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SPECIAL REPORT

The phosphoinositide code is read by a plethora of protein domains

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ABSTRACT

Introduction: The proteins that decipher nucleic acid- and protein-based information are well known, however, those that read membrane-encoded information remain understudied. Here, we report 70 different human, microbial and viral protein folds that recognize phosphoinositides (PIs), comprising the readers of a vast membrane code.

Areas covered: Membrane recognition is best understood for FYVE, PH and PX domains, which exemplify hundreds of PI code readers. Comparable lipid interaction mechanisms may be mediated by kinases, adjacent C1 and C2 domains, trafficking arrestins, GAT and VHS modules, membrane-perturbing annexins, BAR, CHMP, ENTH, HEAT, syntaxin and Tubby helical bundles, multipurpose FERM, EH, MATH, PHD, PDZ, PROPPIN, PTB and SH2 domains, as well as systems that regulate receptors, GTPases and actin filaments, transfer lipids, and assemble bacterial and viral particles.

Expert opinion: The elucidation of how membranes are recognized has extended the genetic code to the PI code. Novel discoveries include PIP-stop and MET-stop residues to which phosphates and metabolites are attached to block phosphatidylinositol phosphate (PIP) recognition, memteins as functional membrane protein apparatuses and lipidons as lipid 'codons' recognized by membrane readers. At least 5% of the human proteome senses such membrane signals and allows eukaryotic organelles and pathogens to operate and replicate.

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Phosphoinositide code; lipid trafficking; signal transduction; FYVE domain; pleckstrin homology domain; phox homology domain; membrane reader; structural biology

1. Introduction

Membranes are read by protein domains that selectively bind the lipids that uniquely mark each subcellular organelle and plasma membrane compartment. These conserved domains mediate reversible attachment of proteins to membranes in order to facilitate the assembly and disassembly of signaling and trafficking complexes in response to changes in lipid concentration and locality. The best characterized are the FYVE, PH, and PX domain superfamilies, which exemplify the PI code that controls eukaryotic membrane recognition [1–3]. This overview of the literature reveals a growing array of soluble, folded domains, which are known to recognize various PI lipids and thus localize and mediate signaling, macromolecular assembly, and trafficking functions on most eukaryotic membrane-bound compartments (Table 1).

Here we review all protein families that recognize dynamic PI-containing membrane surfaces. While some mechanisms remain unclear or controversial, this analysis suggests how extensively the PI code organizes the cell's inner workings. These 2D codes within lipid bilayers are more complex than the 1D templates of nucleotide sequences used to replicate and translate genetic information. The PI code has remained elusive by virtue of the non-covalently attached, diffusible lipid sets that move inside and between the bilayers of organelles which are also challenging to purify together with their partners. Moreover, PI-binding domains may rely on coincidence detection to amplify otherwise weak membrane proclivities which remain difficult to detect. New facets have only recently been described including how membrane readers are regulated by PIP-stop and MET-stop residues to which phosphates and metabolites are covalently attached to toggle lipid-binding functions [19,20]. Elaborating the PI code continues to be a challenge but has been aided by the identification of new membrane readers, lipid-binding modes, and post-translational modifications (PTMs) as well as advances in biophysical, structural, and computational technologies for resolving complexes.

2. Tools for Unraveling the PI Code

The development of new tools to visualize and manipulate membrane readers bound to lipids and membranes at atomic resolution has revolutionized our understanding of biology. The recognition mechanisms involve hundreds of electrostatically polarized domains ranging from 50 to 300 residues. They present basic surfaces that bind patches of lipid molecules while inserting hydrophobic groups into bilayers of various fluidities and curvatures. Complementary insights of these multivalent engagements are provided by NMR spectroscopy, X-ray crystallography, cryo-electron microscopy (cEM) and cryo-electron tomography (cET), thus exposing their complex mechanisms as outlined here. The application of computational tools including Membrane Optimal Docking Area

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Table 1. PI binding domain abbreviations, full names, and number of host proteins based on Interpro and Prosite [4] databases. The number of domain-containing proteins found in homo sapiens (*H.s.*) are listed, as are several domains in bacterial and viral proteins. All PI ligands are abbreviated by the positions of phosphates on their inositol rings, e.g. '1' or '345' columns indicate recognition of PtdIns or $PI(3,4,5)P_3$, respectively, by at least one family member.

Abbrev.	Full Domain Name	*H.s.*	1	3	4	5	3 4	3 5	4 5	345	Selected PDBs entries	References
ADF-H	actin-depolymerizing factor homology	10									1TVJ	[181]
Annexin	Annexin	14							45		5LPU	[119–121]
AP	Adaptor Protein	6							45		2VGL, 6YAF	[5,104,105]
APT1	Aberrant Pollen Transmission 1	4		3	4	5					-	[189–191]
Arrestin	Arrestin	4					34		45	345	1ZSH, 5TV1	[116–118]
BAR	Bin–Amphiphysin–Rvs	44							45		4WPC	[89,90,93]
BATS	Barkor/Atg14 autophagosome targeting sequence	1		3					45		7BL1	[122,124]
BD	Basic Domain (of Diaph2)	1							45		-	[175,176]
BH	Break-point cluster region Homology	66		3	4	5			45	345	-	[147]
C1	protein kinase C conserved region 1	60							45	345	-	[82]
C2	protein kinase C conserved region 2	145			4		34		45	345	5LO8	[83]
CapG	Macrophage-capping protein	1							45		1JHW, 1J72	[183,184]
CH	Calponin Homology	68							45	345	-	[6,178,179]
CHMP-BD	CHarged Multivesicular body Protein Basic Domain	10					34	35	45		-	[7,111–113]
DHDD	Asp-Asp-His-Asp motif-containing	6			4						-	[162,163]
DHR-1	Dock Homology Region-1	11							45	345	7CLY, 3L4C	[87,88]
EH	Eps15 Homology	11			4		34	35	45	345	-	[136–138]
ENTH	Epsin N-terminal homology	8							45	345	1H0A, 1INZ, 5ON7	[101–103]
FACPB	F actin capping beta	1		3					45		-	[185,186]
FERM	Band 4.1 protein, ezrin, radixin, moesin	48						35	45	345	6MFS	[80,81]
F-BAR	Fes/Cip4 homology Bin/amphiphysin/Rvs	25							45		2EFK	[93,96,97]
FGF	fibroblast growth factor	20							45		-	[165]
FYVE	Fab1, YOTB, Vac1 and EEA1	34		3			34		45	345	1JOC, 1HYI	[8–10]
GasderminN	Gasdermin N-terminal	5							45		5B5R	[100]
GAT	GGA and Tom1	6		3	4						1NAF	[106,107]
GBD	GTPase binding domain	18							45	345	-	[175]
GLA	γ-carboxyglutamate-rich	7			4						-	[11]
GLTP	GlycoLipid Transfer Protein	5							45		-	[159]
GLUE	GRAM-Like Ubiquitin-binding in EAP45	1		3						345	2HTH, 2DX5	[77–79]
GOLPH3	Golgi phosphoprotein 3	2			4						3KN1	[188]
GRAM	Glucosyltransferases, Rab-like GTPase activators & myotubularins	21		3		5		35		345	-	[75]
GRK	G-protein coupled receptor kinase	7							45		4TNB, 4TND, 6PJX	[143,144]
HEAT	Huntingtin/Elongation factor 3/phosphatase 2A A subunit/TOR1	2					34	35	45	345	6X9O, 7DXK, 7K1Y	[12,125,127,128]
I-BAR	Inverse Bin–Amphiphysin–Rvs	5							45		1WDZ, 4NQI	[93–95]
IPK	Inositol PhosphoKinase	7							45		5W2I	[148]
JM	Juxtamembrane domain (of epidermal growth factor receptors)	3							45		2M20	[23]
LBD	Ligand Binding Domain (of nuclear receptors)	48							45	345	1YOW, 4QK4, 4RWV	[160,161]
MATH	Meprin And TRAF Homology	12							45	345	3ZJB	[139]
NBB	N-terminal Bundle and Brace	1							45	345	-	[149,150]
ORD	Oxysterol binding protein-Related Domain	12							45		5ZM8	[164]
PDZ	Postsynaptic density 95, Disk large, Zonula occludens	149					34		45	345	4Z33	[129,130]
PH	Pleckstrin Homology	247		3	4		34	35	45	345	2MDX, 5C79	[64,65]
PHD	Plant HomeoDomain finger	72		3	4	5		35	45		-	[13,62,63]
PI 3-/4-K	Phosphatidylinositol 3/4-kinase	13	1						45		4OVV, 4HND, 4HNE, 4PLA, 6BQ1	[14,15,145,146]
PPR	PentatricoPeptide Repeat	7						35			-	[38]
PROPPIN	β-propellers that bind PIs	4		3				35			6IYY, 4EXV	[140–142]
PTB	PhosphoTyrosine Binding	43							45		-	[76]
PX	Phox homology	49		3	4	5	34	35	45	345	6KOJ, 2MXC, 2RAK	[3,16,44,48]
Ras	Rat sarcoma	36							45		-	[166]
REM-CA	REM C-terminal anchor	0			4						-	[123]
RGS	Regulators of G-protein signaling	35								345	-	[172]
RhoGAP	Rho GTPase activating protein	66			4				45	345	5MY3	[173]
Sec14	Sec14	26	1	3	4	5	34	35	45		3W67, 3W68	[157]
Septin	Septin	13							45	345	2QAG, 5CYP, 6WBP, 6WSM	[168–171]
SH2	Src Homology 2	107							45	345	4XZ1, YGK	[131–134]
SPFH	Stomatin/Prohibitin/Flotillin/HflK/HflC	11								345	6IQE	[114,115]
START	StAR-related lipid transfer domain	15	1								1UW5	[158]
SYLF	SH3YL1, Ysc84p/Lsb4p, Lsb3p and plant FYVE proteins	1								345	-	[17]
Syntaxin	Syntaxin	15							45		-	[98]
TBC	Tre2–Bub2–Cdc16	52							45	345	5HJQ	[177]
Tubby	Tubby	5							45		1I7E, 3C5N	[174]
VHS	Vps-27, Hrs and STAM	9				5					1ELK	[18,108,109]
Vinculin	Vinculin	2							45		5LOC	[180]
WD	Trp-Asp repeats	169							45		-	[182]
Total		1906										
PI binding domains in proteins of pathogens (abbreviated and full names)												
3 C	3C/3CL protease (picornaviral)						34	35	45	345	1L1N	[192]

(*Continued*)

Table 1. (Continued).

Abbrev.	Full Domain Name	*H.s.*	1	3	4	5	3 4	3 5	4 5	345	Selected PDBs entries	References
BPD	Bacterial phosphoinositide-binding domain								45		-	[204]
BteA-NTD	Bordetella TTSS effector A N-terminal domain								45		6RGN	[203]
H7	H7 (Poxvirus viral membrane assembly protein)			3	4				45		4W60, 4W5X	[195]
LidA	Legionella pneumophila protein			3	4						4H5Y	[197,202]
LpnE	Legionella pneumophila protein			3							-	[201]
MA	Matrix (retroviral protein)								45		2H3Z	[193]
MavQ-CTD	MavQ C-terminal domain			3				35			-	[200]
P4C	PI(4)P binding of SidC				4						4ZUZ	[196]
P4M	PtdIns(4)P binding of SidM/DrrA				4						4MXP	[197,198]
RavZ-CT	RavZ C-terminal (*Legionella pneumophila* protein)			3							-	[199]

(MODA) [21] and HADDOCK [22] allow modeling of protein: membrane complexes, while the trajectories of their multi-component assemblies are becoming accessible by molecular dynamic simulations [23]. Amphipathic polymers such as styrene maleic acid (SMA) enable detergent-free purification analysis of native assemblies of proteins with minimum perturbation of their associated asymmetric lipid bilayer environments [24], and reveal how PIs modulate signaling inside membranes [25]. Cell imaging of fluorescent proteins using PI-specific probes allows the subcellular recruitment of many targets to be monitored. Mutagenesis of PI-binding determinants combined with selective inhibition of PI kinases allows unambiguous determination of cognate PI ligands in cells. Quantitative binding assays including biolayer interferometry, NMR, and isothermal titration calorimetry can cross-validate novel lipid ligands in micelles, bicelles, nanodiscs, and liposomes [26]. These advances are allowing closer examination of how membrane readers engage the organelle-specific lipid combinations in order to mediate localized membrane trafficking, assembly, and signaling events. Our cross-examination of their mechanisms reveals common features. The consensus is that PI code readers stereospecifically recognize a phosphorylated inositol headgroup while simultaneously engaging a few neighboring lipids and inserting hydrophobic elements into the membrane interior, subject to control by covalent modifications of both protein and lipid components.

3. Origins of the PI code and membrane readers

Decades of effort by many groups have revealed how various domains target proteins to PIs on subcellular compartments to mediate localized trafficking and signaling events (Figure 1).

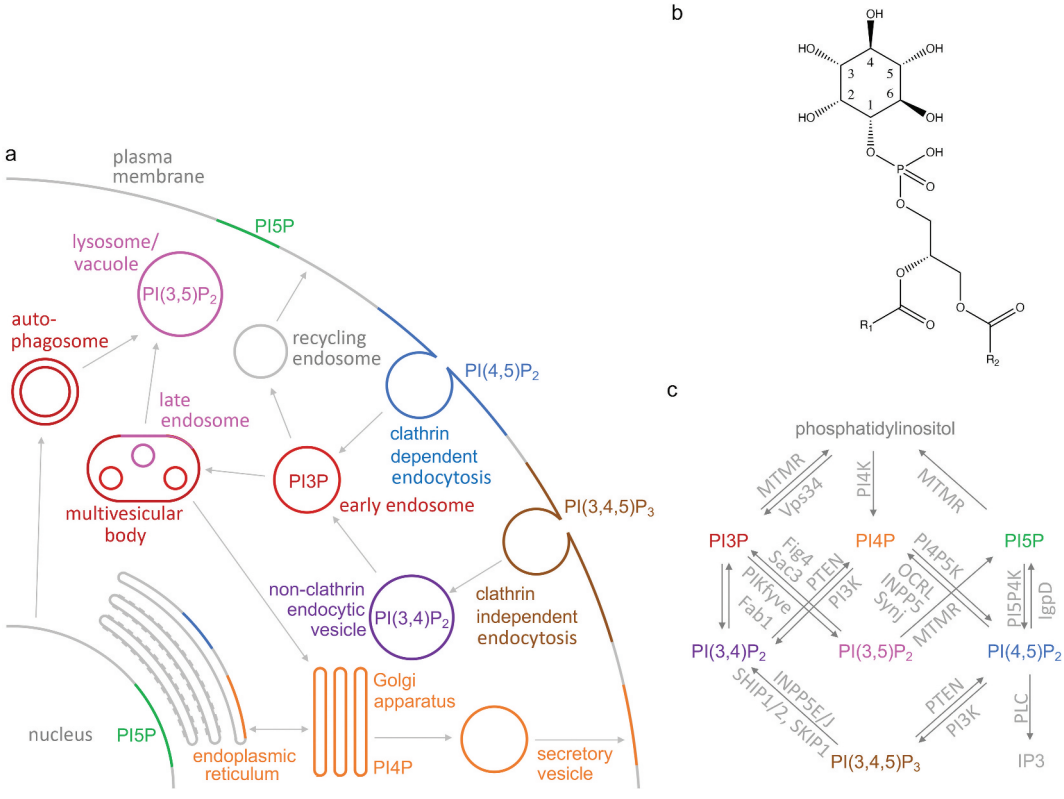

Figure 1. Cellular localizations and structures of PI signaling lipids. a) The distributions of PI molecules on organelle and plasma membranes are color-coded in the cartoon of a cell, with gray arrows indicating the direction of intracellular membrane trafficking pathways. b) The structure of phosphatidylinositol is shown including the hydroxyl positions which are phosphorylated on the 3, 4 and 5 positions of the inositol ring. c) The network of differentially phosphorylated PI lipids generated through the action of various PI kinase and phosphatase enzymes, as indicated by the bidirectional arrows.

These pathways appear in all three kingdoms and evolved before the divergence of Archaea and Eukarya [27]. Eight inositol-containing phospholipids have been found which are physically associated with different organelles within eukaryotic cells, although such distributions are simplifications of the complex dynamics and multiple functions of these lipids [28]. The history of the field grew from the realization that myo-inositol (Ins) is essential for growth and from the identification of the prevalent phosphatidylinositol (PtdIns) molecule as well as its derivative phosphatidylinositol 4,5-bisphosphate, abbreviated as PI(4,5)P_2, which is abundant in the brain [29]. This signature lipid of the plasma membrane is converted to PI(3,4,5)P_3 upon receptor stimulation. Early endosomes and autophagosomes are demarked by phosphatidylinositol 3-monophosphate (PI3P), while PI4P lines the route from Golgi to plasma membranes. Endocytic vesicles contain pools of PI(3,4)P_2. Late endosomes and lysosomes are enriched in PI(3,5)P_2, while PI5P is more widely distributed, being found in nuclear membranes as well as endosomes and autophagosomes. These PI molecules comprise a code that is generated by localized PI kinases and phosphatases on organelle surfaces and is detected by modular domains in order to trigger downstream events, as reviewed previously [1–3].

As a master currency of organelle information, PtdIns and its network of PI isoforms organize cells into dynamic and responsive membrane-bound compartments (Figure 1). The simplicity of this PI code is energetically advantageous and much more efficient than creating and regulating unrelated molecular species for each interconnected organelle. In contrast, nucleic acid- and protein-based information is encoded in linear polymers of nucleotides and amino acid residues that, being 1-dimensional and covalently linked, stably maintain information and are relatively simple to copy and purify. As membrane-bound information is embedded in asymmetric 2D bilayers of diffusible lipids, there are more technical and logistical challenges for the cell and investigator. Lipids pool in semifluid bilayers, exhibit gradients of synthesis and degradation, and are moved *via* interorganelle lipid transfer as well as membrane fission and fusion processes. Importantly, lipid assemblies are destroyed by conventional detergents used to isolate and study such systems, and hence have remained largely invisible. The purification and identification of intact membrane nanodomains and memteins has historically depended on reconstitution systems that cannot simulate asymmetric bilayers or isolate protein complexes of several different lipids. Only recently has the field been revolutionized by native nanodiscs such as SMA lipid particles (SMALPs), and cEM analyses of *ex vivo* structures of biologically intact protein assemblies [24].

Notwithstanding such challenges, detailed studies of FYVE, PH, and PX domains suggest that PIs are signature but not complete elements recognized by membrane readers. As discussed below, membrane readers typically interact stereospecifically with a PI molecule in conjunction with an acidic phospholipid, such as phosphatidylserine (PS) or phosphatidic acid (PA) *via* an adjacent-binding site, while also inserting hydrophobic bulk into a bilayer, which is rich in phosphatidylcholine (PC) molecules. We propose the term lipidon to describe the unique collection of co-located lipids that distinguish biological membrane nanoenvironments and which provide the context for in vivo PI recognition. The evidence for detection of lipidons by FYVE, PH, PX, and related domains is outlined below, and may be extendable to many other membrane readers.

The lipidon concept builds on the PI code and is based on recent structural, molecular, and cellular characterization of several membrane readers, and complements other hypotheses related to machines that assemble on membranes. Signaling pixels were proposed by Grabon et al. in 2019 in order to define a phosphatidylinositol (PtdIns) presentation subunit composed of a PI transfer protein (PITP) and an associated an PtdIns 4-kinase (PI4K) enzyme that interacts with PI4P effectors [30]. Balch and colleagues proposed the existence of trafficking proteostasis networks for guiding protein folding on organelle surfaces [31] as well as membromes, which are collections of proteins that coat, target, tether, and fuse membranes as modeled on studies of Rab GTPases [32]. In 2018, Hancock's group proposed a combinatorial code for lipid binding that is mediated by the prenylated C-terminal polybasic domain of K-Ras [33]. The membron concept was hypothesized by Pitot in 1969 to represent the functional unit comprised of a polyribosome on the membrane of the rough endoplasmic reticulum that selects mRNA molecules for translation into proteins [34]. Coincidence detection has been proposed as a mechanism for simultaneous interaction of proteins with multiple ligand molecules, such as IP_3 and Ca^{2+} which occasionally happen to co-locate, stimulating downstream events [35]. In contrast, lipidons refer to sets of proximal lipids in membranes that are cooperatively recognized by adjacent pockets and surface features of individual membrane readers. Upon integrating into a memtein, lipidons can elicit a wide variety of signaling and trafficking effects and are preferentially formed in organelles to embody spatially and temporally specific information. They can be generalized to a wider membrane code through which combinations of phospholipids, glycolipids, and sterols are recognized by diverse proteins in order to preserve and communicate organelle- and pathway-specific signals.

As the source lipid of PIs, the PtdIns molecule is the basis of a singular molecular currency that encodes cellular information *via* the distribution of its derivatives to various organelle destinations (Figure 1b). We propose that this efficiency gave rise to eukaryotic interior organization and is essential for trafficking and replicating living matter on organelle surfaces. Given the centrality of the PI code, its readers are multitudinous, as reviewed here for the first time. We count 70 types of PI recognition domains in hundreds of human proteins as well as innumerable bacterial and viral proteins, some of which are listed in Table 1. Additionally, there are other rare lipids that serve as unique organelle signals, such as lysobisphosphatidic acid, which is recognized on endosomes by the Alix Bro1 domain [36]. More candidate membrane readers are being identified by proteome-wide screens [37–41], and many have yet to be validated experimentally. The PI code is clearly a vital and widespread cornerstone of the biological code that determines both how proteins function on organelle surfaces, how intracellular compartments

work and how entire cells and virions organize and replicate their contents. As such, the PI code is pivotal to the translation of proteins and the design of organelles, and merits a review 20 years after its discovery [1–3].

3.1. PX domains recognize all PI signals

Of the diverse array of membrane readers, the phox homology (PX) domain family is unique in its ability to recognize all seven PIPs. The PX superfamily spans fungi, protists, viridiplantae and metazoa [42] and includes 49 distinct members in humans. All contain a similar structure consisting of ~120 residues folded into a three-stranded antiparallel β sheet that packs against an α helical subdomain. Both termini extend away from the membrane-binding surface thus allowing unobstructed access to membranes (Figures 2a&3a) [3,22]. A common recognition site is apparent in the complexed structures of PX domains of Grd19, p40^{phox}, p47^{phox}, Snx3, Snx9, Snx11 and Vam7 proteins [3,22,43–49]. A patch of basic and hydrophobic residues specifically recognizes PI headgroups and inserts into lipid bilayers (Figure 2b). The membrane docking surface comprises a hairpin that connects the β1 and β2 strands, the β3-α1 junction and a segment connecting a proline-rich element (PRE) and α2 helix. Its consensus PI3P binding motif is R[Y/F]$X_{23–30}$K$X_{13–23}$R, where X is any residue. The guanidinium group of the first arginine binds the 3-phosphate, the aromatic group stacks against the inositol ring, the lysine residue interacts with the 1-phosphate and the last arginine forms a pair of hydrogen bonds with the 4 and 5-hydroxy groups of the inositol. Attraction to acidic membranes is enabled by the domain's electrostatic dipole. Synergistic bilayer binding is afforded by interdigitation with PC molecules by residues in the β1-β2 loop and a helical element between the PRE and α2 elements as seen in Snx3 [47] and Vam7 structures (Figure 3b) [3,22]. Sorting nexins employ this mechanism to anchor retromer complexes and attach cargo to curved membranes, as resolved recently by cET [50]. The membrane anchoring surface includes a second pocket that can also bind PA or PS, as evidenced by the structure of PI(3,4)P_2-bound PX domain of p47^{phox} [45]. The Snx11 protein, which is found in the late endosome, contains a PX domain that similarly recognizes PI(3,5)P_2, with the 5-phosphate being contacted by the lysine in the conserved set of PI binding motifs (Figure 4a) [49]. Functionally divergent PX domains bind cargo proteins in the vicinity of the canonical PI binding pocket [51,52] or exhibit distinct PI preferences *via* variant motifs [26]. For example, the crystal structure of the Snx9 PX domain bound to PI3P reveals a comparatively large pocket that accommodates its cognate PI(4,5)P_2 ligand [48]. Together these studies provide structurally consistent

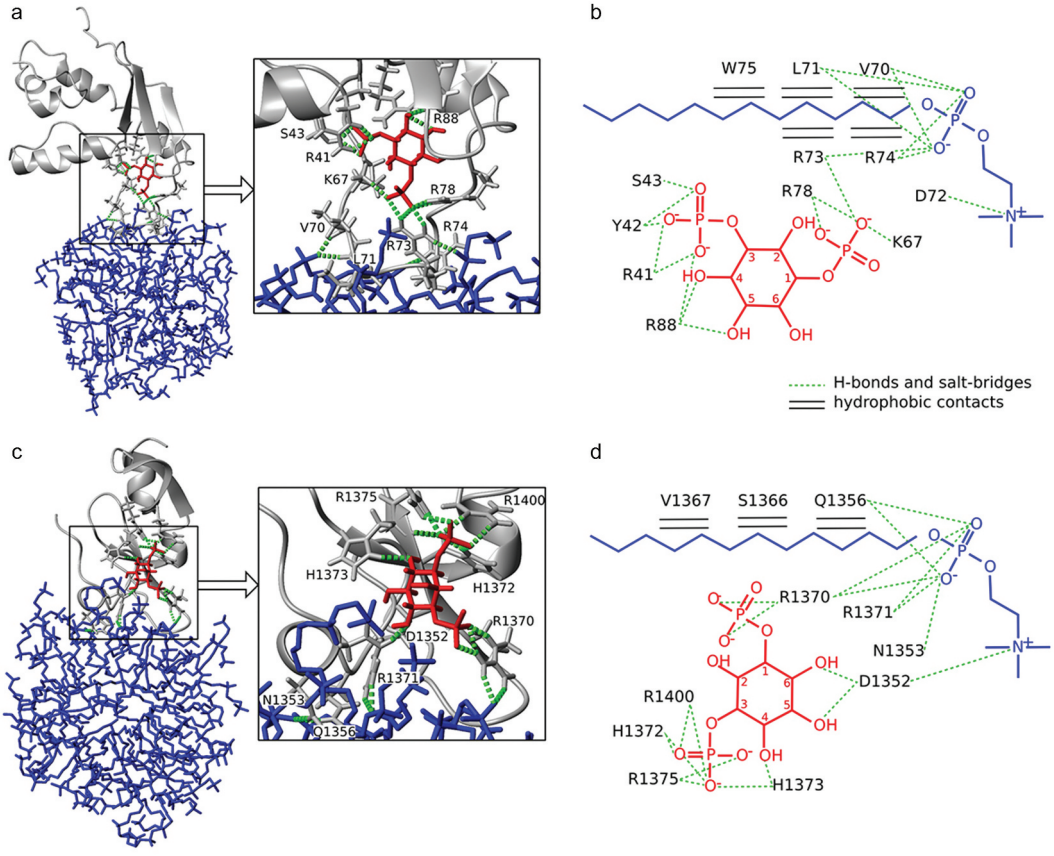

Figure 2. Structures of PX and FYVE domains bound to PI3P-containing micelles. The Vam7 PX domain (PDB: 1KMD [208]) inserted into a dodecylphosphocholine (DPC) micelle (a) is shown, as are the contacts with its PI3P ligand (b), as derived from [22]. The EEA1 FYVE domain complex with a DPC micelle and PI3P molecule (PDB: 1JOC) (c,d) show analogous contacts based on NMR data and HADDOCK calculations. The PI3P, micelle and protein molecule are shown in red, blue and silver. Interacting residues are labeled, with sidechains depicted, with intermolecular hydrogen bonds and salt bridges as green dotted lines and the intermolecular hydrophobic contacts as pair of horizontal black lines alongside the interacting DPC moieties. Used with permission from [22].

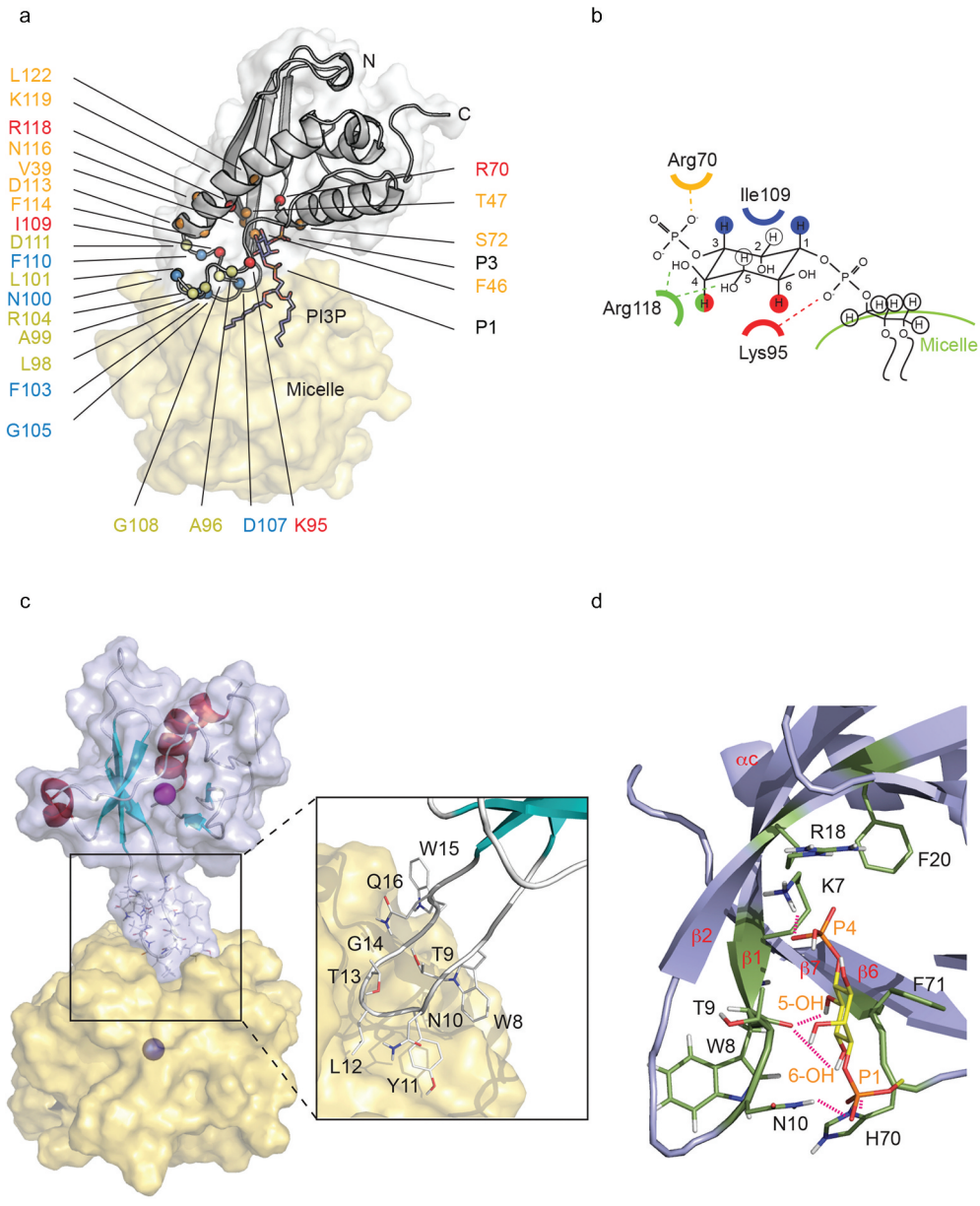

Figure 3. Structures of the Snx3 PX and Fapp1 PH domains bound to PI ligands and micelles (PDB: 2MXC & 2KCJ). (a) The Snx3 backbone is gray with DPC micelle shown as a yellow surface; key residues are labeled and interactions with the PI3P headgroup are shown in (b). Residues which exhibit significant chemical shift perturbations upon interaction with the PI3P headgroup or micelles are colored orange or yellow, respectively, while those exhibiting intermolecular interactions with PI3P or DPC are also displayed in red and blue, respectively, as derived from [47]. The phosphate groups of PI3P are indicated by P1 and P3, and hydrogen bonds are shown with dotted lines. (c) Structure of the PH domain of FAPP1 bound to a DPC micelle and PI4P [71]. The helices and β-strands are shown in aqua and red, respectively, under a translucent silver molecular surface, with the micelle surface shown in yellow. The centers of the protein and micelle are indicated with magenta and blue spheres, respectively, and are separated by 40 Å. The micelle-inserted MIL residues in the β1–β2 loop labeled. (d) The bound inositol and phosphates (labeled P1 and P4) are colored yellow and orange, respectively. Intermolecular hydrogen bonds are shown with dashed lines to key residues, which are labeled and shown with sidechains. Used with permission from [47] and [71].

explanations for how PX domains read the PI signals embedded in a variety of subcellular membranes.

Several additional patterns are apparent in the PX family. All its members contain a membrane interacting module as a terminal domain (typically but not exclusively a PX domain), thus minimizing obstructions for further upstream or downstream anchoring into a lipid bilayer. Various enzymes including kinases, acetyltransferases and methyltransferases attach phosphates and metabolites to residues within the PX domain's membrane-binding surfaces to down-regulate their membrane-reader activities [19,20]. Multimerization of PX proteins is mediated by adjacent coiled coil, leucine repeat and BAR domains, and members often also contain lipid-binding BAR, C2, or transmembrane domains, thus reinforcing membrane association. Together, the specific lipid recognition, membrane avidity, and regulatory mechanisms of PX domains

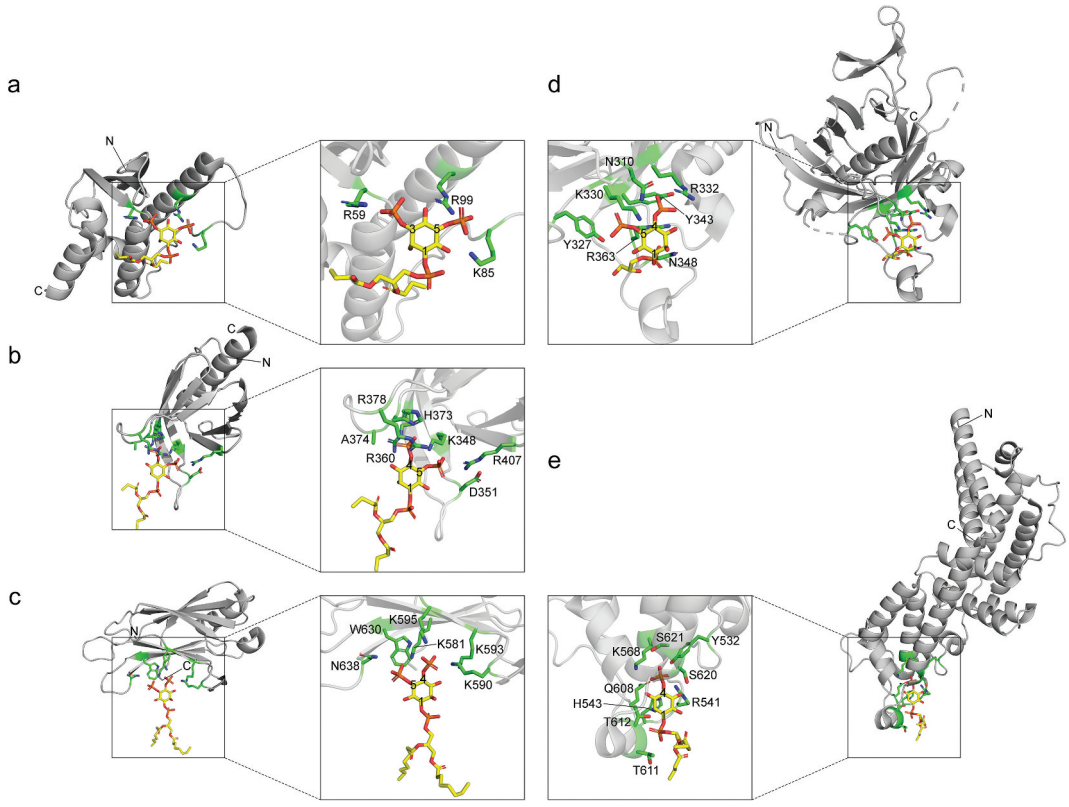

Figure 4. Structures of representative structures of membrane readers bound to PI ligands. The structures of (a) SNX11 PX:PI(3,5)P_2 (PDB: 6KOJ) [49], (b) ASAP1 PH:PI(4,5)P_2 (PDB: 5C79) [69], (c) rabphilin 3A C2:PI(4,5)P2 (PDB: 5LO8) [84] (d) Tubby:PI(4,5)P_2 (PDB: 1I7E) [174] and (e) DrrA P4M:PI4P (PDB: 4 MXP) [198] complexes are shown. PIs and PI-binding residues are shown with carbon atoms colored yellow and green, respectively, while backbone ribbons are colored silver. Created using PyMOL.

converge to enable high fidelity reading of lipidons, providing a set of principles that can be extrapolated to other domains.

3.2. FYVE domains generally recognize PI3P

The FYVE domain family is named after the Fab1p, YOTB, Vac1, and EEA1 proteins. These cysteine-rich domains are stabilized by two zinc atoms and generally bind specifically to PI3P [53]. Unlike PX domains, they are more widespread in green plants, and are also encoded by some viruses [42]. These domains comprise ~70 residues that form a pair of two-stranded anti-parallel β-sheets and a C-terminal α-helix that together present three PI3P binding elements (Figure 2 c,d) [54]. The central R(R/K)HHCR motif directly recognizes the 3-phosphate with its pair of histidines enhancing membrane affinity at the low pH levels found in endocytic pathways [55]. The aspartic acid in the N-terminal WXXD motif precludes interactions with PIs having phosphates at 4- or 5-positions of the inositol ring, while the tryptophan undergirds the lipid-binding pocket [53,56]. The arginine in the C-terminal R(V/I)C motif forms hydrogen bonds with a PI ligand. The FYVE domain of protrudin diverges from these canonical motifs and binds instead to multiply phosphorylated PIs in the plasma membrane [57]. Endosome targeting involves the electrostatic approach of the dipolar domain followed by selective lipid recognition and insertion of a membrane insertion loop (MIL) into a bilayer containing PI3P and accessory PS and PC molecules [58]. This multivalent binding mechanism mirrors that of PX domains, with PIs again serving as primary localization determinants while accessory lipids help stabilize the membrane-bound form.

The interactions of FYVE domains with lipid bilayers are dynamic and tunable. Affinity for membranes is reduced by inclusion of an acidic residue in the MIL, as shown in the FYVE domain of ALFY, which specifically binds PI3P but localizes the protein to autophagosomes to facilitate aggregate clearance [59]. Phosphorylation of a serine or threonine in a membrane binding element of a FYVE domain is also predicted to compromise interactions with phospholipid bilayers [60] in a manner analogous to the PIP-stops discovered in PX domains [20,47]. Membrane avidity can be enhanced by juxtaposing a pair of FYVE domains within parallel dimers, as exemplified by EEA1's coiled coil [56], or by adjacency with another membrane reader, such as a pleckstrin homology (PH) domain that also binds PI3P, as shown in Pfafin2 [61]. This supports the existence of generally applicable mechanisms for positively and negatively regulating diverse membrane readers by avidity enhancement and PTM-based inhibition, respectively.

The plant homeodomain (PHD) finger is found in 72 human proteins and contains a pair of zinc-binding sites within a fold reminiscent of FYVE domains. Specific recognition of PI5P by

the PHD finger along with C-terminal polybasic element of the ING2 protein is apparent by dot blot, surface plasmon resonance, and subcellular localization assays, suggesting that this lipid interation regulates chromatin association [62]. The PHD finger of the ING1 protein also binds specifically to PI5P-containing liposomes, while another nuclear protein, UHRF1, instead binds PI5P *via* a polybasic region, thus modulating recognition of unmodified histone protein sequences by its PHD finger [63]. PI interactions have been reported for 17 additional human PHD fingers including that of the TAF3 transcriptional regulator, which modulates binding to histone tail peptides [63].

3.3. PH domains comprise the largest family of membrane-binding modules

The largest superfamily of membrane readers are the PH domains, which are found in at least 247 human proteins either singly or in sets of up to 5 repeats. This domain consists of ~110 residues that form a seven stranded antiparallel β-sheet sandwich followed by one or two α-helices (Figures 3&4). The PI interactions of PH domains were the first to be documented [64], with at least 61% of these modules now predicted to be membrane interacting [65], as is supported by liposome-binding data [66]. Their β1-β2 loops insert into membranes and binds PIs through a KX_n(K/R)XR binding motif [67]. The most common mode of PI recognition on the "closed" side of this loop is exemplified by the PH domain of FAPP1, which also engages parts of the β3, β4, and β7 strands in order to recognize disordered, fluid PI4P-containing bilayers and induce membrane tubulation (Figure 3 c,d) [68]. The PH domain of ASAP1 binds PI(4,5)P_2 similarly *via* its β1/β2, β3/β4, and β5/β6 loops, and displays a polarized surface with a second nonspecific lipid-binding interaction that would reinforce membrane interactions (Figure 4b) [69]. The latter-binding site is seen in ARHGAP9 to recognize a PI(3,4)P_2 molecule [70], suggesting that both lipid-binding pockets engage membrane surfaces. The main auxiliary lipid, which cooperatively stabilizes PH:membrane complexes appears to be PS [66,71], reminiscent of the lipidons detected by FYVE and PX domains. Like the latter modules, the PH domain's membrane interactions can also be controlled by the addition of metabolite groups to their membrane-binding surfaces [72] or hyperphosphorylation of neighboring sequences [73,74], supporting a universal process for regulating PI recognition.

Several modules with folds similar to PH domains also bind PIs (Table 1). The structurally related GRAM domains of myotubularin PI phosphatases binds to a variety of PIs and possesses an exposed basic patch that could conceivably contact membranes [75], although complexed structures are lacking. The PTB domain was named for its ability to bind phosphotyrosine-containing peptides, but can also bind unphosphorylated peptides as well as PI(4,5)P_2, hinting at the functional plasticity of its PH-like fold [76].

The GRAM-like Ub binding in EAP45 (GLUE) domain is similar to a PH domain, and in the case of the Vps36 protein also binds PI and localizes to late endosomes [77]. The structure of the GLUE domain contains a long loop that mediates ubiquitin and ESCRT proteins, while a distinct basic site is reminiscent of the secondary lipid-binding site found in PH domains, suggesting cooperative interactions that could downregulate ubiquitinated membrane proteins [78,79].

There are 48 human FERM domains, which contain ~300 residues that form a clover leaf-like arrangement of three subdomains. The radixin FERM domain has been co-crystallized with a PI(4,5)P_2 headgroup molecule bound in a basic groove between two subdomains (one of which resembles a PH domain), potentially modulating interactions with membrane protein partners [80]. The crystal structure of the atypical FERM domain of talin bound to PI(4,5)P_2 reveals a different site and suggests induced conformational changes, release of the rod domain and opening of the protein to activate integrin partners [81]. Further investigation is needed to resolve how such modules engage membrane surfaces to induce downstream signaling and focal adhesion formation, but enough is known to suggest how lipid-binding functions can be propagated across a family of divergent 3D folds.

3.4. Conserved domains of Protein Kinase C

There are ~60 human proteins that contain C1 domains, which are named for their presence in protein kinase C (PKC). These ~50 residue modules are composed of two β-sheets and an α-helix that form a fold stabilized by a pair of zinc coordination sites. Although there are no ligand-bound structures yet available, the atypical C1 domain of RAS guanyl-releasing protein 2 utilizes a set of basic residues to specifically recognize PI(4,5)P_2 and PI(3,4,5)P_3, thus directing the protein to the plasma membrane to activate downstream signaling [82]. These modules can bind diacylglycerol, PI lipids, and proteins, providing opportunities for coincidence detection.

Like C1 domains, the C2 domain was originally identified in PKC. It is found in 145 human proteins, with its eight stranded antiparallel β-sandwich fold able to bind lipids including PI and PS, often in a calcium-dependent fashion [83]. The simultaneous interaction of calcium and PI(4,5)P_2 with the Rabphilin-3A C2 domain interaction could initiate plasma membrane bending and fusion events [84]. The mechanism of concerted C2 domain binding to both PI(4,5)P_2 and PS ligands in a calcium-independent manner suggests that the synaptotagmin-like protein 4 recognizes a corresponding plasma membrane lipidon (Figure 4c) [85]. Modification of a lysine compromises its membrane interactions *in vitro*, suggesting that phosphorylation of the nearby Tyr396 might also block binding [86].

A family of 11 human proteins contain a Dock Homology Region-1 (DHR-1) domain that resembles a C2 domain. Its crystal structure has been determined, allowing the position of PI(4,5)P_2 and PI(3,4,5)P_3 to be modeled in a basic groove between β hairpin loops [87]. The crystal structure of DOCK8's DHR-1 domain bound to its unresolved PI(4,5)P_2 ligand reveals that the lysine and arginine residues reorient their sidechains accordingly [88]. These calcium-independent PI interactions target guanine nucleotide exchange factor

functions to the plasma membrane to help control the organization and dynamics of the actin cytoskeleton and cell migration.

3.5. Helical bundles engage PI-rich membranes

The classical Bin–Amphiphysin–RVs (BAR) domain is found in 16 human proteins including several sorting nexins. Like PX domains, it can bind PS and PI lipids, and deforms bilayers to induce membrane tubulation [89]. BAR domains form dimers that have been crystallized with IP_6, revealing a basic patch formed by two helices that offer electrostatic rather than stereoselective interactions, with their banana-shaped dimers also being sensitive to membrane curvature [90]. Such domains often work in concert with other modules that help mediate lipid bilayer selectivity and insertion. This has been illustrated by the structures of Snx9 [48], Snx3 and Vps5 [50,91], where BAR and PX domains work together within dynamin GTPase and retromer assemblies to tubulate membranes and traffic cargo. BAR domains are phosphorylated on sites that regulate membrane interactions and tubulation properties, as shown with the syndapin 1 protein [92] and reminiscent of the PIP-stops identified in PX domains [20,47]. The structurally related F-BAR and I-BAR domains are also comprised of bundles of three long, kinked helices that form antiparallel dimers, but differ in the helix lengths, twists, and curvatures that differentiate their abilities to sense or induce curvatures of $PI(4,5)P_2$-containing membranes [93–97].

The fusion of membranes within eukaryotic cells relies on fusogenic helical proteins that recognize specific lipids in target organelle surfaces. The role of lipids is less well-understood than the protein–protein interactions that form such fusion machines. However, binding of PA as well as several PIs occurs within the juxtamembrane region of Syntaxin1A. Mutations of basic residues here compromise both lipid interactions, fusogenicity, and secretory functions in cells [98]. Resolution of the kinetics of membrane fusion pore-forming processes could benefit from continued development of nanodiscs, which allow the key intermediate states to be resolved [99]. Large pores in the plasma membrane are formed by the structurally unrelated gasdermin protein through oligomerization and $PI(4,5)P_2$ interactions mediated by its N domain, thus eliciting cytotoxic effects [100].

A set of adapter proteins interact with clathrin to initiate endocytic events at the plasma membrane. Several contain epsin N-terminal homology (ENTH) domains that bind $PI(4,5)P_2$ to trigger α helix insertion into the membrane, thus bending the bilayer. A pair of sites bind this lipid cooperatively, with NMR and crystal structures showing how the ligand is recognized and how helices reposition in multimeric states to prepare for membrane insertion [101–103]. The adaptor protein complex AP2 helps to assemble endocytic clathrin-coated vesicles used to internalize cargo. A pair of binding sites for $PI(4,5)P_2$ and its α and μ subunits is apparent from a co-crystal structure with inositol-hexaphosphate [104], and mediate recruitment of the closed form, thus allowing subsequent opening on plasma membrane surfaces [105]. Another type of clathrin adaptor is found in the Golgi-localized, γ-ear containing, Arf-binding proteins (GGAs), which mediate trafficking between the trans-Golgi network and endosomes. The crystal structure of the GAT domain of the GGA1 protein reveals a basic site on its four helical bundle that can accommodate PI4P ligands [106,107]. The VHS domain is a cargo-sorting module that is found in target of Myb1 (TOM1) proteins. The structure of the VHS domain of Tom1 consists of eight α helices [108] and binds to PI5P [109] as well as to ubiquitin chains on cargo proteins [110]. These examples highlight the role of adaptors in connecting proteins and lipids on membrane surfaces.

The charged multivesicular body protein (CHMP) family participates in formation of endosomal sorting complexes for transport-III (ESCRT-III) assemblies on membranes. Binding of their N-terminal basic domains to $PI(4,5)P_2$-containing membranes through the formation of helical multimers provides localization to the necks of membrane tubules [111]. The structure of CHMP3 reveals a helical dimer with a highly basic flat surface that could contact membranes, with variations across the family suggesting functional divergence [112]. Indeed, some CHMP relatives prefer either flat or positively curved membranes, or participate through heterodimerization [113].

Prohibitins are a family of proteins that associate with the mitochondrial inner membrane and bind $PI(3,4,5)P_3$ in a phosphorylation-dependent manner [114]. The crystal structure of the heptad repeat region of prohibitin 2 reveals a dimeric antiparallel coiled-coil [115]. Based on our analysis with the MODA program [21] this structure presents an extensive membrane docking area that could help tether it to the mitochondrial membrane, with avidity being enhanced by further oligomerization.

Signaling through G protein-coupled receptors (GPCRs) involves the recruitment of arrestin proteins, which uncouple these transmembrane proteins from heterotrimeric G proteins and also engage clathrin to initiate internalization. This involves the binding of arrestins to $PI(3,4,5)P_3$ *via* a basic site, which modulates the internalization of the receptors [116]. In contrast, binding of soluble inositol hexaphosphate (IP_6) molecules to multiple sites on arrestin structures modulates the interaction of its domains with kinases [117] as well as oligomerization, which leads to its release from membranes into the cytosol [118].

The annexin A2 protein consists of four repeats, each containing five helices, which present sites of membrane-dependent interaction with actin. Direct binding of annexin A2 to $PI(4,5)P_2$ mediates its recruitment to pinosomes that bud from the plasma membrane [119] as well as sites of actin-rearrangement where enteropathogenic *E. coli* bacteria attach [120]. Our MODA analysis [21] of available structures reveals a predicted membrane binding element spanning the RRTK motif in repeat 1, the interactions of which may be regulated by a cluster of N-terminal phosphorylated residues [121].

The ~80 residue BATS domain contains an amphipathic α helix and binds preferentially to membranes containing PI3P or $PI(4,5)P_2$ while sensing curvature in order to target autophagosomes [122]. The plant-specific remorin proteins contain

a C-terminal helical domain that binds PI4P and sterol to mediate plasma membrane targeting [123]. The structure of the Vps34 complex II, which is involved in endocytic sorting, has recently been determined by cET and shows the position of a fused BATS domain near the membrane interacting surface [124].

The PIKfyve protein is a FYVE domain-containing kinase that is present in all eukaryotes and may constitute their sole source of PI(3,5)P_2 production. It forms a complex with Fig4 phosphatase, which regulates its kinase activity, as well as the Vac14 scaffolding protein, as resolved recently by cEM [125]. The latter protein consists of a series of helical HEAT repeats and assembles a cup-shaped pentameric star that undergoes conformational changes on membranes to control active site access. The Vac14 protein localizes to endocytic organelles in fibroblasts and neurons, where it controls postsynaptic function by regulating the endocytic cycling of receptors [126]. Basic elements in the HEAT repeats of the huntington protein also interact with PI(4,5)P_2 and mediate plasma membrane localization [127]. Several of its HEAT repeats are positioned to form a membrane binding surface that is visible in cEM structures [128], suggesting that PI binding may be a recurrent feature of this family in addition to their more established role in mediating protein interactions.

3.6. Dual purpose modules that bind proteins and PIs

A number of domains that usually bind proteins have also been found to act as membrane interacting modules. The PDZ domain is a ~ 90 residue α/β module found in 149 different human proteins, where it typically mediates interactions with C-terminal tails of receptors in the vicinity of the plasma membrane. A study of 2000 PDZ domains from 20 species estimated that 30% bind membranes, with 95 shown to bind membranes, and several demonstrating selectivity for PIs [129]. The crystal structure of syntenin's pair of PDZ domains in a ternary complex with a receptor peptide ligand and PI(4,5)P_2 reveals cooperative binding *via* adjacent hydrophobic and basic pockets, demonstrating how coincidence detection can work in a single domain [130].

Src Homology 2 (SH2) domains are 120 residue α/β modules that are present in 107 human proteins, where they typically recognize phosphotyrosine-containing peptide sequences. However, some can also bind PI(4,5)P_2 or PI(3,4,5)P_3, as discovered in 1995 and later applied to 76 human SH2 domains by surface plasmon resonance and NMR analysis [131–134]. These studies suggest that 90% of SH2 domains bind to plasma membrane lipids including PIs. The positions of the candidate basic sites for binding lipids in SH2 domains vary, either being outside of or overlapping the phosphopeptide binding groove, so as to either promote or compete with engagement of signaling proteins.

A family of 11 human proteins contain Eps15 homology (EH) domains, which comprise a pair of calcium-binding EF hand motifs that form a binding site for Asn-Pro-Phe motifs, as resolved by NMR [135]. In the cases of EHD1, EHD3 and EHD4 and the second EH domain of Eps15, the ability to bind weakly to PI lipids in a calcium-independent fashion has been demonstrated [136] and could provide targeting to endocytic compartments and tubular membranes [137]. The EHD1 residues involved in binding PI and PA lipids have been mapped by NMR and are found far from the part of the domain that recognizes NPF motifs. In contrast, the second EH domain of Eps15 interacts with PI(3,5)P_2 via a site near where NPF peptides bind to provide membrane targeting [135,136]. The oligomerization of the EHD2 protein juxtaposes the basic-binding sites where PIs are thought to engage, forming an extensive binding surface for curved membranes [138].

The Meprin and TRAF Homology (MATH) domain is a ~ 180 residue module that mediates interactions with a variety of cell surface receptors. It forms an 8-stranded β sandwich fold that assembles into trimers resembling mushrooms when viewed along with the preceding coiled-coil region. The crystal structure of the MATH domain of the TRAF4 protein was used to model a PI binding pocket based on effects of mutating several conserved basic residues [139]. The three pockets of the trimer suggest that multivalent plasma membrane interactions mediate its recruitment to tight junctions involved in cell migration.

A family of four human WIPI proteins contain a PROPPIN domain that binds both PI3P and PI(3,5)P_2 [140]. The crystal structures of yeast [141] and human [142] PROPPIN domains have been determined. Both contain seven bladed β-propeller folds with bound sulfates by 'L/FRRG' motifs that suggest two distinct PI3P and PI(3,5)P_2 binding sites in blades 6 and 5, respectively, while other blades mediate interactions with proteins involved in autophagosome biogenesis.

3.7. Kinase interactions with PI lipids

Kinases display a diversity of PI-binding mechanisms that influence their activities. The GRK5 kinase contains terminal basic elements that mediate recruitment to PI(4,5)P_2-enriched membranes where they can also engage G protein coupled receptors [143,144]. The juxtamembrane domain of epidermal growth factor receptor (EGFR) forms a dimeric antiparallel helix that interacts with PI(4,5)P_2. This complex contributes to the formation of an asymmetric receptor dimer, which activates the kinase domain and leads to downstream signaling. Clusters of basic residues in the helical structure suggest a PI(4,5)P_2 binding site that explains how mutating these residues compromises lipid binding [23]. The cEM structure of the PI4Kα enzyme that converts phosphatidylinositol to PI4P at the plasma membrane reveals an extensive basic surface positioned to engage acidic phospholipid bilayers in conjunction with other subunits within its hexameric assembly [145]. The crystal structure of PI3-kinase α (PI3Kα) reveals a PI(4,5)P_2 molecule bound in a basic crevice between domains. The two terminal phosphates bridge the activation loop and the inter-SH2 domain, which connects its two SH2 domains. This arrangement suggests that PI(4,5)P_2 inhibits the kinase while oncogenic mutations could increase membrane binding and PI kinase activity [146]. The BH domain, which is found in the p85α subunit of this kinase, binds weakly to PIs *via* a binding site that overlaps that of the lipid phosphatase PTEN, suggesting a regulatory intersection [147]. Inositol phosphate multikinase (IPMK) participates in a variety of signaling

pathways by phosphorylating substrate molecules. Its crystal structure shows how a PI molecule is bound within its active site by residues including a key arginine that engages both terminal phosphates [148]. A pseudokinase known as Mixed Lineage Kinase domain-Like (MLKL) contains an N-terminal Bundle and Brace (NBB) domain that forms a six-helix bundle with a basic PI(4,5)P_2 binding site that mediates recruitment of it and its partner kinase RIPK3 to the plasma membrane to induce cell death [149,150]. In addition, many kinases employ PI-interacting modules such as C1, C2, FYVE, PDZ, PH, PX, RGS, and SH2 domains to target them to their organelle destinations.

3.8. Phosphoinositide transfer proteins

Two structurally distinct families of PITPs are named for their ability to transfer PI molecules between membranes *in vitro*. The Sec14-like PITPs can accommodate either phosphatidylinositol or PC in separate but overlapping sites [151–153], while the StAR-related lipid transfer (START) domain captures PI or PC in an eight-stranded β sheet surface that coordinates lipid headgroups [30,154,155]. It has been argued that their *in vitro* lipid transfer capabilities do not translate to *in vivo* function, and instead their biological role is to act as 'nanoreactors' for stimulating PI4P biogenesis by fetching PI from the endoplasmic reticulum and presenting it to PI4Ks [30,151,156]. In the case of the α-tocopherol transfer protein, a vitamin E molecule is exchanged with a PI and may allow delivery of the former molecule to the plasma membrane. Crystal structures of its Sec14 domain with PI(3,5)P_2 or PI(4,5)P_2 bound to a basic cleft indicate how lipid binding can open a lid to facilitate release of the vitamin into the membrane [157]. The intracellular transfer of PtdIns molecules may be mediated by the START domain of PITPα, which is proposed to shuttle lipids between plasma and nuclear membranes. The lipid headgroup is bound by conserved polar residues within a deep pocket located between its eight-stranded β sheet and overlaid α helices [158]. The mobile C-terminal helix acts as a gate for the ligand, with proximal residues inserting into membranes to assist in the exchange and release of lipids. Structurally unrelated glycolipid transfer proteins (GLTPs) may ferry lipids between organelles, some of which contain known PI binding modules such as C1, C2, and PH domains. The ceramide-1-phosphate transfer protein (CPTP) does not contain such modules yet localizes to Golgi and plasma membranes. Its lipid transport activity may be stimulated by direct binding of PI4P and PI(4,5)P_2 to an exposed basic-binding surface on its all-helical GLTP domain fold [159], potentially explaining its localization to sites where ceramide is produced and delivered.

The ability of nuclear receptors to regulate gene expression can be influenced by their interactions with PI lipids. This is illustrated by the steroidogenic factor 1, which has been crystallized with bound PI(4,5)P_2 and PI(3,4,5)P_3 molecules, revealing how their dipalmitoyl chains insert into the core of the domain, which is formed by 12 α helices. The acyl chains stabilize helical packing and increase ligand affinity, while the headgroup is exposed and rotatable. This PI binding mode is atypical among membrane readers but allows ready access of the substrate headgroup by the IPMK kinase [160]. A related nuclear receptor known as the Liver Receptor Homolog-1 binds PI(3,4,5)P_3 tightly in a similar manner, stabilizing the ligand-binding domain and influencing interactions with co-regulatory proteins [161].

A set of intracellular phospholipase A_1 (iPLA_1) enzymes that hydrolyze phospholipids as well as several PI transfer proteins are known to contain a ~ 190 residue DDHD domain of unclear structure and function. This module's signature AspAspHisAsp motif is thought to represent a metal-coordination site. The DDHD2 protein is localized in cis-Golgi and ER compartments, and specifically recognizes PI4P through its DDHD domain [162], while the related DDHD1 protein is locallized in ER exit sites and recognizes PI4P through the concerted action of its adjacent SAM and DDHD domains [163].

The oxysterol binding protein-related protein 2 (ORP2) is responsible for the selective delivery of cholesterol molecules to the plasma membrane and simultaneous removal of a molecule of PI(4,5)P_2. A crystal structure of the Oxysterol binding protein-Related Domain (ORD) reveals a continuous β sheet fold in which long-chain tails of PI(4,5)P_2 molecules are positioned to straddle subunits of the tetramer [164]. Pairs of histidine and arginine residues engage the inositol headgroup which sits inside an open helical lid that is proposed to close upon cholesterol binding based molecular dynamics simulations, providing a mechanism for shuttling these lipids between plasma and endosomal membranes.

The secretion of fibroblast growth factor (FGF) proteins across the plasma membrane leads to the stimulation of fibroblast and endothelial cell growth. In the case of FGF2, this process involves binding of PI(4,5)P_2 to induce the formation of disulfide-linked oligomers that cross the membrane, whereupon the same interface binds instead to heparin sulfate on the cell surface [165]. Simulations based on NMR and mutational data suggest how PI-dependent dimers and trimers form and then assemble into larger complexes with 8–12 subunits that can translocate and potentially also form membrane pores.

3.9. G-protein signaling on membranes

A family of GTPase enzymes act as membrane-bound switches that hydrolyze guanosine triphosphate (GTP) into guanosine diphosphate (GDP), as have been characterized by NMR. The unmodified K-Ras protein binds PI(4,5)P_2-containing liposomes *via* its structural domain and C-terminal unstructured basic region. The latter site adds affinity while the former site spans β strands 1, 2, 3, and 5 and α3. The presence of 2 distinct sites infers flipping between membrane-bound orientations [166]. The farnesylated and carboxy-methylated form of K-Ras dimerizes in a membrane-dependent fashion on nanodiscs [167]. The GDP and GTP-bound states differ in their respective conformations on PS-containing lipid bilayers, with altered packing of the α4 and α5 helices in their respective dimer interfaces. Both states engage lipid bilayers *via* C-terminal basic residues. However, the membrane orientations differ, with the α3 loop becoming more protected and the effector-binding site being more exposed upon membrane

binding. This reveals how PTMs can stabilize and select particular membrane-bound orientations of protein domains to elicit specific signaling effects.

The septin family of GTPases form hetero-oligomeric filaments on the plasma membrane in order to facilitate cytokinesis. They contain a conserved basic region next to a GTP-binding motif that specifically binds $PI(4,5)P_2$ and $PI(3,4,5)P_3$ [168]. A comparison of the crystal structures of the GTP and GDP complexes of septin 9 suggests that a membrane-interacting N-terminal helix is either free to engage membranes or occluded by a polyacidic region, respectively [169]. A filamentous structure formed by a hetero-trimer of septins 2, 6 and 7 suggests an elongated surface that could engage the membrane by presenting an array of coiled coil extensions [170], which are resolved in recent crystal structures of septins 6 and 8 [171]. Our MODA analysis [21] reveals a set of membrane-interacting sites in these structures, which in the polymeric forms could generate the PI-bound concentric rings around the mother-bud neck to elicit cell division. A set of regulators of G-protein signaling (RGS) accelerate the activity of GTPases and, in the case of the RGS4 protein, are inhibited by $PI(3,4,5)P_3$ interactions, with a candidate basic-binding site identified by mutagenesis being conserved [172]. The Rho GTPase activity protein (RhoGAP) family of proteins can also bind to PIs, as shown by structural studies of Rgd1p, explaining how it stimulates its G protein partners to establish cell polarity [173], underscoring the diversity of PI regulatory mechanisms in such pathways.

A family of Tubb-like proteins (TULPs) respond to signals from receptors including activated heterotrimeric G proteins with which they interact at the cell surface. These proteins are found on the plasma membrane and in the nucleus, where they regulate gene expression. They share a conserved 260-residue domain that forms a closed 12-stranded β barrel surrounding a buried α helix. This Tubby domain binds $PI(4,5)P_2$ in a basic pocket at the end of an unoccupied binding groove for double stranded DNA (Figure 4d). Multiple lipid-binding sites are evident by MODA analysis [21] of this structure as well as in human TULP1, suggesting multivalent PI binding. Lipid release presumably triggers a conformational change that leads the protein to translocate to the nucleus to mediate transcriptional activation [174].

3.10. PI-regulated actin assemblies

A variety of actin-binding proteins are regulated by PI interactions. The GTPase binding domain of N-WASP binds tightly to membranes containing $PI(4,5)P_2$ and $PI(3,4,5)P_3$ lipids, thus enabling actin filament assembly on the plasma membrane in response to cell stimulation [175]. The Basic Domain (BD) at the N-terminus of the diaphanous-related 2 (Diaph2) protein also binds $PI(4,5)P_2$, with models showing how distributions of this lipid are selectively bound through multivalent complexes with cholesterol [176]. Another module which interacts with both GTPases and PIs is known as a Tre2–Bub2–Cdc16 (TBC) domain, which generally serves to regulate vesicle trafficking. This domain mediates the electrostatically-driven interactions of the Skywalker protein with $PI(4,5)P_2$- and $PI(3,4,5)P_3$-containing membranes, with a crystal structure showing how the PI headgroup is bound by basic residues in a functionally critical pocket [177].

Actin filaments are connected to integrin adhesion receptors through α-actinin. This helical protein forms antiparallel homodimers with a pair of actin-binding calponin homology (CH) domains at each end. The second CH domain binds $PI(4,5)P_2$ and $PI(3,4,5)P_3$ in the vicinity of helix F based on H/D exchange data, inducing conformational changes and inhibiting actin filament interactions [178]. Docking of $PI(4,5)P_2$ to the crystal structure of full length α-actinin-2 is consistent with a binding site involving three arginine residues, which could then trigger opening of the multidomain protein and binding of its partner titin [179]. Vinculin proteins are cytoskeletal adaptors that connect actin to adhesion receptor complexes. Metavinculin possesses two basic sites where $PI(4,5)P_2$ binds with high affinity, as evidenced by the complexed crystal structure as well as mutagenesis studies [180]. These lipid interactions induce the dimerization of the protein, which contributes to the formation of an organized actin cytoskeleton.

The actin depolymerizing factor-homology (ADF-H) domain is found in cofilin and similar actin-binding proteins, which are ubiquitously expressed in eukaryotic cells in order to accelerate the turnover of actin filaments. The binding site and non-specific interactions of cofilin with $PI(4,5)P_2$ have been characterized by NMR and indicate mutually exclusive binding of actin and independence from phosphorylation [181]. The gelsolin protein also engages $PI(4,5)P_2$ through its ADF-H domains, with ATP binding dislodging the complex and membrane interactions to initiate recycling of actin filament remodeling [182]. The similar CapG protein also binds this lipid, with MODA analysis [21] suggesting binding sites in its structure [183,184]. The F-actin capping protein (FACP) encoded by CapZ contains a 280 residue beta subunit, with our MODA analysis [21] as well as hydrophobicity analysis suggesting sites that could be responsible for binding to biological ligands including $PI(4,5)P_2$ [185] and PI3P [186] at the plasma and omegasome membranes, respectively.

Proteome-wide screening for acidic phospholipid binding proteins yielded another set of candidates, including Coronin 1A, which binds $PI(4,5)P_2$ and localizes to the plasma membrane where it disassembles Arp2/3-containing actin filament branches [182]. This membrane interaction is mediated by its N-terminal Trp-Asp (WD) repeats (which are also found in the proppin fold), and is enhanced by oligomerization via a C-terminal coiled coil region while being hampered by F-actin binding.

The Golgi apparatus contains PI 4-kinases that are key regulators of Golgi structure and function. Their effectors include proteins that bind PI4P through Golgi phosphoprotein 3 (GOLPH3) domains. A crystal structure of the GOLPH3 domain contains a sulfate ion that suggests the position of a bound PI4P ligand molecule within a conserved pocket [187]. This interaction may allow such proteins to recuit mannosyltransferases to the Golgi for processing of glycoproteins and glycolipids as well as unconventional myosin proteins and F-actin to influence the development of Golgi-ribbon morphologies [188].

The ~250 residue APT1 domain of the yeast Atg2 protein binds specifically to PI3P in a calcium-dependent manner, and contributes to its essential role in autophagy [189]. This structurally uncharacterized module is also found in four human proteins including Vps13A, where it engages the actin cytoskeleton and mediates trafficking within the endosomal membrane system [190]. Interestingly, it can also bind PI4P and PI5P without dependency on divalent cation interactions, suggesting multiple PI-dependent processes [191].

3.11. Pathogen proteins that bind PIs

The replication of virions in eukaryotic host cells often takes place on membranes that provide platforms for particle assembly. For example, the picornavirus 3 CD protein accumulates on membranes within infected cells, and its 3C protease domain contains a pocket that binds nonspecifically to PIs [192]. In a similar vein, the matrix proteins of HIV and Ebola bridge genomic and membrane components during viral budding by specifically binding PI(4,5)P_2. In the former case, this interaction triggers the release of myristoylated N-termini for insertion into the plasma membrane [193] while in the latter it leads to multimerization [194]. The helical H7 protein of vaccinia virus resembles PX domains and contains a distinct basic pocket, which binds PI3P and PI4P and contributes to a viral membrane assembly process that is essential for replication of complete particles [195].

The Gram negative bacterium *Legionella pneumophila* delivers almost 300 protein effectors into host cells to establish a vacuolar structure on which to replicate. The crystal structure of one of these reveals four domains, including the PI4P binding of SidC (P4C) domain, which comprises a four-helix bundle with a basic pocket that could accommodate a PI molecule [196]. The SidM protein of this bacterium contains a PtdIns4P binding of SidM/DrrA (P4M) domain, which consists of six helices that form a basic binding site for a PI4P molecule while positioning leucine sidechains to insert into the lipid bilayer, thus tethering the protein to vacuolar membranes (Figure 4e) [197,198]. The RavZ protein contains a five-helix bundle domain that recognizes PI3P and membrane curvature in order to target autophagosomes where it proteolytically cleaves its substrates [199]. The MavQ protein binds PI3P and PI(3,5)P_2 via its C-terminal domain, while its N-terminal PI3K domain coordinates with the PI 3-phosphatase SidP to form traveling PI waves that undulate over regions of the endoplasmic reticulum, leading to vesicles and tubules [200]. Two other *L. pneumophila* proteins, LidA and LpnE, also bind specifically to PIs in order to anchor to vacuoles [201,202]. Another Gram negative bacterium, *Bordetella pertussis*, expresses the BteA protein, which localizes to the plasma membrane of host cells *via* PI(4,5)P_2 interactions of its four helix bundle domain [203], as does an analogous domain of the *Vibrio parahaemolyticus* protein VopR [204]. Together these selected examples demonstrate how pathogens exploit the PI code to organize and replicate their components on host cell membranes.

4. Expert opinion

The PI code that determines how cells are organized into dynamic membrane-bound organelles and associated protein machines depends on hundreds of membrane readers. The presence of so many readers in eukaryotes highlights the fundamental importance of the PI code while also demonstrating common principles underlying their coordinated contributions to cell biology. These have been painstakingly studied by many researchers, revealing the mechanisms of membrane recognition in a way that can now be extended to other proteins and folds. It is increasingly apparent that these domains simultaneously detect PIs and other co-located lipids within what we term a lipidon, rather than just recognizing a solitary PI headgroup, with further potential selectivity controlled by the fluidity and curvature of the bilayers along with protonation states induced by local pH microenvironments. These modules also act in concert with other protein domains and partners through cooperative interactions, coincidence detection, and complex regulatory influences. Their interactions are controlled by kinases and phosphatases that act on exposed hydroxyl groups of lipids and amino acid residues with recognition surfaces, as well as metabolite-based modifications of basic sidechains that populate membrane binding surfaces. The dynamic equilibrium of these modifications in response to external stimuli and cell cycle controls presumably allows for accurate maintenance and replication of the cell's components, and merits further analysis amongst the growing universe of membrane readers.

The illumination of FYVE, PH, and PX domain binding mechanisms reveals how multi-lipid ligands are engaged in membrane mimics, such as micelle and bilayer systems. However, much remains to be done. The molecular identities and conformations of the lipidons of most members of these superfamilies remains unknown and cannot yet be reliably predicted. How multivalent networks of several lipids in an asymmetric bilayer are recognized and reshaped by even well-characterized membrane readers remains murky. How proteins change in structure and signaling state in response to lipid binding and local PTMs is unclear. This is in contrast to our deeper understanding of how specific conformations of nucleic acid and protein motifs are recognized by transcriptional, translational and signaling machines and then elicit molecular responses that change cellular behavior. Improved ways to detect, represent and use lipidons and membrane readers are needed. Whether lipids will need to be covalently linked into lipidon mimetics to stabilize them or encircled in polymers designed to retain native assemblies of membrane reader:lipidon complexes remains to be explored. In the meantime, the measuring stick for progress in this field may be the identification and resolution of multi-component, biologically accurate lipid:protein complexes, of which there are currently only a handful, whether peripheral or integral to the membrane [205].

There is a growing need to characterize the increasing number of families of membrane readers, many of which lack experimental structures, let alone of their lipid-bound or post-translationally modified states. High-throughput screening efforts are yielding additional candidate PI binding domains from eukaryotic proteomes that merit closer examination [37–41,206] and may benefit from screening of native membrane sections using SMALP technology [205]. Moreover, there are innumerable membrane readers among pathogenic species that take advantage of the PI code. Their diverse binding sites, lipid specificities, and localizations necessitate further analysis and cross-validation to discern their true biological lipid ligands and complete binding and regulatory sites. This information is difficult to obtain due to in part to the challenges of making and analyzing such labile complexes. Hence quantitative datasets that allow comparison of lipid binding specificities and affinities within or between superfamilies are generally lacking. Nonetheless, new technologies including BLI are positioning to enable accurate and cost-effective profiling of lipid binding using smaller amounts of material. Continued development of structural biology tools will allow the recognition of lipids by diverse folds to be visualized. However, there are limitations. While NMR can be used to study small, reconstituted domains with isotope labels, large native complexes are inaccessible. While X-ray crystallography can occasionally resolve proteins bound to detergents or lipids, artifacts have appeared and complexes with multiple different lipids are lacking. While cEM can be used to resolve stable ex vivo states of larger assemblies, the identities of bound biologically-sourced lipids have not yet been evident. The convergence of cEM and SMALP technology may eventually allow routine elucidation of memtein structures including bound lipidons. In the meantime, full experimental characterization will continue to require several complementary techniques to understand the nature of biologically relevant complexes of lipid bilayers with proteins.

Predictive methods continue to improve, although the multidomain architectures and flexible sequences, sidechains, and PTMs involved in membrane binding remain difficult to model accurately. Protein structure and membranebinding site prediction algorithms including I-TASSER and MODA are already sufficiently robust to allow identification of conserved membrane docking surfaces in protein domains, thus allowing extrapolation across families of homologous proteins as well as analogous domains [20]. Expansion of the number of structurally characterized PI-specific domains will allow programs like MODA to be refined to discern more binding features. While lipid specificity and affinity cannot typically be fully and accurately predicted, the presence of PI-specific binding motifs and regulatory elements can already be inferred. Another limitation is the lack of adequate models of heterogeneous membrane environments, with hydrophobic slabs or single PI molecules being poor proxies for the lipid compositions recognized in vivo. More robust computational tools, such as molecular dynamics simulations of lipid mixtures using improved force fields are needed to allow prediction of amino acid sidechain and bilayer conformations if they are to be useful for lipidon representation. Increases in computing power may allow the multiple membrane-interactive sites in larger structures of tandem domains, oligomers, multisubunit complexes, and fibrils, such as prions [207] to be discerned. The accumulation of more high-quality experimental data from bilayers (rather than detergents) and from live cells will allow the evaluation, comparison, and refinement of predictions from such simulations as well as derivation of a more quantitative basis for modeling specific protein–lipid interactions in various membrane environments. This in turn will be crucial for understanding reversible, regulated lipid binding in normal cellular contexts, as well as protein misbehaviors in their pathological states, which often involve misfolding on membrane surfaces.

Large databases of 3D structures and PTMs, as well as open access tools such as MODA, have proven invaluable for enabling advances including elucidating how the PI code is regulated. Recently these tools were used to reveal how PIP-stops and MET-stops control PI code recognition and pervade all kingdoms of life [19,20]. Moreover, they provide any investigator with the ability to check their protein target for membrane-binding sites and regulatory switches. The identification of PIP-stops and MET-stops also provides an array of targets for therapeutic intervention as well as improved biomarker data for diagnostic and prognostic tests. The enzymes that block PI code readers, such as acetyltransferases and methyltransferases, represent untapped targets for intervention, while inhibitors of protein kinases that phosphorylate their membrane docking surfaces can now be more rationally designed.

The ongoing challenge now is to resolve the diverse lipidons and memteins at atomic resolution, including quantifying the dynamic interactions between lipid and protein molecules as they exist in vivo, including the regulated states. Such complexes are the best representations of many therapeutic targets and encompass not only the membrane readers described here but also integral membrane systems. The design of improved native nanodisc systems to extract, purify, and measure such assemblies with minimal perturbation or bias remains an ongoing goal of the SMALP network. Being able to extract and present intact assemblies of membrane readers bound to lipidons from cells and tissues will allow screening of drug-like molecules and antibodies that are more specific for biologically relevant states. Disruption of protein:PI interactions through PTMs or mutations of membrane readers reprograms cellular states and contributes to many diseases including cancer. Indeed, proteins bound to membranes and signaling lipids including PIs represent the most valuable drug targets, and their exploitation will be accelerated by understanding their signaling and regulatory mechanisms in greater predictive detail.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert

testimony, grants or patents received or pending, or royalties. M.O. volunteers as director of the SMALP Network and invented copolymers used to produce native nanodiscs. Funder of this work had no influence on its content.

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