVs were transferred to hypotonic binding solution (without proteins). Images were acquired as described in *Recombinant Protein-Binding Experiments*. For fast acquisition, only a single Z-slice of the GUV midsection was acquired per field. Images were acquired every 10 s for 25 min.

**Image Processing and Analysis.** All image processing and analysis was conducted with the Anaconda distribution of Python (Python ≥ 3.5). Specifically, custom Python 3.7 scripts were written based on the Numpy (36), SciPy (37), Scikit-image (38), Allen Cell Structure Segmenter (39), Cellpose (40) and Napari libraries (41).

To quantify binding of recombinant proteins to GUVs, a semiautomated Python custom script was developed. Analysis comprised the following steps: First, a line was manually drawn by the user from the center of GUV to the edge to crop a selected GUV from the whole image stack. Afterward, intensity normalization and a three-dimensional Gaussian blur (3-pixel radius) were applied to the cropped GUV stack to remove background noise. A marker-controlled watershed algorithm was used on each slice of the cropped stack to segment the GUV. The slice with largest segmented GUV area was defined as the middle section. Based on the watershed segmentation result, a 3-pixel-wide contour of the GUV was drawn onto the GUV middle section. Lastly, the rim-binding intensity was calculated as the median of all intensities along the contour. To account for fluorescence variabilities across multiple purification batches, raw rim-binding of each domain was normalized by the fluorescence signal of a 1-μM protein solution from same purification batch. To this end, the same confocal settings and contours were used as for the GUV measurements. Both GUV radius and circularity were calculated from the two-
dimensional contour of the GUV middle section. Circularity was defined as $1 - \frac{c}{a}$, where $c$ is distance between foci and $a$ is the length of major axis (circularity is equal to 1 when GUV is in circular shape). Detailed explanations and codes are available at https://github.com/joeshen123/GUV-Protein-Binding-Analysis-Program.

An autonomous, custom Python script was used to quantify cPla2-mKate2 binding to intact NMs of A549 or HeLa cells. Maximum-intensity projection images were generated from 3D confocal stacks, followed by a Gaussian blur (3-pixel radius) to remove background noise. A generalist, deep learning-based method (Cellpose) was applied to segment nuclei and generate 3-pixel (3-pixel radius) to remove background noise. A generalist, deep learning-based method (Cellpose) was applied to segment nuclei and generate 3-pixel-radius masks. A single Z-plane image was acquired every 25 s for 15 min. Following imaging, the fluorescence decay data were fitted with a double-exponential model to calculate the lifetime for each pixel, and the lifetime image was exported as 512 × 512 single Z-plane image for each time point. For quantification, the fluorescence lifetime for each GUV was calculated by averaging the fluorescence lifetimes on its rim.

### Table 2. Buffer compositions

| Δ ΔMOSM (Inside – Outside) | Salt concentration | Sucrose concentration |
|---------------------------|--------------------|-----------------------|
| –400 mOsm                 | 107.5 mM KCl, 1 mM MgCl₂, 5 mM EGTA, and 10 mM Hepes | 667 mM |
| 0 mOsm                    | 107.5 mM KCl, 1 mM MgCl₂, 5 mM EGTA, and 10 mM Hepes, and varying CaCl₂ | 246 to 267 mM |
| 100 mOsm                  | 107.5 mM KCl, 1 mM MgCl₂, 5 mM EGTA, and 10 mM Hepes | 167 mM |
| 240 mOsm                  | 110.5 to 121 mM KCl, 1 mM MgCl₂, 5 mM EGTA, and 10 mM Hepes, and varying CaCl₂ | 0 mM |

Equilibrium Binding Experiments. Concentrated GUVs were transferred to binding buffer containing domains at varying concentrations in BSA-coated glass-bottom dishes. GUVs were left in the binding solution for 2 h until protein recruitment approached an equilibrium. Afterward, GUV membrane and protein fluorescence images were simultaneously acquired using a 40x objective as described in Recombinant Protein-Binding Experiments. Fluorescence signals on GUV membrane were normalized as described in Image Processing and Analysis. For C2-like domains, an apparent dissociation constant $K_{d}$ and maximum binding intensities $B_{max}$ were determined by nonlinear least-square analysis of bound-protein fluorescence ($B_{bound}$) versus total domain concentration ([Domain]) using a Langmuir-type adsorption isotherm as previously described (42):

$$B_{bound} = B_{max} \frac{[\text{Domain}]}{[\text{Domain}] + K_{d}}$$

For ARFGAP1-ALPS domains, the apparent dissociation constant $K_{d}$, maximum binding intensity $B_{max}$, and the apparent Hill coefficient $n$ were determined by nonlinear least-square analysis of bound-protein fluorescence ($B_{bound}$) versus the total domain concentration ([Domain]) using anisotropy assay with Hill expansion (43), which fitted the experimental data better than the above equation (assuming no cooperativity):

$$B_{bound} = B_{max} \frac{[\text{Domain}]^{n}}{[\text{Domain}]^{n} + K_{d}^{n}}$$

To compare the Ca²⁺-sensitivities of C2-like domains, concentrated GUVs were transferred to binding buffer containing 1-μM domain and varying concentrations of free Ca²⁺ in BSA-coated glass-bottom dishes. GUVs were left in the binding solution for 2 h until protein recruitment approached equilibrium. Images were acquired and analyzed as described in Recombinant Protein-Binding Experiments and Image Processing and Analysis. The Ca²⁺ concentration required for half-maximal membrane-binding ($[\text{Ca}^{2+}]^{1/2}$) was obtained by fitting a Hill equation:

$$B_{bound} = B_{max} \frac{[\text{Ca}^{2+}]^{n}}{[\text{Ca}^{2+}]^{n} + \text{Ca}^{2+}^{2n}}$$

where $B_{bound}$, $B_{max}$, $n$, and $[\text{Ca}^{2+}]$ refer to the bound-protein fluorescence signal, the maximum protein-binding intensity, the apparent Hill coefficient, and the free Ca²⁺ concentration, respectively. Positive cooperativity ($n > 1$) of Ca²⁺-binding was previously known for C2-like domains (e.g., ref. 28). Hence, we restricted the range of the apparent Hill coefficient to ≥1 for better fitting.

Detailed explanations and MATLAB codes are available at: https://github.com/joeshen123/Cell-Nucleus-Membrane-Binding-2D.

Flipper-TR Measurements. Membrane tension Flipper-TR probe was purchased from Cytoskeleton, Inc (CY-SC020). GUVs composed of 99% DPPC + 1% Flipper-TR in dimethyl sulfoxide (DMSO) were prepared using electroformation. Shortly before starting the experiment, GUVs were added to isotonic solution ($\Delta \text{MOSM} = 0$ mOsm, 0 μM free Ca²⁺) or hypotonic solution ($\Delta \text{MOSM} = 100$ mOsm, 0 μM free Ca²⁺) in BSA-coated glass-bottom wells. For fluorescence lifetime imaging, GUVs were imaged on a Leica SP8 Falcon microscope equipped with Plan Apo 40X/1.1 water lens. GUV membranes were excited with 488 nm laser at 23% laser power and emission sampled with 580- to 625-nm filters. A single Z-plane image was acquired every 25 s for 15 min. Following imaging, the fluorescence decay data were fitted with a double-exponential model to calculate the lifetime for each pixel, and the lifetime image was exported as 512 × 512 single Z-plane image for each time point. For quantification, the fluorescence lifetime for each GUV was calculated by averaging the fluorescence lifetimes on its rim.

### Generation of Stable A549 and Hela Cell Lines. A549 human lung cancer cells (CCL-185, ATCC) were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% Penicillin-Streptomycin. Lipofectamine 3000 (Thermo Fisher Scientific) was used to transfect A549 cells according to the manufacturer’s instructions. To establish stable cell lines expressing cPla2-mKate2-P2A-eGFP-Alexa, A549 cells were transfected with SB100X Sleeping Beauty transposase and a pSP/EF-1 α /MCS/Puro transposon vector encoding the genes of interest. A total 48 h after transfection, cells were grown in complete growth medium with 1 μg/ml puromycin for 1 to 2 wk. mKate2 and eGFP double-positive cells were sorted by flow cytometry. The stable HeLa (CCL-2) cell line expressing cPla2-mKate2 had been prepared for a previous publication (4).

cPla2-mKate2 Binding to Intact NM. The following medium (“base medium”, BM) was prepared for intact NM-binding experiments: 123 mM KCl, 12 mM NaCl, 1 mM KH₂PO₄, 1.94 mM MgCl₂, 10 mM MOPS, 0.28 mM CaCl₂, and 1 mM EGTA/NaCl at pH 7.2 to 7.3. Additional CaCl₂ was added into BM to change free-Ca²⁺ concentration as previously described (35). Cell membranes were permeabilized in BM supplemented with 25 μg/ml digitonin (Cayman Chemical) and 2.5% PVP360 (MiliporeSigma) for 15 min as previously described (4). Changing PVP360 concentration allowed to control NM-stretch. To measure NM-binding of cPla2-mKate2 with increasing osmotic stretch, permeabilized cells were washed two times with phosphate-buffered saline (PBS), followed by 2 h incubation in BM supplemented with ~200 mM free Ca²⁺ and a PVP360 concentration ranging from 0 to 2.5%. Z-stacks (1.2-μm steps over 40 μm) were acquired and cPla2-mKate2 membrane-binding quantified as described in Recombinant Protein-Binding Experiments and Image Processing and Analysis.

To measure the Ca²⁺-sensitivity of cPla2-mKate2 adsorption to intact NM as a function of NM-tension, CaCl₂ was added into BM to vary free [Ca²⁺] from 0 to 1.4 μM. Different PVP360 concentrations were chosen to compare the effects of increasing NM-stretch: 0%, 1.7% (A549 cells only), and 1.9%. Cells were first permeabilized in BM supplemented with 25 μg/ml digitonin, 2.5% PVP360, and varying free [Ca²⁺]. After washing two to three times with PBS, the permeabilized cells were incubated in BM supplemented with the indicated concentration of PVP360 (0%, 1.7%, or 1.9%) and free Ca²⁺ (0 to 1.4 μM) for 3 h until binding reached an equilibrium. Z-stacks (1.4 μm z-steps over 30 μm) were acquired, and [Ca²⁺]^{1/2} was determined by fitting a Hill-type equation as described in Equilibrium Binding Experiments.

### Structure and Multiple Sequence Alignment. Multiple-protein sequence alignment of the cPla2-C2 domain was performed using CLUSTALW and Jalview Version 2 (44, 45). Figures of domain structures were generated by PyMOL Molecular Graphics System, Version 2.5.1 Schrödinger, LLC (https://pymol.org/2).

**Statistics.** For bar-plot comparisons of fitted coefficients (e.g., [Ca²⁺]^{1/2}), the error bars denote the 95% CI as calculated by MATLAB’s confint function. Otherwise, error bars represent the 3D. Additional repeats of zebrafish cPla2-C2 equilibrium binding experiments with (DPPC-16%PS) are listed in Dataset S1. Detailed sample sizes for the experiments are provided in Dataset S2 and omitted from the figures for clarity. A two-tailed Students t test assuming unequal variances was used to determine P values using the Microsoft Excel t test function.
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