

1 Structural basis for the association of PLEKHA7 with membrane- 2 embedded phosphatidylinositol lipids

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15 **Running Title:** PLEKHA7 interaction with PIP membranes

16 **Keywords:** PLEKHA7; PH domain; PIP; nanodisc; structure; NMR

17⁴**Abbreviations:** IP(4,5)P₂: inositol (1,4,5) triphosphate; IP(3,4)P₂: inositol (1,3,4) triphosphate; IP(3,4,5)P₃:
18 inositol (1,3,4,5) tetraphosphate; ITC: isothermal titration calorimetry; MD: molecular dynamics; NMR:
19 nuclear magnetic resonance; PH: pleckstrin homology; PIP: phosphatidyl inositol phosphate; PI(4,5)P₂:
20 phosphatidyl inositol (4,5) diphosphate; PI(3,4)P₂: phosphatidyl inositol (3,4) diphosphate; PI(3,4,5)P₃:
21 phosphatidyl inositol (3,4,5) triphosphate; PLEKHA7: pleckstrin homology domain containing family A
22 member 7; HSQC: heteronuclear single quantum coherence

23 24 25 26 27 28 29 30 31 32 33 34 35 **Summary**

36 PLEKHA7 (pleckstrin homology domain containing family A member 7) plays key roles in intracellular
37 signaling, cytoskeletal organization and cell adhesion, and is associated with multiple human cancers. The
38 interactions of its pleckstrin homology (PH) domain with membrane phosphatidyl-inositol-phosphate (PIP)
39 lipids, are critical for proper cellular localization and function, and their inhibition is an attractive target for
40 anti-cancer therapy. While structural data can provide insights in this area, little is known about the way in
41 which PLEKHA7 and other PH domains interact with membrane-embedded PIPs. Here we report atomic-
42 resolution structures of the PLEKHA7 PH domain and describe the molecular mechanism for its recognition of
43 membrane-bound PIPs. Using X-ray crystallography, nuclear magnetic resonance (NMR), molecular
44 dynamics (MD) simulations, and isothermal titration calorimetry (ITC), we show – in atomic-level detail –
45 that the interaction of PLEKHA7 with PIPs is multivalent and induces PIP clustering. The PIP binding
46 mechanism is distinct from a discrete one-to-one interaction. Our findings reveal a central role of the
47 membrane assembly in mediating protein-PIP association and provide a roadmap for the design of
48 PLEKHA7-PIP inhibitors.

50 PLEKHA7 (pleckstrin homology domain containing family A member 7)⁴ is a major component of the
51 cytoplasmic region of epithelial adherens junctions that functions to ensure cell-cell adhesion and tight
52 junction integrity through its interactions with cytoskeleton proteins (Meng et al., 2008; Paschoud et al.,
53 2014; Pulimeno et al., 2010; Rouaud et al., 2020). Increased levels of human PLEKHA7 are associated with
54 hypertension (Levy et al., 2009) and glaucoma (Awadalla et al., 2013), and PLEKHA7 protein staining has
55 been reported in several human cancers, including advanced breast, renal and ovarian cancer (Kourtidis et
56 al., 2015; Tille et al., 2015), with highest occurrences in colon cancer (Castellana et al., 2012). A recent
57 study (Nair-Menon et al., 2020) showed that disruption of the apical junction localization of RNA
58 interference machinery proteins correlates with loss of PLEKHA7 in human colon tumors and poorly
59 differentiated colon cancer cell lines, while restoration of PLEKHA7 expression restores proper localization
60 of RNA interference components and suppresses cancer cell growth in vitro and in vivo. Moreover, we have
61 observed (Jeung et al., 2011) that PLEKHA7 associates with proteins of the membrane-bound KRAS
62 signaling nanocluster in colon cancer cells, and that *plekha7* gene knock down inhibits the proliferation of
63 colon cancer cells with mutated KRAS, but not normal cells with wild-type KRAS. PLEKHA7, therefore,
64 represents an attractive druggable target for the selective inhibition of signaling and its tumor-related
65 functions in colorectal cancer. Despite its importance, the molecular basis for the role of PLEKHA7 in these
66 disorders is poorly understood.

67 PLEKHA7 is one of nine family members characterized by a 120-residue N-terminal pleckstrin
68 homology (PH) domain. PH domains are found in more than 100 different proteins, they are known for their
69 ability to bind phosphatidyl-inositol-phosphate (PIP) lipids within cell membranes, and their association with
70 PIPs is essential for intracellular signaling, cytoskeletal organization and the regulation of intracellular
71 membrane transport (DiNitto and Lambright, 2006; Lemmon, 2007). Notably, PH domains can be selectively
72 targeted by small molecules that inhibit their signaling function (Indarte et al., 2019; Meuillet et al., 2003;
73 Meuillet et al., 2010). In the case of PLEKHA7, the PH domain is required for establishing proper
74 subcellular localization through its interactions with PIPs (Wythe et al., 2011), and thus, could offer a new
75 avenue for attacking cancer by inhibiting PLEKHA7 localization.

76 Following the first NMR structures of pleckstrin (Yoon et al., 1994) and β -spectrin (Macias et al., 1994),
77 the structures of many other PH domains have been reported, both in their free state or complexed with
78 soluble inositol phosphate (IP) small molecules (Moravcevic et al., 2012). These have provided important
79 functional insights, but little is still known about the way in which PH domains associate with full-length
80 PIPs incorporated in membranes, and nothing is known about the structural basis for PIP binding by
81 PLEKHA7. Using a multidisciplinary approach that combined X-ray crystallography, NMR, all-atom MD
82 simulations, and ITC binding measurements, we have determined the structures of the PLEKHA7 PH domain
83 and characterized its interactions with membrane-embedded PIPs with atomic-level detail. The data reveal
84 three PLEKHA7 binding sites for PIP, and demonstrate that confinement of PIP molecules by the membrane
85 assembly is essential for promoting the multivalent association of one PH domain with multiple PIPs leading
86 to membrane surface localization.

87

88 Results and discussion

89 **Structure of the PLEKHA7 PH domain.** We obtained crystals for two states of the PH domain of
90 PLEKHA7 – one ligand-free (PHA7_{apo}) and the other bound to sulfate from the crystallization buffer
91 (PHA7_s) – and we determined two structures, which refined to 2.80 Å and 1.45 Å resolution, respectively
92 (Fig. 1A, B; Fig. S1A-C; Table S1). Each structure crystallized in a different space group and has two copies
93 of the protein per crystallographic asymmetric unit, but the protein-protein interfaces differ in each case, and
94 do not appear to reflect biologically functional dimers. PLEKHA7 adopts the PH domain signature fold
95 (Lemmon, 2007; Lietzke et al., 2000): a seven-stranded β -barrel, capped at one end by a 16-residue α -helix.
96 The barrel opening is highly positively charged and lined by the β 1- β 2, β 3- β 4 and β 6- β 7 loops which are
97 hypervariable in both length and sequence across the PH domain family. The K173-K183-R185 motif, at the
98 end of β 1 and start of β 2, forms the canonical IP binding site of PH domains (Moravcevic et al., 2012). In the

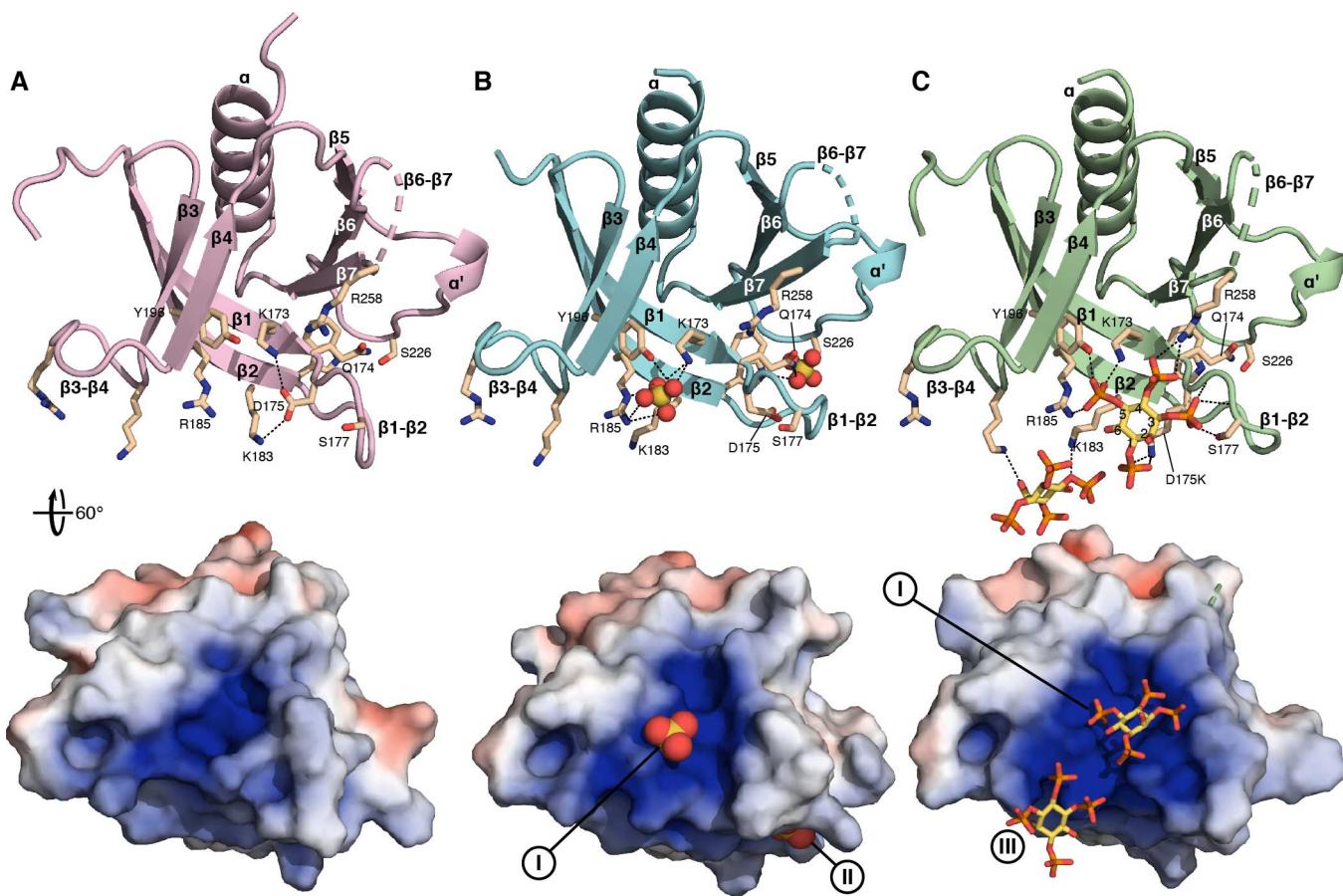


Fig. 1. Structure of the PLEKHA7 PHD. (A) PHA7_{apo}. (B) PHA7_s bound to sulfate (yellow/orange spheres). (C) Mutant PHA7-D175K bound to soluble IP(3,4,5)P₃ (yellow/orange sticks). Key residues are shown as sticks. Dashes denote protein-protein, protein-sulfate or protein-IP(3,4,5)P₃ polar contacts (<4 Å). The 60° surface representations are colored by electrostatic potential from -5 kT/e (red) to +5 kT/e (blue). The positions of the three binding sites (I-III) for sulfate or phosphate are marked.

99 case of PHA7_s, this site – denoted here as site I – is occupied by one of two sulfate anions resolved in the
 100 structure. No electron density was observed for either the β6-β7 loop or C-terminal residues S285-R298,
 101 which were included in the sequence of PHA7_s (residues 164-298) but deleted in PHA7_{apo} (residues 164-
 102 285). The structures show that the PH domain is confined to residues 164-285, and subsequent NMR, ITC
 103 and MD simulation studies utilized this trimmed sequence, henceforth referred to as PHA7 (Fig. S1A).

104 To further examine the protein conformation and dynamics in solution, we prepared ¹⁵N/¹³C labeled
 105 PHA7 for NMR experiments. The assigned NMR chemical shifts (¹HN, ¹⁵N, ¹³CA and ¹³CB) show that the
 106 protein adopts the same structure in solution as in its crystalline form, and that the long β6-β7 loop forms a
 107 random coil (Fig. S2A). The ¹H/¹⁵N nuclear Overhauser effect (NOE) data show that the β-strands, and
 108 helices (α1, α') all have similar (>0.8) NOE intensities, consistent with uniform backbone dynamics and
 109 conformational order (Fig. S2B). By contrast, the termini and loops have distinctly lower NOE intensities,
 110 and hence greater extents of dynamics and lower conformational order. High flexibility is observed for β6-
 111 β7 where the intensities approach zero, and also for β1-β2 and β3-β4, loops that are important for defining
 112 the recognition of specific PIP phosphorylation states by other PH domains (Lemmon, 2007).

113 PLEKHA7 shares 50-75% amino acid sequence identity with the other PLEKHA family members (Fig.
 114 S3A), but it is unique in containing a 20-residue insertion between β6 and β7. Long β6-β7 loops are found in
 115 other members of the larger PH domain superfamily. In GRP1 and ARNO (Cronin et al., 2004; Lietzke et al.,

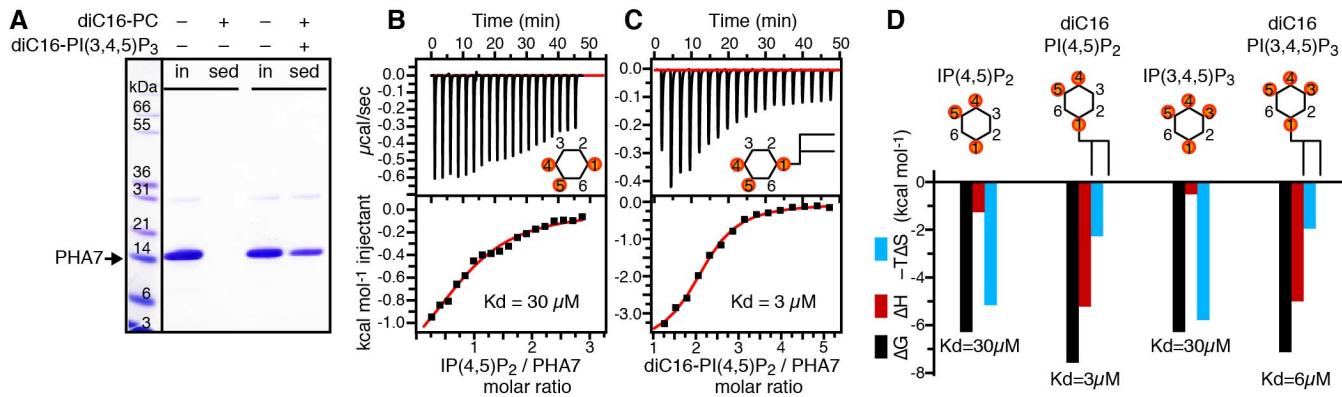


Fig. 2. Interactions of PHA7 with PIP lipids and soluble IPs. (A) SDS-PAGE analysis of PHA7 co-sedimentation with diC16-PC liposomes prepared with or without 10% molar diC16-PI(3,4,5)P₃. Monomeric PHA7 (arrow) migrates with apparent molecular weight of ~15 kDa. (B-D) Representative ITC binding isotherms (B, C) and free energies (D) measured for titrations of PHA7 with soluble IP(4,5)P₂ and IP(3,4,5)P₃, or nanodiscs containing 10% molar diC16-PI(4,5)P₂ and diC16-PI(3,4,5)P₃. Continuous red lines are the best fits of the data to a single-site binding model, used to extract the values of the dissociation constant (K_d).

116 2000), the β 6- β 7 loop forms a β -hairpin that elongates the β -barrel, generating a more extensive pocket for
 117 binding IP, while in the case of ANLN, the loop does not appear to be involved in IP binding. The β 6- β 7
 118 sequence of PHA7 differs from those of GRP1, ARNO and ANLN.

119 **Interactions with soluble IP heagroups and membrane-embedded PIPs.** To assess the affinity of
 120 PHA7 for membrane-embedded PIP lipids, we performed co-sedimentation experiments with PIP-containing
 121 liposomes. While PHA7 does not sediment with pure dipalmitoyl-phosphatidyl-choline (diC16-PC)
 122 liposomes, incubation with liposomes containing 10% molar diC16-PI(3,4,5)P₃ resulted in abundant co-
 123 sedimentation (Fig. 2A). Notwithstanding this evident high affinity for PIP-rich membranes, our attempts to
 124 co-crystallize PHA7 with soluble IP headgroups – IP(4,5)P₂, IP(3,4)P₂ or IP(3,4,5)P₃ – resulted in the apo
 125 form of the protein, without bound ligand, regardless of crystallization or soaking conditions. This is in line
 126 with the μM -range dissociation constants (K_d) that we measured by ITC for the interaction of PHA7 with
 127 soluble IPs (Fig. 2B; Fig. S4; Table 1). The combined data indicate that PHA7 has modest affinity for
 128 soluble IPs but appreciably higher affinity for PIP lipids incorporated in membranes. A similar effect has
 129 been reported for the PH domain of kindlin 3 whose binding affinity for PIP was enhanced 1,000 fold upon
 130 PIP incorporation in nanodiscs (Ni et al., 2017).

131 To analyze this effect quantitatively, we performed ITC experiments with PHA7 and PIP-enriched lipid
 132 nanodiscs – nanometer-size discoidal membranes that are stabilized by two copies of a membrane scaffold
 133 protein (MSP) derived from the apolipoprotein ApoA1 (Bayburt et al., 2002). We prepared nanodiscs with
 134 either pure diC16-PC or a 1/9 molar mixture of diC16-PIP and diC16-PC. Remarkably, the ITC binding
 135 affinity measured by titrating the protein with PIP-nanodiscs is enhanced by more than one order of
 136 magnitude relative to soluble IP headgroups (Fig. 2C; Fig. S4; Table 1), in line with the co-sedimentation
 137 results. Moreover, PHA7 now appears to display some – albeit very modest – selectivity ($p < 0.05$) for
 138 PI(4,5)P₂ > PI(3,4,5)P₃ > PI(3,4)P₂ lipid membranes.

139 The enhanced affinity for membrane-incorporated PIPs suggests that additional factors – beyond a pure
 140 one-to-one PHA7-IP interaction – play a role in recruiting the protein to the membrane surface. The binding
 141 free energies derived from ITC offer some insights in this regard (Fig. 2D). The association of PHA7 with
 142 free IPs is highly entropy driven, with binding thermodynamics characterized by very small values of
 143 favorable enthalpy (ΔH) and a much greater entropic component (ΔS). The situation is reversed for the
 144 association of PHA7 with membrane-embedded PIPs, where the enthalpic component dominates. This effect

Table 1. ITC values of K_d (μM) for the interactions of wild-type PHA7 or mutant PHA7-D175K with soluble IP headgroups or diC16-phosphatidyl inositol (diC16-PI) incorporated in lipid nanodiscs.

	IP(4,5)P ₂ or PI(4,5)P ₂		IP(3,4,5)P ₃ or PI(3,4,5)P ₃		IP(3,4)P ₂ or PI(3,4)P ₂
	PHA7	PHA7-D175K	PHA7	PHA7-D175K	PHA7
soluble IP	30.5 \pm 0.6	10.7 \pm 0.2	30.1 \pm 2.3	3.5 \pm 0.5	–
nanodisc diC16-PI	3.0 \pm 1.2	0.5 \pm 0.1	6.2 \pm 0.2	5.5 \pm 0.1	9.0 \pm 0.3

145 may reflect a reduction of PIP conformational flexibility imposed by the membrane scaffold, as well as the
146 interaction of one PHA7 molecule with multiple PIP molecules incorporated in the nanodisc membrane. The
147 additional free energy observed for membrane-integrated PIPs may be attributed to a change in the
148 probability of binding that results from confinement of multiple PIPs by the membrane, according to the
149 principles of binding free energy additivity (Jencks, 1981). Such superadditivity is well known in drug
150 development, where chemical linking of weakly-binding fragments results in molecules with binding free
151 energy greater than the sum of the individual fragments. In the case of PHA7-PIP, membrane confinement
152 replaces chemical linking to yield enhanced affinity.

153 **Structural basis for binding soluble IPs.** In the absence of co-crystals, the structure of PHA7s provides
154 useful insights about the protein's interactions with the IP moiety. In PHA7s (Fig. 1B; Fig. S5A) one sulfate
155 anion occupies site I, where it is coordinated by the side chains of K173 in β 1, R185 in β 2 and Y196 at the
156 end of β 3. A second sulfate anion binds on the opposite side of the β 1- β 2 loop – denoted here as site II –
157 where it is coordinated by the side chains of Q174 and W182, which define the start and the end of the β 1- β 2
158 loop, and S226 in the middle of the β 5- β 6 loop. While IP binding at this second site is considered "atypical",
159 and thought to occur only in PH domains that lack the canonical binding site (Moravcevic et al., 2012), it has
160 been observed experimentally for the PH domains of spectrin (Hyvonen et al., 1995), Tiam1 and ArhGAP9
161 (Ceccarelli et al., 2007), and ASAP1 (Jian et al., 2015), where both canonical and atypical sites are
162 simultaneously bound to short-chain PI(4,5)P₂.

163 Notably, while the structures of PHA7_{APO} and PHA7s are superimposable, with root mean square
164 deviation (rmsd) of 0.49/0.52 Å (chains A-A/B-B) for CA atoms in the barrel core, they differ appreciably in
165 the β 1- β 2 loop where the rmsd jumps to 2.54/2.42 Å (chains A-A/B-B) (Fig. S5B). In the apo structure, the
166 β 1- β 2 loop folds towards the barrel opening and adopts a β -turn conformation that is restrained by a network
167 of hydrogen bonds involving the K173 and K183 amino groups, the S177 hydroxyl, and the D175
168 carboxylate oxygens (Fig. 1A; Fig. S5C, D). The D175 side chain points into binding site I, at the center of a
169 clasp-like structure formed by the K173 and K183 side chains. The temperature factors of the β 1- β 2 loop are
170 twice greater than for the rest of the protein, and the loop has a distinct conformation in each of the two
171 molecules of the crystal structure, reflecting its flexibility (Fig. S1B). In the sulfate-bound structure, on the
172 other hand, the β 1- β 2 loop folds away from the barrel opening (Fig. 1B; Fig. S5C, D). The D175 side chain
173 is disengaged from the K173-K183 clasp: It points into the loop, forming hydrogen bonds with its main
174 chain atoms, and away from the sulfate anion bound in site I. The β 1- β 2 temperature factors, while higher
175 than those of the barrel proper, are lower than those observed in PHA7_{APO}, and the loop has the same
176 conformation in both molecules of the structure, indicating that the bound sulfate stabilizes the β 1- β 2 loop
177 conformation (Fig. S1C).

178 The structures point to D175 and the β 1- β 2 loop as important mediators of the interaction of PHA7 with
179 IP headgroups. A previous study (Carpent et al., 2007) identified a "sentry" Glu residue in the PH domain of
180 AKT positioned to disfavor binding of a phosphate group at the 3-position of the inositol ring and define a
181 preference for IP(4,5)P₂ over IP(3,4)P₂ and IP(3,4,5)P₃. Changing this sentry Glu to Lys, an AKT mutation
182 that occurs in many tumors, reverses the preference and increases the affinity for IPs by ~10-fold.

183 To examine the role of D175 in PHA7, we generated the D175K mutant and characterized its structure
 184 and IP binding properties. ITC measurement show that PHA7-D175K has a three-fold greater affinity for
 185 IP(4,5)P₂ ($K_d=10.7\text{ }\mu\text{M}$) and nine-fold greater affinity for IP(3,4,5)P₃ ($K_d=3.5\text{ }\mu\text{M}$) compared to wild-type
 186 (Fig. S4; Table 1). Notably, PHA7-D175K co-crystallized with soluble IP(3,4,5)P₃. The structure refined to a
 187 resolution of 2.43 Å (Fig. 1C, Table S1) with two copies of the protein per crystallographic asymmetric unit.
 188 Once again, no electron density was observed for the long β 6- β 7 loop, indicating that it remains mobile and
 189 disordered, without apparently contributing to IP binding.

190 The two molecules of PHA7-D175K within the crystallographic asymmetric unit coordinate two
 191 IP(3,4,5)P₃ moieties sandwiched between them: One at the site I, and the second associated more
 192 peripherally with yet another electropositive patch – denoted here as site III – formed by the side chains of
 193 K183, R185, K198, and R201 that protrude from β 2 and from the β 3- β 4 loop (Fig. S6). The structure reflects
 194 at least four distinct modes of IP binding by PHA7. In molecule A of the asymmetric unit the phosphate
 195 group at the fifth position of the inositol ring occupies the same position as the sulfate anion in the structure
 196 of wild-type PHA7s, while in molecule B the binding geometry is flipped such that it is the phosphate group
 197 in first position that coincides with the sulfate binding site (Fig. S6B, C). Binding of the peripheral
 198 IP(3,4,5)P₃ at site III is similarly mirrored in each copy of PHA7-D175K, resulting in two possible binding

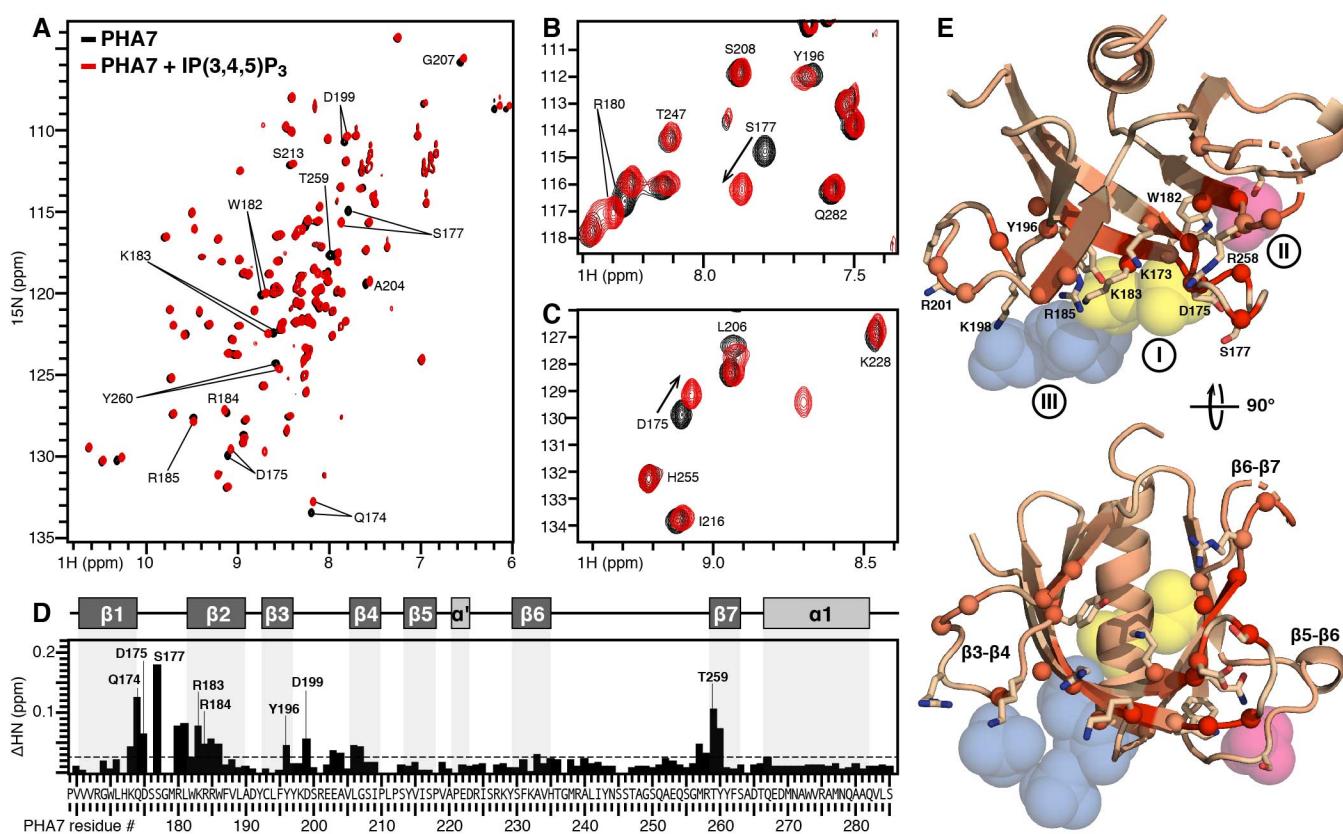


Fig. 3. Mapping the interaction of PHA7 with soluble IP(3,4,5)P₃. (A-C) $^1\text{H}/^{15}\text{N}$ HSQC NMR spectra of ^{15}N labeled wild-type PHA7 domain acquired with (red) or without (black) 1.5 molar equivalents of soluble IP(3,4,5)P₃. The spectra were acquired at 15°C. Selected regions of the spectra are expanded (B) to highlight specific perturbation sites. (D) Profile of $^1\text{H}/^{15}\text{N}$ chemical shift perturbations induced by IP(3,4,5)P₃ across the sequence of PHA7. Bars represent the combined difference (ΔHN) of amide ^1H and ^{15}N chemical shifts. The protein secondary structure is outlined at the top. (E) Orthogonal views of the structure of PHA7_{APo}. Colors reflect the magnitude of ΔHN from 0 ppm (wheat) to the maximum value (red). Highly perturbed sites (> 1 standard deviation of the values of ΔHN) have CA atoms shown as spheres. Key side chains of three IP binding sites (I-III) are shown as sticks. The positions of sulfate or phosphate groups identified in the structures of PHA7s and PHA7-D175K are shown as spheres, superimposed on the structural model. They denote binding sites I (yellow), II, (pink) and III (blue).

199 geometries. Moreover, the involvement of K183 and R185 in both sites I and III reflects plasticity in the
200 PHA7-IP interaction.

201 To further examine the interaction of wild-type PHA7 with IPs, we performed NMR experiments in
202 solution. Addition of free IP(3,4,5)P₃ into ¹⁵N-labeled PHA7 resulted in specific ¹H/¹⁵N chemical shift
203 perturbations that map to the PHA7 barrel opening (Fig. 3A-B). In line with the ITC data, NMR reflects a
204 weak binding interaction with fast exchange binding dynamics (Williamson, 2013). No major structural
205 reorganization of either the core or the β 6- β 7 loop of PHA7 is observed. Notably, however, signals from all
206 three binding sites identified by crystallography are perturbed (Fig. 3D, E), with the largest changes observed
207 for the β 1- β 2 and β 3- β 4 loops and residues at the start of β 7, including key residues (Q174, D175, S177,
208 K183, R185, Y260) identified in the structures of PHA7_S and PHA7-D175K. We conclude that the
209 interaction of PHA7 with soluble IP headgroups is structurally plastic, with three potential binding sites for
210 inositol phosphate localized across the electropositive barrel opening, and low selectivity for a specific
211 orientation of the IP(3,4,5)P₃ moiety.

212 **Structural basis for binding membrane PIPs.** The structural and ITC binding data show that PHA7 has
213 modest affinity for freely soluble IP ligands but appreciably higher affinity for PIP-rich lipid bilayers,
214 pointing to the importance of the PIP membrane environment. To explore the interaction with PIP
215 membranes at the atomic level, we performed NMR experiments with ¹⁵N labeled PHA7 and PIP-nanodiscs.
216 Incubation of PHA7 with diC16-PC nanodiscs, resulted in no detectable NMR spectral changes (Fig. 4A;
217 S7A). This is consistent with the liposome co-sedimentation results and confirms the inability of PHA7 to
218 bind membranes devoid of PIP. Incubation with nanodiscs containing 10% molar diC16-PI(3,4,5)P₃ gave a
219 dramatically different result: The NMR spectrum was effectively obliterated, except for peaks from sites in
220 the termini and the flexible β 6- β 7 loop (Fig. 4B; S7B). We interpret such massive peak suppression to reflect

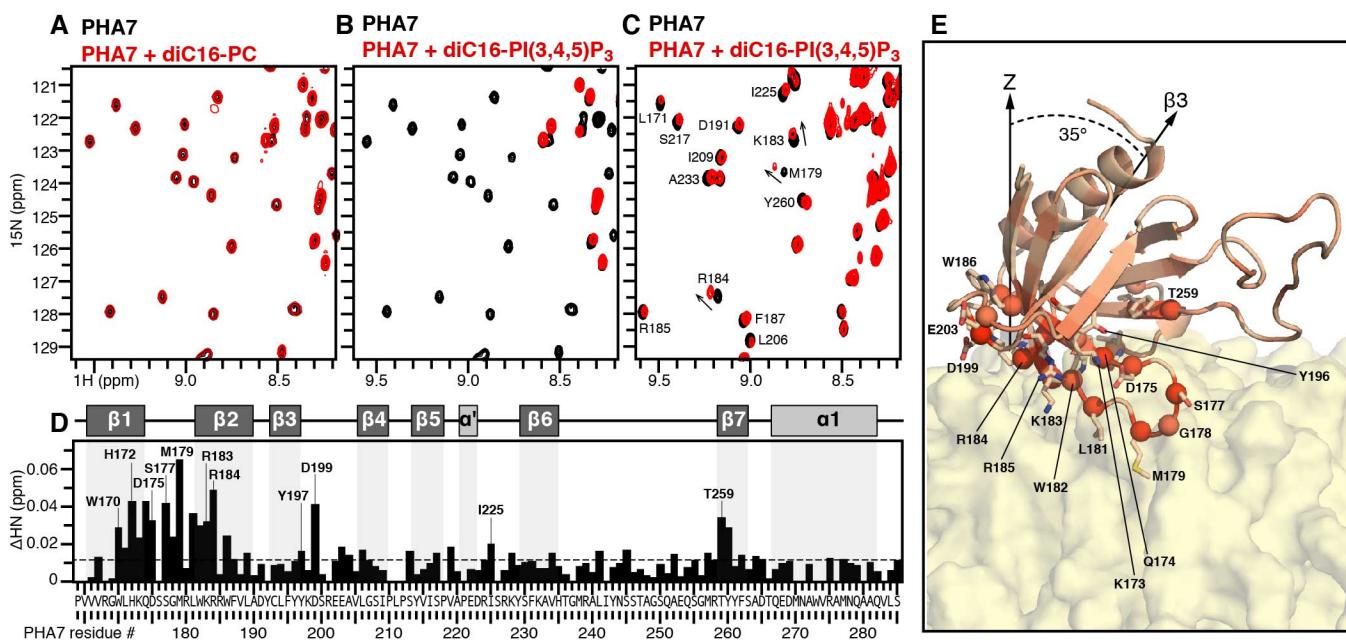


Fig. 4. Mapping the interaction of PLEKHA7 with diC16-PI(3,4,5)P₃ nanodiscs. (A-C) ¹H/¹⁵N HSQC NMR spectra of ¹⁵N-labeled wild-type PHA7 obtained before (black) or after (red) incubation with 1.5 molar equivalents of lipid nanodiscs prepared with 100% diC16-PC (A), or 9/1 molar diC16-PC and diC16-PI(3,4,5)P₃ (B, C). The spectra were acquired at 15°C (A, B) or 45°C (C). (D) Profile of ¹H/¹⁵N chemical shift perturbations induced by the association of PHA7 with diC16-PI(3,4,5)P₃ nanodiscs, at 45°C, across the protein sequence. (E) Snapshot (taken at 890 ns) of a MD simulation of PHA7 with a diC16-PI(3,4,5)P₃-rich lipid bilayer membrane. PHA7 colors reflect the magnitude of NMR chemical shift perturbation from 0 ppm (wheat) to the maximum value (red). Highly perturbed sites (> 1 standard deviation of the values of Δ HN) have CA atoms shown as spheres and side chains shown as sticks. The membrane is represented as a molecular surface.

221 immobilization of the relatively small, 125-residue protein, caused by its association with the comparatively
 222 large nanodisc membrane. Moreover, since both PC and PIP nanodisc preparations have similar and
 223 homogeneous size distributions (~8 nm radius; Fig. S8), we attribute the peak suppression effect to a PIP-
 224 dependent association of the protein with the nanodisc membrane that is long-lived on the msec time scale of
 225 $^1\text{H}/^{15}\text{N}$ correlation NMR experiment.

226 Increasing the temperature restored the NMR spectrum (Fig. 4C; Fig. S7C), and while this is expected to
 227 enhance the binding exchange dynamics, it also allowed us to map specific chemical shift perturbations that
 228 reflect the interaction of PHA 7 with the PIP nanodisc membrane. The perturbations (Fig. 4D) mirror the
 229 profile observed with free IP(3,4,5)P₃ (Fig. 3C), but map to more extended regions of β 1 and β 2, and include
 230 sites in β 5- β 6. The largest perturbations map to the β 1- β 2 hairpin, the β 3- β 4 loop, and the start of β 7,
 231 implicating binding sites I, II and III in PIP-mediated membrane association, and indicating that PHA7 uses
 232 all three to dock onto the PIP-membrane surface.

233 All atom MD simulations provide molecular context for the NMR data. We performed ten independent,
 234 unrestrained, 1- μ s MD simulations of PHA7 for each of three different lipid bilayer membranes similar to
 235 the experimental nanodiscs (Table S2). All ten simulations with either PI(4,5)P₂ or PI(3,4,5)P₃ resulted in
 236 rapid membrane surface association of PHA7, which remained membrane-bound throughout the course of 1-
 237 μ s simulation, while little evidence of binding to PC-only membranes was observed (Fig. S9). In all cases,
 238 PHA7 adopts a preferred orientation at the PIP membrane surface, with the β 3 strand at an average angle of
 239 35° from the membrane normal, the C-terminal helix exposed to bulk water, and sites I, II and III docked on
 240 the membrane (Fig. 4E; Fig. S10).

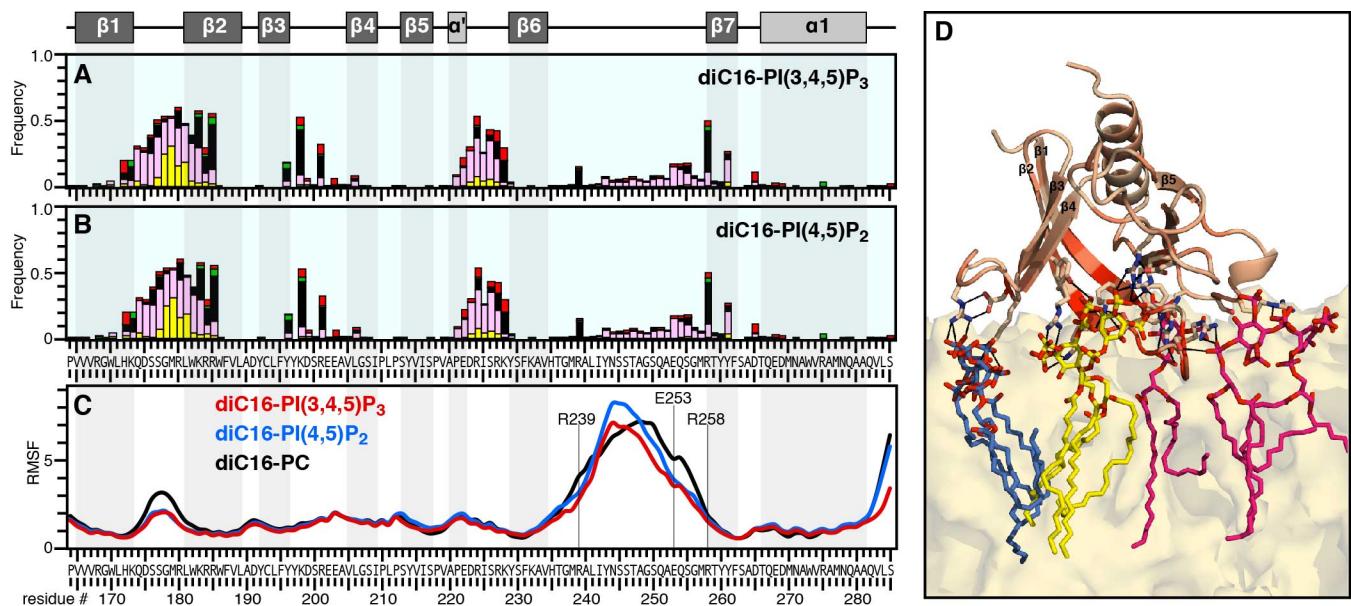


Fig. 5. Structure and dynamics of the PLEKHA7 PH domain. (A, B) MD interaction profile of PHA7 with diC16-PI(3,4,5)P₃ or diC16-PI(4,5)P₂ membranes. The bars represent frequency of occurrence within 4 Å of water (light blue), Na⁺ (red) or Cl⁻ (green) ions, phospholipid tails (yellow), PC headgroups (pink), or PIP headgroups (black). Each data point is the average of ten independent MD simulations over the last 500 ns of 1- μ s MD trajectories. (C) Time-averaged RMSF calculated for PHA7 heavy atoms over the last 500 ns of 1- μ s MD trajectories for ten independent simulations in diC16-PI(3,4,5)P₃ (red), diC16-PI(4,5)P₂ (blue), or diC16-PC (black) membranes. (D) Snapshot (taken at 890 ns) of MD simulation of PHA7 with a diC16-PI(3,4,5)P₃ membrane. PHA7 colors reflect the magnitude of NMR chemical shift perturbation from 0 ppm (wheat) to the maximum value (red). Key side chains with close (< 4 Å) contacts to PIP headgroups are shown as sticks. The membrane is represented as a molecular surface (yellow). Colors of bound PIP molecules (lines) represent their association with binding sites I (yellow), II (pink), or III (blue).

241 Notably, the interaction patterns from unrestrained MD simulations mirror the experimental NMR
242 perturbation profile (Fig. 5A, B). Protein sites with NMR signals that are sensitive to PIP-nanodiscs also
243 interact with the PIP membrane surface in the MD simulations. Viewed in a snapshot of MD simulation with
244 a PI(3,4,5)P₃ membrane (Fig. 4E), the NMR perturbations are associated with membrane-binding sites.
245 These include conserved hydrophobic residues (L181, M179) in the β 1- β 2 loop that insert deeply into the
246 hydrophobic core of the membrane, thereby providing added stability to the interaction. The PH domain
247 orientation at the membrane surface is remarkably similar to that observed from EPR-guided MD
248 simulations of GPR1 with PIP membranes (Lai et al., 2013). In that study, hydrophobic interactions between
249 GPR1 side chains and the membrane core were also observed, indicating that superficial membrane
250 penetration helps stabilize a specific geometry of PH domain association with the membrane surface.

251 In line with this observation, the time-averaged root mean-squared fluctuations (RMSF), calculated for
252 heavy atoms over the last 500 ns of 1- μ s MD trajectories, show that the β 1- β 2 loop experiences a marked
253 reduction in conformational flexibility upon binding PIP membranes (Fig. 5C). By contrast this effect is not
254 observed for PC membranes. Moreover, while the long β 6- β 7 loop remains highly flexible in both the
255 soluble and membrane-associated states of PHA7, the width of its flexible region is significantly reduced. At
256 the molecular level, the loop fluctuations are damped at its edges, where R239 and R258 engage with a
257 PIP phosphate group and E253 helps stabilize the interaction.

258 Finally, closer examination of the PHA7-PIP membrane assembly reveals multivalent interactions of the
259 protein with PIPs. A total of seven PI(3,4,5)P₃ molecules establish close contacts ($> 4\text{\AA}$) with the protein:
260 Two PIP molecules associate with each of sites I and III, and three associate in the periphery of site II (Fig.
261 5D; Fig. S11). In this way, PHA7 recruits a cluster of PIP molecules to its periphery. The data demonstrate
262 that PHA7 established multivalent interactions with membrane PIPs. These are primarily electrostatic,
263 involving basic sidechains and inositol ring phosphate groups, but also include hydrogen bonds from polar
264 side chains, and hydrophobic contacts between nonpolar side chains and the lipid acyl chains. These results
265 explain the enhanced affinity of PHA7 for PIP membranes compared to freely soluble IPs.

266
267

Conclusions

268 PLEKHA7 relies on the interactions of its PH domain with PIPs for proper localization to the plasma
269 membrane surface and for its normal functions (Wythe et al., 2011). Using a multidisciplinary approach, we
270 have defined the major elements of PIP recognition by the PLEKHA7 PH domain and demonstrated the
271 important role of the membrane scaffold in mediating its interaction with PIPs.

272 Although PH domains share a common fold and sequence homology, their PIP affinities and selectivities
273 vary broadly. ITC shows that PLEKHA7 has modest affinity for free IP headgroups and no detectable
274 selectivity for phosphorylation at specific inositol ring sites. By contrast, the binding affinity for PIP
275 membranes is highly enhanced. Compared to freely soluble IP headgroups, the interaction with membrane
276 PIPs is enthalpy-driven and exhibits slight selectivity for PI(4,5)P₂ ($> \text{PI}(3,4,5)P_3 > \text{PI}(3,4)P_2$) relative to the
277 other phosphorylation types. PI(4,5)P₂ is the major PIP lipid present in the inner leaflet of the plasma
278 membrane of mammalian cells. Its phosphorylation by PI 3-kinase (PI3K) produces PI(3,4,5)P₃, a key
279 second messenger in pathways related to cell survival and metabolism, while dephosphorylation of
280 PI(3,4,5)P₃ by phosphatases produces PI(3,4)P₂ (Cantley, 2002). Low PIP selectivity is in line with the
281 observations (Wythe et al., 2011) that PLEKHA7 membrane targeting is not linked to the PI3 kinase-
282 dependent conversion of PI(4,5)P₂ to PI(3,4,5)P₃ in cells, and that its PH domain is promiscuous for PI(4)P₁,
283 PI(4,5)P₂, PI(3,4,5)P₃, as well as other non-inositol phospholipids with accessible phosphate groups, in lipid
284 overlay assays.

285 The crystal structures reveal three positively charged binding sites for the phosphate groups of the PIP
286 headgroup moiety, and the NMR data show that all three sites engage with soluble IP headgroups, forming
287 an extended binding surface at the open end of PH domain β -barrel. Notably, NMR studies with PIP
288 nanodiscs show that the same binding sites, plus conserved hydrophobic residues in the β 1- β 2 loop, engage

289 with membrane-embedded PIPs leading to membrane surface association of the PH domain that is long-lived
290 on the msec time scale. Moreover, the MD simulations show that PLEKHA7 interacts with the PIP
291 membrane in two ways: by engaging the phosphate groups of multiple PIP molecules through electrostatic
292 interactions with its three binding sites, and by inserting hydrophobic side chains into the hydrophobic core
293 of the lipid bilayer membrane. This multivalent binding interaction results in long-lived association of PH
294 domain with the PIP membrane, on the μ s time scale of the MD simulations.

295 Our results indicate that PLEKHA7 induces membrane PIP clustering. We note that our simplified
296 nanodiscs and membrane systems have both restricted geometries and PIP concentrations that are ten times
297 higher than cells – conditions that are likely to favor cluster formation. In cells, the reverse situation, where
298 PLEKHA7 binds to pre-formed PIP clusters, is also possible. While the cellular levels of PI(4,5)P₂ are low
299 (~1% of total lipid), clustering is thought to increase its local concentration at specific membrane sites. A
300 recent study (Wen et al., 2018) demonstrated that physiological levels of divalent and trivalent metal ions
301 can induce cluster formation of very low PIP concentrations (< 0.05 mol% of total lipids). The ability to
302 form such cation-bridged PIP clusters at extremely low concentrations reveals an important property of this
303 central lipid and provides evidence for the formation of distinct pools of PIP in cellular membranes, with
304 fundamental consequences for biological function. As noted (Wen et al., 2018), cation-induced PIP
305 clustering would dampen the functions of proteins that bind free PIP lipids while enhancing the functions of
306 proteins that bind clusters preferentially.

307 Clustering of PIP molecules around PH domains has also been observed in coarse-grained MD
308 simulations, and has been proposed to contribute to the binding free energy (Yamamoto et al., 2020).
309 Notably, two cooperative PIP-binding sites have been observed experimentally in the PH domain of ASAP1,
310 and this has been proposed to enable rapid switching between active and inactive states during cellular
311 signaling (Jian et al., 2015). For PLEKHA7, we identified three binding sites, and found that all three are
312 engaged upon addition of either soluble IPs or PIP membranes, leading us to conclude that multivalent
313 association is operative in both settings. Nevertheless, the membrane assembly is fundamentally important.
314 PLEKHA7 establishes additional hydrophobic interactions with the membrane hydrocarbon core and adopts
315 a preferred orientation at the membrane surface. Moreover, confinement of PIP molecules by the membrane
316 scaffold reduces the degrees of freedom of the system to a single binding interface. We propose that these
317 factors cooperate to lower the binding free energy and enhance the affinity of protein association with PIP
318 membranes.

319 While most experimental studies have focused on the interactions of PH domains with soluble IP
320 headgroups, our results provide atomic-level insights about the affinity of PLEKHA7 for full-length
321 membrane-embedded PIPs. They highlight the central function of the membrane assembly and provide a
322 roadmap for understanding how the functions of the PH domain integrate with the signaling, adhesion and
323 nanoclustering functions of full-length PLEKHA7 in cells.

324 **Methods**

326 **Protein preparation.** Protein sequences (Fig. S1) of human PLEKHA7 were cloned into the BamHI
327 and XhoI restriction sites of the pGEX-6P-1 plasmid, and expressed in *E. coli* BL21 cells. For
328 crystallography and ITC the bacterial cells were grown at 37°C, in LB media. For NMR studies, the bacteria
329 were grown in M9 minimal media containing (¹⁵NH₄)₂SO₄ and/or ¹³C-glucose (Cambridge Isotope
330 Laboratories), to obtain isotopically labeled protein. Protein expression was induced by adding 1 mM
331 isopropyl-1-thio- β -D-galactopyranoside to the culture when the cell density reached OD₆₀₀ = 0.6. After
332 growing the cells for an additional 15 hrs at 18°C, they were harvested by centrifugation (6,000 x g, 4°C,
333 15 min) and stored at -80°C overnight.

334 Cells harvested from 2 L of culture were suspended in 35 mL of buffer A (25 mM Na/KPO₄, pH 7.4,
335 200 mM NaCl, 2 mM DTT, 1 mM EDTA), supplemented with protease inhibitors (cComplete Mini EDTA-
336 free cocktail; Roche), and lysed using a French Press. The soluble fraction was isolated as the supernatant
337 from centrifugation after cell lysis, and purified by passing first on Glutathione Sepharose beads (GE

338 Healthcare), and second by cation exchange chromatography (HiTrap SP column, 5 ml, GE Healthcare) with
339 a linear gradient of NaCl in buffer B (20 mM Tris-Cl, pH 8, 1 mM DTT, 1 mM EDTA). The protein was
340 concentrated to 10 mg/ml and stored frozen at -80°C. Purified protein was transferred to appropriate buffers
341 by size exclusion chromatography (Superdex 75 10/300, GE Healthcare) and stored at 4°C.

342 **Nanodisc preparation.** Briefly, the phospholipids were dissolved in 1 mL of nanodisc buffer (20 mM
343 Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA) supplemented with Na-cholate to obtain a final 1:2 molar ratio
344 of lipid to cholate. MSP1D1 Δ h5 was produced in *E. coli*, as described (Hagn et al., 2013), dissolved in
345 700 μ L of nanodisc buffer, and combined with the lipid solution. After incubation at room temperature for
346 1 hr, Biobeads SM-2 (Biorad; 2 g; prewashed in nanodisc buffer) were added, and the mixture was further
347 incubated at room temperature, with gentle mixing, for 12 hr. The Biobeads were removed by centrifugation
348 and the resulting nanodiscs were washed twice with one sample volume of nanodisc buffer. The nanodisc
349 solution was concentrated using a 10 kD cutoff Vivaspin concentrator (Viva Products) and replaced with
350 NMR buffer to obtain 500 μ L of 0.2 mM nanodiscs. The nanodisc concentration was estimated by measuring
351 A₂₈₀ of MSP1D1 Δ h5, of which there are two copies per nanodisc. The distribution of PIP molecules among
352 nanodiscs is assumed to be homogeneous, but this may not be the case. Nanodiscs were prepared with 100%
353 diC16-PC, or 9/1 molar mixtures of diC16-PC with diC16-PI(3,4,5)P₃, diC16-PI(4,5)P₂, or diC16-PI(3,4)P₂.
354 Analytical size exclusion chromatography (Superdex 75 10/300 GL column, GE Healthcare) was performed
355 in NMR buffer to assess nanodisc size homogeneity (Ding et al., 2015).

356 **ITC experiments.** ITC experiments were performed at 23°C, with all components in ITC buffer
357 (15 mM Tris pH 7.6, 70 mM NaCl, 0.5 mM TCEP), using an iTC200 instrument (MicroCal). Titrations with
358 soluble IP headgroups were performed with 50 μ M PHA7 in the ITC cell and 0.5 mM soluble IP molecules
359 in the injection syringe. Titrations with PIP nanodiscs were performed with 15 μ M nanodiscs in the ITC cell
360 and 0.5 mM PHA7 in the injection syringe. The protein concentrations were estimated by measuring
361 absorbance at 280 nm (A₂₈₀). Nanodisc concentrations reflect the A₂₈₀ measurement of MSP1D1 Δ h5.
362 Integrated heat data were processed and analyzed with ORIGIN software (Microcal). The data were fit to a
363 single-site binding model to extract the values of the dissociation constant for each titration.

364 **Liposome co-sedimentation assays.** Dry lipids, either 100% diC16-PC, or a 9/1 molar mixture of
365 diC16-PC and diC16-PI(4,5)P₂, were suspended in 2 mL of buffer (20 mM MES pH 6.0, 100 mM NaCl,
366 2 mM DTT, 1 mM EDTA) at a concentration of 3 mg/mL, then sonicated in a bath sonicator until the
367 suspension became translucent, marking the formation of small unilamellar vesicles. A 200 μ L solution of
368 vesicles was mixed with a 20 μ M solution of PLEKHA7 and incubated at room temperature for 2 h.
369 Liposomes were then harvested by centrifugation in a BECKMAN Airfuge (A-100/30 rotor, 91,000 rpm) for
370 20 h. The supernatant and sediment fractions were separated and analyzed by SDS-PAGE.

371 **Crystallization, X-ray data acquisition and structure determination.** All PHA7 crystals were
372 obtained using the sitting drop method. For ligand-free PHA7_{APO}, protein solution (13 mg/ml in 180 mM
373 NaCl, 20 mM Tris pH 8, 50 mM BisTris pH 6.0, 0.7 mM TCEP, 6 mM Na azide) was mixed with an
374 equivalent volume of crystallization solution (20% PEG 3350, 20 mM MgCl₂, 20 M NiCl₂, 100 mM HEPES
375 pH 7) and equilibrated at room temperature. For PHA7_s, protein solution (30 mg/ml in 180 mM NaCl,
376 20 mM sodium phosphate pH 6.5, 5 mM DTT) was mixed with an equivalent volume of crystallization
377 solution (25% glycerol, 2 M (NH₄)₂SO₄) and equilibrated at room temperature. Crystals were frozen without
378 the addition of a cryoprotectant. For PHA7-D175K, 1.5 mM IP(3,4,5)P₃ and protein solution (15 mg/ml in
379 180 mM NaCl, 20 mM Tris pH 8, 30 mM BisTris pH 6, 0.5 mM TCEP, 6 mM Na azide) were mixed with an
380 equivalent volume of crystallization solution (20% PEG 3350, 200 mM Na acetate) and equilibrated at room
381 temperature. Crystals were frozen after addition of glycerol to final concentration 20% v/v.

382 X-ray diffraction data for PHA7_{APO} and PHA7_s were collected at the Advanced Light Source (Berkeley,
383 CA) beamline 8.3.1, with a wavelength 1.116 Å and temperature of 100 K. The data for PHA7-D175K were
384 collected on a Rigaku diffractometer with R-axis detector, with a wavelength of 1.54 Å and temperature of
385 100 K. The data were processed using the CCP4 suite (Winn et al., 2011), to resolution of 2.80 Å
386 (PHA7_{APO}), 1.45 Å (PHA7_s) and 2.43 Å (PHA7-D175K). The structure of PHA7_s was solved first, with

387 molecular replacement guided by the structure of PEPP1 (PDB: 1UPR; 52% identity). Phenix.AutoBuild
388 (Adams et al., 2010) was used for initial model building, followed by several rounds of manual model
389 inspection and correction in Coot (Emsley et al., 2010) and refinement by phenix.refine (Adams et al., 2010)
390 and Refmac5. The structures of PHA7_{APO} and PHA7-D175K were solved using Phaser (52) using the
391 structure of PHA7_S as molecular replacement model, and refined as for PHA7_S.

392 Molprobity (Chen et al., 2010) and the PDB validation server were used for structure validation
393 throughout refinement. All structures had Ramachandran statistics with more than 95% of residues in
394 favored positions and less than 1% outliers. Poisson-Boltzmann electrostatics were calculated in PyMOL
395 using APBS (Baker et al., 2001). Illustrations were prepared using PyMol.

396 **NMR experiments.** Samples for NMR studies were transferred to NMR buffer (20 mM MES pH 6.0,
397 100 mM NaCl, 1 mM TCEP, 1 mM EDTA). For studies of PLEKHA7 with nanodiscs, the purified PH
398 domain was added directly to preformed nanodiscs prepared in NMR buffer. Assignments of the solution
399 NMR resonances from N, HN, CA and CB were obtained using HNCA (Grzesiek and Bax, 1992) and
400 HNCACB (Wittekind and Mueller, 1993) experiments. Chemical shifts were referenced to the H₂O
401 resonance (Cavanagh et al., 1996). Secondary structure was characterized by analyzing the chemical shifts
402 with TALOS+ (Cornilescu et al., 1999; Shen et al., 2009). The total differences (Δ HN) in amide ¹H and ¹⁵N
403 chemical shifts due to the C-terminus or peptide binding were calculated by adding the changes in ¹H (Δ H)
404 and ¹⁵N (Δ N) chemical shifts using the equation Δ HN= (1/2) [(Δ H)² + (Δ N/5)²]. The NMR data were
405 processed and analyzed using NMRPipe (Delaglio et al., 1995), Sparky (Goddard and Kneller, 2004) and
406 NMRView (Johnson and Blevins, 1994). The NMR pulse sequences are described in detail in the literature
407 (Bax and Grzesiek, 1993; Cavanagh et al., 1996; Clore and Gronenborn, 1998; Ferentz and Wagner, 2000;
408 Fesik and Zuiderweg, 1990; Kay, 2001).

409 **Molecular dynamics simulations.** All-atom MD simulations were performed using the
410 CHARMM36(m) force fields for protein and lipids (Brooks et al., 2009; Klauda et al., 2010), with the TIP3P
411 water model (Jorgensen et al., 1983) in 100 mM NaCl. All systems were prepared using CHARMM-GUI
412 *Solution Builder* and *Membrane Builder* (Jo et al., 2008; Jo et al., 2009; Lee et al., 2019), and equilibrated
413 with the CHARMM-GUI standard protocol. The temperature and pressure were maintained at 318.15 K and
414 1 bar. MD production simulations were conducted with OpenMM (Eastman et al., 2013) for 1 μ s, and the
415 last 500 ns of trajectories were used for analysis. The initial structural model was taken from the crystal
416 structure of PHA7_S; all ligands were removed and residues 236-254 were modeled using GalaxyFill.

417 Ten MD simulations were performed for each of the three membrane systems (Table S3), for a total of
418 thirty independent simulations. The initial system size of each replica was 90 Å x 90 Å x 175 Å to
419 accommodate for all initial components, yielding a total of approximately 125,000 atoms per replica. All
420 analyses were performed using CHARMM, and models were visualized with VMD and PyMOL.

421

422 Data availability

423 The structural atomic coordinates have been deposited in the Protein Data Bank with accession codes
424 7KK7, 7KJO and 7KJZ. The assigned NMR chemical shifts have been deposited in the BMRB databank
425 with accession code 50512.

426

427 Acknowledgments

428 This work was supported by grants from the National Institutes of Health (GM118186, CA179087,
429 CA160398, CA030199) and the National Science Foundation (MCB1810695).

430

431 **Declaration of interests**

432 The authors declare that they have no conflicts of interest with the contents of this article. The content is
433 solely the responsibility of the authors and does not necessarily represent the official views of the National
434 Institutes of Health.

435

436 **Author contributions**

437 F.M.M., R.C.L. and G.P. conceived and guided the project.
438 A.E.A. performed crystallography and determined the structures.
439 Y.Y and J.Y. performed NMR experiments and data analysis.
440 A.E.A. G. C., M.G.K. and L.A.B. prepared samples
441 A.A. B. performed ITC experiments and data analysis.
442 W.I. guided the MD simulations.
443 A.I. and C.D. performed and analyzed the MD simulations.
444 F.M.M. prepared figures and wrote the manuscript.
445 All authors discussed the results and contributed to manuscript preparation.

446

447 **Additional information**

448 **Supplementary information** is available for this paper.
449 **Correspondence and requests for materials** should be addressed to F.M.M.

450

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