Structural basis for phosphatidylinositol-phosphate biosynthesis

Oliver B. Clarke1,*, David Tomasek2,*, Carla D. Jorge3,*, Meagan Belcher Dufrisne2, Minah Kim2, Surajit Banerjee4, Kanagalaghatta R. Rajashankar4, Lawrence Shapiro1, Wayne A. Hendrickson1, Helena Santos3 & Filippo Mancia2

Phosphatidylinositol is critical for intracellular signalling and anchoring of carbohydrates and proteins to outer cellular membranes. The defining step in phosphatidylinositol biosynthesis is catalysed by CDP-alcohol phosphotransferases, transmembrane enzymes that use CDP-diacylglycerol as donor substrate for this reaction, and either inositol in eukaryotes or inositol phosphate in prokaryotes as the acceptor alcohol. Here we report the structures of a related enzyme, the phosphatidylinositol-phosphate synthase from Renibacterium salmoninarum, with and without bound CDP-diacylglycerol to 3.6 and 2.5 Å resolution, respectively. These structures reveal the location of the acceptor site, and the molecular determinants of substrate specificity and catalysis. Functional characterization of the 40%-identical ortholog from Mycobacterium tuberculosis, a potential target for the development of novel anti-tuberculosis drugs, supports the proposed mechanism of substrate binding and catalysis. This work therefore provides a structural and functional framework to understand the mechanism of phosphatidylinositol-phosphate biosynthesis.
In eukaryotes, phosphatidylinositol (PI)-based lipids (phosphoinositides) play important roles in numerous aspects of intracellular signalling and in the anchoring of glycosylphosphatidylinositol-linked proteins to the membrane. In prokaryotes, PI is produced by mycobacteria, as well as some other bacterial genera, where it is required for the biosynthesis of key components of the cell wall. For example, the cell walls of mycobacteria carry complex lipoglycans such as lipomannan and lipoarabinomannan, which are tethered to the membrane via a common PI anchor that constitutes their first building block. In Mycobacterium tuberculosis, these lipids function as important virulence factors and modulators of the host immune response.

Mycobacterium tuberculosis (Fig. 1a) generate PIP using L-inositol-1-phosphate (PIP synthase) has been characterized in archaea, which like the PI biosynthesis (class I family members only). All (class I) CDP-alcohol phosphotransferases (CDP-APs) are peripheral membrane proteins of an unrelated fold, and are not involved in PI biosynthesis (class II enzymes will not be discussed here, and we will use the term CDP-APs as referred to class I family members only). All (class I) CDP-APs are integral membrane enzymes that catalyse the transfer of a substituted phosphate group from a CDP-linked donor, CDP-DAG for PI and PIP biosynthesis, to an acceptor alcohol to generate a phosphodiester-linked product.

Two recent structure reports on CDP-APs from Archaeoglobus fulgidus (Af), a protein termed Af2299 and a di-myoinositol-phosphate phosphatase synthase (AfDIPP synthase) have offered a first glimpse of the transmembrane (TM) architecture and catalytic machinery of this enzyme family. However, neither Af2299 nor AfDIPP synthase process lipids, leaving unanswered the key question of how membrane-embedded substrates are recruited and processed by CDP-APs, to generate glycerophospholipids such as PI and PIP. To shed light on this question at a molecular level, we decided to focus on the PIP synthases for three reasons. First, they are the closest evolutionary relatives to Af2299 and AfDIPP synthase, an advantage for the crystal engineering approach we adopted here and which is described below. Second, PIP synthases bind CDP-DAG as donor substrate, a feature in common with eukaryotic PI and cardioliipin synthases, as well as all prokaryotic CDP-APs involved in glycerophospholipid biosynthesis. Third, they have possible medical relevance, as genetic ablation of mycobacterial PIP synthase is lethal.

We have determined the crystal structures of PIP synthase from Renibacterium salmoninarum, in the apo form and with bound CDP-DAG to 2.5 and 3.6 Å resolution, respectively. These structures show how CDP-DAG binds to the enzyme, and reveal the molecular determinants of substrate specificity and catalysis. Functional assays performed on PIP synthase from M. tuberculosis, which is 40%-identical to the ortholog from R. salmoninarum, and a potential target for the development of novel anti-tuberculosis drugs, supports the proposed mechanism of substrate binding and catalysis. This work provides both a structural and a functional framework to investigate and understand PIP biosynthesis.

**Figure 1 | Transmembrane architecture of a PIP synthase from R. salmoninarum.** (a) PIP synthases catalyse the transfer of a diacylglycerol-substituted phosphate group (purple/red) from the CDP-DAG donor to the inositol phosphate acceptor (green), generating PIP and CMP. (b) Structure of the RsPIPS-D6N homodimer in ribbon representation viewed from two orthogonal orientations (in the plane of the membrane on the left; towards the cytosol down the dimer axis on the right). One protomer is coloured grey, and the helices of the other are depicted in spectral colouring, from blue (JM1) to red (TM6). The Af2299 extramembrane domain used to facilitate crystallization is not shown here.
Results

Crystal engineering and structure determination. Initial attempts to express and crystallize several mycobacterial PIP synthases and close bacterial homologues were unsuccessful. We rationalized that this might be due, at least in part, to the lack of a crystal contact forming soluble domain. Unlike most other CDP-APs, both Afi2299 and AfDIPP synthase have an N-terminal cytosolic cytidylyltransferase-like domain14,15, which provided the essential contacts in the crystal lattice between layers of molecules. We reasoned that the Afi2299 extramembrane domain might be able to improve the performance in crystallization experiments of other CDP-APs. To test this hypothesis, we generated chimeric constructs by fusing the Afi2299 cytosolic domain to the N-terminus of 14 different PIP synthases. We also introduced mutations at six positions in all 14 PIP-synthese sequences to mimic the interface between soluble and TM domains observed in the structure of Afi2299 (Supplementary Fig. 1). Attachment of this domain led to a significant increase in expression levels for all the constructs tested, and proteins from several species of mycobacteria and bacteria yielded crystals by the lipidic cubic phase technique16. We determined the structure of a chimeric construct of PIP synthase from R. salmoninarum (RsPIPS)—the causative agent for bacterial kidney disease in salmonids, a major threat to these species worldwide17—in which the first six residues of the RsPIPS sequence were omitted (RsPIPS-Δ6N; Supplementary Fig. 1). Crystals of RsPIPS-Δ6N diffracted X-rays to 2.5 Å resolution, and the structure was solved by molecular replacement using the cytidylyltransferase-like domain of Afi2299 as a search model (Table 1). Subsequently, we determined the structure of a construct containing the complete RsPIPS sequence (absent the initiating methionine; RsPIPS-FL) in complex with CDP-DAG at 3.6 Å resolution, again by molecular replacement, in this instance using RsPIPS-Δ6N as search model (Table 1 and Supplementary Fig. 1).

In both structures, the relative disposition of the transmembrane and soluble domains is very similar to that observed in the structure of Afi2299. Of the six mutations introduced at the RsPIPS–Afi2299 interface, two appear particularly important in limiting flexibility between the two domains. L75 and F77, which are both located in the loop between TM2 and TM3, are buried in hydrophobic pockets on the surface of the cytidylyltransferase-like domain, replicating the interactions observed in the crystal structure of Afi2299 (Supplementary Fig. 1).

Transmembrane architecture and active site in RsPIPS-Δ6N. RsPIPS adopts a homodimeric architecture similar to those previously observed in Afi2299 and AfDIPP synthase (Fig. 1b and Supplementary Fig. 2), with each protomer possessing six TM helices surrounding a large polar cavity. Sequence alignment with eukaryotic CDP-APs that process a lipid acceptor substrate, such as choline/ethanolamine phosphotransferase (CEPT1; Supplementary Fig. 3) suggests that these eukaryotic enzymes may possess an additional three to four TM helices at the C terminus, perhaps serving as an additional TM module to accommodate the bulkier, hydrophobic acceptor. In RsPIPS, the central polar cavity is located at the cytosolic boundary of the membrane, and contains three distinct regions, which together form the active site (Fig. 2a). The nucleotide-binding site is delineated by TMs 1, 2 and 3, and is characterized by a signature sequence featuring eight absolutely conserved residues (D1xxD2G3xxAR…G4xxD5xxD6) (ref. 18), five of which are located on TM2 and three on TM3. As observed previously18, the first three of the conserved aspartate side chains coordinate a metal, and D4 likely acts as the catalytic base. The four other signature amino acids either provide structural flexibility or line the binding site that accommodates the pyrimidine ring of the CDP18.

Proximal to the nucleotide-binding site, and within the membrane-spanning region is a pocket wedged between TMs 4, 5 and 6, which probably represents the inositol phosphate acceptor-binding cavity (Fig. 2). Several conserved residues line this cavity, including two arginine residues (R153 and R191) that in the structure of RsPIPS-Δ6N coordinate a SO42− ion present at high concentration in the crystallization solution. R153 and R191 are absolutely conserved amongst all PIP synthases, but not in eukaryotic PI synthases. We hypothesize that these residues coordinate the phosphate of inositol phosphate, a moiety unique to PIP synthases.

Directly above the nucleotide-binding site, we observed a gap between JM1 and TM2, creating a hydrophobic groove that is open to the membrane. In contrast, the structures of Afi2299 and AfDIPP synthase displayed a small, hydrophilic pocket in this region, which in the case of Afi2299 was shown to accommodate the glycerol moiety of the CDP-glycerol donor. The difference in the nature of the donor substrate, CDP-DAG for RsPIPS and CDP-glycerol for Afi2299 is most likely responsible for the differences observed in the architecture of the donor-substituent site in these two enzymes.

Structure of RsPIPS in complex with CDP-DAG. We initially engineered RsPIPS chimeric constructs with alternative junctions to the N-terminal domain (Supplementary Fig. 1), and crystals were obtained of two of these, RsPIPS-Δ6N and RsPIPS-FL. The crystals of RsPIPS-Δ6N diffracted well in the apo-state (2.5 Å), but attempts to obtain co-crystal structures with CDP-DAG were unsuccessful. In contrast, although crystals of RsPIPS-FL only diffracted to lower resolution (3.6 Å), a structure of the complex with CDP-DAG was obtained readily. The structure of the complex of CDP-DAG bound to RsPIPS-FL revealed strong density for CDP, with the nucleotide ring wedged between TMs 2 and 3, and the diphosphate moiety coordinated by a bound magnesium ion that also interacts with conserved aspartate residues of the CDP-AP signature sequence (Fig. 2b). In all four protomers in the asymmetric unit, density was also observed for the acyl chains of the CDP-DAG, which lie against the TM region in a groove formed by JM1, TM2 and TM5 (Fig. 3, Supplementary Fig. 4 and Supplementary Movie 1). This groove is entirely absent in the two previous structures of CDP-APs, which have a small, hydrophilic pocket in this location, consistent with their preference for soluble donor moieties such as CDP-glycerol and CDP-inositol (Supplementary Fig. 1).

Functional validation of crystallization constructs. Functional characterization of RsPIPS-FL, performed by measuring incorporation of [14C]inositol-1-phosphate into membranes derived from RsPIPS-FL-expressing Escherichia coli cells, revealed that although this enzyme from R. salmoninarum exhibits significant Mg2+–dependent PIP-synthese activity (Supplementary Fig. 1), we judged the activity level to be too low to provide the basis for a reliable assay system. By contrast, equivalent constructs of the close PIP-synthese homolog from M. tuberculosis (MtPIPS; 40% identity to RsPIPS) showed robust specific activity (Supplementary Fig. 1). We therefore used MtPIPS, which has high homology to RsPIPS in its active site region (Supplementary Fig. 5), as an assay system for structure-based functional characterization of PIP synthases.

Functional characterization of the chimeric MtPIPS proteins revealed that the activity of MtPIPS-Δ6N was substantially lower than that of MtPIPS-FL (Supplementary Fig. 1). We hypothesize that this diminished activity of MtPIPS-Δ6N is due to compromised binding of CDP-DAG, as JM1 is truncated and distorted in RsPIPS-Δ6N, potentially interfering with CDP-DAG
binding (Supplementary Fig. 1i). This could provide an explanation as to why we were unable to obtain the structure of RsPIPS-Δ6N in complex with its cognate lipid substrate. The length of the linker appears to be the primary cause of the reduced activity of the Δ6N construct, not the addition of the Afl299 domain, nor the interface mutations. Indeed, the activity of MtPIPS-FL is comparable to the activity of MtPIPS constructs lacking the extramembrane domain and interface mutations (Supplementary Fig. 1j). All proteins tested expressed at levels comparable to WT protein.

Functional characterization of PIP synthase. We selected mutants expected to have compromised substrate binding or catalytic activity based on residue conservation and based on our functional assay. All mutants analysed were expressed at levels comparable to WT MtPIPS-FL. Mutation to alanine in RsPIPS of the two arginine residues (R155 and R195) that bind to SO4 in complex with its cognate lipid substrate. Both PO4− and SO4− inhibit activity at of a crystallization chaperone fusion combined with engineering of the interface between the two domains, prerequisites for successful crystallization and structure determination, did not substantially impact the capability of the enzyme to function as compared with the wild-type (WT) protein.

Table 1 | Data collection and refinement statistics.

|                        | RsPIPS-Δ6N | RsPIPS-FL |
|------------------------|------------|-----------|
| Wavelength (Å)         | 0.979      | 0.979     |
| Resolution range collected (Å) | 103.92–2.50 (2.60–2.50) | 167.3–3.613 (3.97–3.613) |
| Resolution used in refinement(Å) | 14.92–2.502 (2.591–2.502) | 167.3–3.613 (3.742–3.613) |
| Space group            | P 2, 21, 2 | P 21     |
| Unit cell              | 48.63      | 89.00     |
| a                      | 94.069     | 62.49     |
| b                      | 103.92     | 169.77    |
| c                      | 99.77      | 103.92    |
| Total reflections       | 320,975 (21,993) | 78,622 (18,367) |
| Unique reflections      | 16,784 (1,453) | 21,268 (1,996) |
|Multiplicity            | 19.0 (13.6)| 3.7 (3.8) |
| Completeness (%)        | 98.2 (84.5)| 98.9 (97.9)|
| Mean I/σ(I)            | 9.9 (1.7)  | 5.0 (1.4) |
| Wilson B-factor         | 43.74      | 79.46     |
| Rmerge                  | 0.267 (1.654) | 0.252 (0.930) |
| Rfree                   | 0.281 (1.775) | 0.294 (1.087) |
| CC(1/2)                | 0.999 (0.636) | 0.991 (0.616) |
| Resolution where I/σ(I) drops below 2.0 (overall) | 2.59 | 3.89 |
| Resolution where I/σ(I) drops below 2.0 (along h) | 2.50 | 4.09 |
| Resolution where I/σ(I) drops below 2.0 (along k) | 3.25 | 4.09 |
| Resolution where I/σ(I) drops below 2.0 (along l) | 2.61 | 3.62 |
| Resolution where CC(1/2) drops below 0.5 (overall) | 2.50 | 3.62 |
| Resolution where CC(1/2) drops below 0.5 (along h) | 2.50 | 3.84 |
| Resolution where CC(1/2) drops below 0.5 (along k) | 3.34 | 4.05 |
| Resolution where CC(1/2) drops below 0.5 (along l) | 2.59 | 3.62 |
| Reflections used in refinement | 16,891 (1,453) | 21,268 (1,996) |
| Reflections used for Rfree | 824 (58) | 1,043 (117) |
| Rwork                  | 0.2284 (0.3326) | 0.2801 (0.3918) |
| Rfree                  | 0.2520 (0.3627) | 0.2997 (0.4246) |
| Number of atoms (all non-hydrogen) | 2,952 | 10,845 |
| Number of atoms (protein) | 2,586 | 10,350 |
| Number of atoms (ligands) | 333 | 495 |
| Protein residues        | 338 | 1,358 |
| RMS (bonds)             | 0.003 | 0.004 |
| RMS (angles)            | 0.72 | 1.07 |
| Ramachandran favoured (%) | 98 | 97 |
| Ramachandran allowed (%) | 2 | 1.9 |
| Ramachandran outliers (%) | 0 | 0.1 |
| Rotamer outliers (%)    | 1.8 | 1.7 |
| Clashscore              | 2.87 | 8.53 |
| Average B-factor (all atoms) | 70.55 | 76.53 |
| Average B-factor (protein) | 70.89 | 77.17 |
| Average B-factor (ligands) | 69.59 | 63.13 |
| Average B-factor (solvent) | 53.66 |
| Number of TLS groups    | 2 |

Statistics for the highest-resolution shell are shown in parentheses; RMS, root mean square; TLS, Translation-Libration-Screw.
concentrations well below those used for crystallization (Fig. 4c), consistent with competition between binding of SO\textsubscript{4}\textsuperscript{2–} and the phosphate group of inositol phosphate. More subtle substitutions (R/Q) at the same positions (R\textsubscript{155} and R\textsubscript{195}) also resulted in substantial reduction in enzymatic activity (Fig. 4b). Kinetic characterization of inositol phosphate and CDP-DAG-dependent activity of R\textsubscript{195Q} (Fig. 4d and Supplementary Fig. 6), which retains \( \approx 40\% \) of WT activity, demonstrated that the mutation had only a mild effect on the \( K_M \) for CDP-DAG (236 \( \mu \)M for WT; 329 \( \mu \)M for R\textsubscript{195Q}), while severely impairing \( K_M \) for inositol phosphate (122 \( \mu \)M for WT; 1,208 \( \mu \)M for R\textsubscript{195Q}). Comparison of WT and R\textsubscript{195A} proteins extracted and purified from isolated membranes with a non-ionic detergent showed that both proteins are membrane inserted and have the same elution profile on size-exclusion chromatography (SEC), strongly suggesting that this point mutation does not compromise folding (Supplementary Fig. 7). Direct single-point measurements of \(^{14}\text{C}\)inositol-1-phosphate binding to liposome-incorporated R\textsubscript{195Q}, WT and D93N (D\textsubscript{4}) MtPIPS-FL proteins were carried out to differentiate the direct effects of these mutations on inositol phosphate affinity from other mechanisms by which catalysis could be impaired (Fig. 4e). These assays were carried out in the presence and absence of CDP-DAG. Intriguingly, inositol phosphate binding was strictly CDP-DAG dependent (Fig. 4e). Determination of the fraction of \(^{14}\text{C}\)PIP in the liposomes after the binding assay was carried out to assess the catalytic activity of the proteins under these conditions. The only sample exhibiting any detectable level of catalytic activity was the WT construct in the presence of CDP-DAG, for which nearly all of the radioactive inositol phosphate above the background level was incorporated in the lipophilic PIP and therefore found in the organic phase (77.8 \( \pm \) 0.6 pmol per assay; \( \pm \) indicates standard error of the mean, \( n = 3 \)). The amount of inositol phosphate bound in total was comparable to WT for the D93N mutant, consistent with the role of this residue in catalysis, as opposed to substrate recognition. In contrast, R\textsubscript{195Q} bound a significantly lower amount of substrate, compatible with its putative function in binding of inositol phosphate. Alanine mutagenesis of a conserved serine (S\textsubscript{132}) that also interacts with the Rs\textsubscript{PIPS}-bound SO\textsubscript{4}\textsuperscript{2–} reduced activity as well, albeit to a lesser extent. Lysine K\textsubscript{135} is located such that it may interact with the inositol ring, and the K\textsubscript{135A} mutation also resulted in a partial loss of activity.

Furthermore, mutation in MtPIPS of P153W, a conserved residue on TM5, which stacks against one of the aliphatic chains

---

**Figure 2 | A large interfacial cavity contains the active site of RsPIPS.** (a) The structure of RsPIPS\textsubscript{Δ6N} is shown in ribbon representation, with one protomer coloured grey and the other coloured by the Kyte-Doolittle hydrophobicity scale\textsuperscript{34}, from \(-4.5\) (most polar, light blue) to 4.5 (most hydrophobic, orange). Two orthogonal representations are shown, on the left is a view in the plane of the membrane and on the right is a view from the cytosol along the dimer axis. A transparent purple surface (calculated using the 3V server\textsuperscript{35}) delineates the borders of the interfacial cavity, which contains three subregions as follows: 1, the inositol phosphate acceptor-binding pocket; 2, the nucleotide-binding pocket between TM2 and TM3; and 3, a hydrophobic groove between TM2 and JM1. (b) Detail of the active site viewed in the plane of the membrane, with side chains that contact the bound \( \text{Mg}^{2+} \) and SO\textsubscript{4}\textsuperscript{2–} ions labelled and depicted in stick representation.
of CDP-DAG, resulted in nearly complete loss of enzymatic activity, consistent with a loss of CDP-DAG binding due to obstruction of the lipid-binding groove by the larger tryptophan side chain (Fig. 4a,b). In agreement with this hypothesis, substitution of P153 with alanine had minimal effects on activity, while substitution with valine resulted in a partial defect in activity. Kinetic characterization of P153V (Fig. 4d and Supplementary Fig. 6) showed $V_{\text{max}}$ to be substantially decreased, consistent with an effect of this mutation on the catalytic efficiency of the enzyme. Unexpectedly, the $K_m$ for inositol phosphate was increased, whilst the $K_m$ for CDP-DAG was decreased for this mutant, possibly suggestive of a more complex role of P153, critically located at the CDP-DAG entrance to the active site, and two residues in sequence away from R155, one of the two key inositol phosphate-binding residues. Mutation of another residue, L70W, near the groove but oriented away from it, did not affect the activity (Fig. 4a,b), while substitution of the directly adjacent M69, which contacts CDP-DAG from TM2, by tryptophan resulted in severely impaired activity. Substitution with residues similarly sized or smaller than the native methionine did not compromise activity (Fig. 4a,b).

Not surprisingly, mutation of D31, a conserved residue on TM1, which forms a hydrogen bond with the primary amine of CDP, to alanine, also severely compromised activity, but did not completely abolish it (Fig. 4a,b). This partial effect of the D31A mutation is likely due to the fact that most of the residues in the CDP-AP signature sequence participate in binding of the nucleotide, and that T34, present in all CDP-APs as S or T as part of a conserved P(D/N)xx(T/S) motif, also binds to the primary amine of the pyrimidine ring, and thus may compensate, at least in part, for the absence of the contribution from D31.

Finally and as expected, even a conservative D to N mutation at the site of the putative catalytic base, D93 (the fourth aspartate in the signature sequence) resulted in near complete abrogation of CDP-AP activity (Fig. 4a,b), without compromising substrate binding (Fig. 4e).

**Discussion**

The defining step in glycerophospholipid biosynthesis is catalysed by CDP-APs. These constitute a large and diverse family of membrane-embedded enzymes characterized by a signature sequence containing eight absolutely conserved amino acids and 6–10 predicted TM segments. The structure of the CDP-AP RsPIPS reported here exhibits a homodimeric, six-TM architecture similar to those described for Af2299 and AfDIPP synthase. This architecture appears to be conserved amongst all the CDP-AP family members that utilize a soluble acceptor substrate such as inositol or inositol phosphate, irrespective of the nature—hydrophobic or hydrophilic—of the CDP-attached donor (Supplementary Fig. 3). These include all characterized prokaryotic CDP-APs, as well as eukaryotic PI synthases. In contrast, CDP-APs that utilize a lipidic acceptor, such as eukaryotic phosphatidylethanolamine (PE) and phosphatidylcholine (PC) synthases, typically have three or four additional TM helices at the C terminus (Supplementary Fig. 3), which are likely required to accommodate the acyl chains of the bulky hydrophobic acceptor substrate.

Fusion of RsPIPS to a crystallization chaperone derived from the extramembrane domain of Af2299 was instrumental in obtaining diffracting crystals. The employment of crystallization chaperones is a well-established technique for obtaining crystals of otherwise recalcitrant membrane proteins and has enjoyed particularly extensive use in the field of G-protein-coupled receptor crystallography. We believe that the extramembrane domain of Af2299 may prove a valuable addition to the complement of membrane protein crystallization chaperones.
although further studies are necessary to demonstrate the general-purpose utility of this fusion partner.

The structure of RsPIPS confirms the locations of the acceptor- and donor-substituent-binding pockets described in the structure of AfJ2299, and identifies a pair of conserved arginine residues (R153 and R191), as involved in the specific recognition of inositol phosphate. Sequence alignment of RsPIPS with human CEP1 (Supplementary Fig. 3), a CDP-AP that utilizes a lipid nucleotide as the substrate, shows that an RxxR motif containing R153 aligns to a motif previously identified in CEP11 as a determinant of acceptor specificity. We suggest that the locations of the acceptor and donor sites are conserved across the entire CDP-AP family, regardless of the identity of the substrates.

A unique feature of the structure of RsPIPS is the presence of a hydrophobic crevice between JM1, TM2 and TM5 (Fig. 2 and Supplementary Fig. 1), which in the structure of RsPIPS-FL accommodates the lipid substrate (Fig. 3 and Supplementary Fig. 4). This groove is directly exposed to the bulk lipid, providing a pathway for lateral diffusion of CDP-DAG into the active site. The nucleotide is wedged between TM2 and TM3 in a pocket, which is also lined by TM1. The CDP interacts with residues from the signature sequence on TM2 and TM3. In addition, D29 and T32, part of a conserved P(D/N)x(T/S) motif at the start of TM1, form hydrogen bonds with polar substituents of the pyrimidine ring. Given the absolute conservation of residues lining the nucleotide-binding pocket, we anticipate this mode of binding will be universally conserved.

PI is an essential lipid for mycobacteria, providing the anchor and first building block of major constituents of their cell wall.

Figure 4 | A homology model of MfPIPS, with functional characterization of selected point mutants. (a) The MfPIPS homology model is shown in ribbon representation with one protomer in grey and the other in light blue, from two views, on the left viewed in the plane of the membrane and on the right from the cytosol, along the dimer axis. The substrates, CDP-DAG (purple) and inositol phosphate (black), are modelled based on the structures of RsPIPS-FL (in complex with CDP-DAG) and RsPIPS-Δ6N (with bound SO₄²⁻). The homology model was generated using the Phyre2 server, in one-to-one threading mode using the sequence of MfPIPS (UniProt accession: P9WPG7) as the target and the structure of RsPIPS-FL (with the A2299 extramembrane domain excised) as the template. Selected residues which are predicted to participate in either inositol phosphate binding (R155, R195, S132, K135), CDP-DAG binding (P153, M69, D31), neither (L70), or catalysis (D93) are shown with side chains in stick representation and coloured as in (b). (b) The activity of point mutants at these positions for the MfPIPS-FL construct is shown compared to wild-type MfPIPS-FL. (c) SO₄²⁻ (closed diamonds) and PO₄³⁻ (open circles) inhibit the activity of MfPIPS-FL with half-inhibitory concentrations of 44 and 22 mM, respectively. (d) Kₘ of MfPIPS-FL WT, R195Q and P153V for inositol phosphate (InsP; white) and CDP-DAG (grey). InsP, ³⁻[¹⁴C]inositol-1-phosphate (inositol phosphate). (e) Quantification of bound ³⁻[¹⁴C]inositol-1-phosphate after incubation of liposomes containing 9 μM MfPIPS-FL (WT, D93N, R195Q or empty liposome control) in the presence and absence of 200 μM CDP-DAG with 40 μM ³⁻[¹⁴C]inositol-1-phosphate. Measurement errors were quantified as s.e.m. (n = 3).
Genetic ablation of PIP synthase in *Mycobacterium smegmatis* leads to a loss of cell viability. This observation, combined with the unique substrate requirements of MtiPPIP, positions this enzyme as a plausible target for the development of novel anti-tuberculosis therapeutics. The structure of RsPPI provides a high-homology model for MtiPPIP (40% identity; Supplementary Fig. 1). We opted to perform experiments aimed at functional characterization of PIP synthase on MtiPPIP, due to its higher intrinsic medicinal interest and the low specific activity of the native RsPPI enzyme (Supplementary Fig. 1). The conserved pocket that accommodates the inositol phosphate acceptor provides a potentially attractive site for future structure-based drug design for two reasons. First, inositol phosphate is not recognized by eukaryotic CDP-APs. Second, the affinity of MtiPPIP for inositol phosphate is relatively low (Fig. 4d), making displacement by a putative inhibitor more feasible, at least theoretically. Mutation of either of the two conserved arginine residues (R155 and R195) in this pocket resulted in gravely impaired enzymatic activity. We hypothesize that these two residues are responsible for binding on the phosphate of inositol phosphate. Interestingly, although mutation of R195 to a glutamine resulted in severely compromised activity, a increased Km for inositol phosphate, the binding of inositol phosphate to liposome-incorporated R195Q was reduced only to a moderate degree (Fig. 4e), suggesting R195 may play an additional role in the reaction mechanism beyond its contribution to inositol phosphate affinity, potentially in positioning this substrate appropriately for catalysis. Mutation of residues that line the diacylglycerol-binding groove on either TM2 or TM5 to bulky tryptophans also compromised enzymatic activity of MtiPPIP, presumably by obstructing the groove that accommodates the acyl chains of CDP-DAG. We suggest that similar mutations in an ancestral lipid-processing CDP-AP may have contributed to the evolution of polar-osmolyte generating CDP-APs, like lipid-processing CDP-AP, which may have contributed to the evolution of eukaryotic CDP-APs. Second, the affinity of MtiPPIP for inositol phosphate is strictly CDP-DAG dependent, and that the prerequisite for inositol phosphate binding (and hence catalysis) (Fig. 4e). The observation that CDP-DAG binding is a prerequisite for inositol phosphate binding GroE, positions this membrane enzyme that processes exclusively soluble products and substrates.

Finally, measurements of the binding of inositol phosphate to liposomes containing incorporated MtiPPIP-FL (WT and D39N), in the presence and absence of CDP-DAG showed that binding of inositol phosphate is strictly CDP-DAG dependent, and that the D39N mutation, while it almost completely abrogated activity of the enzyme, does not substantially impact substrate affinity (Fig. 4e). The observation that CDP-DAG binding is a prerequisite for inositol phosphate binding (and hence catalysis) implies that MtiPPIP follows a sequential ordered bi-bi reaction mechanism in which CDP-DAG binds first, followed by inositol phosphate, and the likely formation of a reactive phosphoryl intermediate through the action of an aspartate residue (D) in the signature sequence) acting as a catalytic base, in this case D93. This is consistent with mechanisms described previously for several other members of the family.R. salmoninarum; and 14: K1EN22, Janbacter hoylesi. PCR was used to amplify the bacterial expression vector pBluescript encoding Af2399 (with an N-terminal 6x His tag and a Tobacco Mosaic Virus (TMV) 3’ leader sequence) without excluding the portion of the gene not encoding the N-terminal soluble domain. Gibson assembly was used to fuse the genes encoding PIP synthases to the linear fragment of the pBluescript-Af2399 vector. All point mutants of MtiPPIP were generated using the QuickChange site-directed mutagenesis kit (Agilent). Sequences of all primers used for cloning and mutagenesis are provided in Supplementary Table 1.

Membrane isolation and protein expression and purification. For protein overexpression, plasmids encoding PIP synthases, generated as described above, were transformed into BL21 (DE3) pLysS E. coli competent cells. Transformed cells were used to inoculate a starter culture (8 ml) of 2xYT medium supplemented with 100 µg ml-1 ampicillin and 50 µg ml-1 chloramphenicol. This culture was grown at 37 °C overnight while shaking (240 r.p.m.). The next day, the starter culture was used to inoculate 800 ml of 2xYT medium supplemented with 100 µg ml-1 ampicillin and 50 µg ml-1 chloramphenicol. Cultures were again grown at 37 °C while shaking (240 r.p.m.). Once the OD600 reached 1.0 (after about 3 h), the shaker temperature was reduced to 22 °C and 15 mL of 10x bacterial growth medium was added with a final concentration of 0.2 mM isopropyl β-1-thiogalactopyranoside. After overnight incubation at 22 °C, cells were collected by centrifugation at 4,000 g for 15 min at 4 °C and stored at –80 °C until needed. Cultures for large-scale protein expression were 800 ml in volume, while 15-ml cultures were grown similarly to test protein expression in small scale.

For large-scale purification of PIP synthases, frozen cell pellets were resuspended in lysis buffer containing 20 mM HEPES-NaOH (pH 7.5), 200 mM NaCl, 20 mM MgSO4, 10 µg ml-1 DNase I, 10 µg ml-1 RNase A, 1 mM TCEP, 1 mM phenylmethylsulfonyl fluoride (PMSF) and Complete Mini EDTA-free Trypsin inhibitor cocktail (Roche) as described in the instructions. Cells were lysed with an Emulsiflex C3 homogenizer (Avanti) and phosphatidylcholine (Avanti) were mixed in a 3:1 ratio (w/w) by dissolving in chloroform. Chloroform was removed under a stream of nitrogen gas to obtain a thin layer of dry lipids. Lipids were resuspended in lysis buffer containing 200 mM HEPES, pH 7.5 and 1.5% (w/v) 1-O-Octyl-β-D-glucopyranoside.

Method 1

**Target identification and cloning.** CDP-alcohol phosphotransferases with predicted involvement in PIP synthesis were identified from 14 prokaryotic organisms by homology to a template of known function. Six mutations were introduced into each one (Supplementary Fig. 1) to replicate the interface between the cytosolic and TM domains observed in the structure of Af2399, and the corresponding genes were synthesized (GenScript). Genes not bearing the mutations at the interface were PCR amplified from the matching genomes. The Uniprot IDs and species of the sequences identified were as follows: 1: Q9FTY9, *M. smegmatis*; 2: G6X547, *M. abscessus*; 3: T0UMF3, *M. tuberculosis*; 7: H6MZX4, *Gordonia polyisoprenivorans*; 8: Q05180, *Rhodococcus sp.* (strain RB1); 9: Q9YTD3, *Nocardia farcinica*; 10: D9UX52, *Streptomyces sp.* AA4; 11: FXF121, *Micromonatius phosphovorus*; 12: K9B2F1, *Brevibacterium casei*; 13: A9WSF5, *R. salmoninarum*; and 14: K1EN22, *Janbacter hoylesi*. PCR was used to amplify the bacterial expression vector pBluescript encoding Af2399 (with an N-terminal 6x His tag and a Tobacco Mosaic Virus (TMV) 3’ leader sequence) without excluding the portion of the gene not encoding the N-terminal soluble domain. Gibson assembly was used to fuse the genes encoding PIP synthases to the linear fragment of the pBluescript-Af2399 vector. All point mutants of MtiPPIP were generated using the QuickChange site-directed mutagenesis kit (Agilent). Sequences of all primers used for cloning and mutagenesis are provided in Supplementary Table 1.

Membrane isolation and protein expression and purification. For protein overexpression, plasmids encoding PIP synthases, generated as described above, were transformed into BL21 (DE3) pLysS E. coli competent cells. Transformed cells were used to inoculate a starter culture (8 ml) of 2xYT medium supplemented with 100 µg ml-1 ampicillin and 50 µg ml-1 chloramphenicol. This culture was grown at 37 °C overnight while shaking (240 r.p.m.). The next day, the starter culture was used to inoculate 800 ml of 2xYT medium supplemented with 100 µg ml-1 ampicillin and 50 µg ml-1 chloramphenicol. Cultures were again grown at 37 °C while shaking (240 r.p.m.). Once the OD600 reached 1.0 (after about 3 h), the shaker temperature was reduced to 22 °C and 15 mL of 10x bacterial growth medium was added with a final concentration of 0.2 mM isopropyl β-1-thiogalactopyranoside. After overnight incubation at 22 °C, cells were collected by centrifugation at 4,000 g for 15 min at 4 °C and stored at –80 °C until needed. Cultures for large-scale protein expression were 800 ml in volume, while 15-ml cultures were grown similarly to test protein expression in small scale.

For large-scale purification of PIP synthases, frozen cell pellets were resuspended in lysis buffer containing 20 mM HEPES-NaOH (pH 7.5), 200 mM NaCl, 20 mM MgSO4, 10 µg ml-1 DNase I, 10 µg ml-1 RNase A, 1 mM TCEP, 1 mM phenylmethylsulfonyl fluoride (PMSF) and Complete Mini EDTA-free Trypsin inhibitor cocktail (Roche) as described in the instructions. Cells were lysed with an Emulsiflex C3 homogenizer (Avanti) and phosphatidylcholine (Avanti) were mixed in a 3:1 ratio (w/w) by dissolving in chloroform. Chloroform was removed under a stream of nitrogen gas to obtain a thin layer of dry lipids. Lipids were resuspended in lysis buffer containing 200 mM HEPES, pH 7.5 and 1.5% (w/v) 1-O-Octyl-β-D-glucopyranoside.

**Preparation of liposomes and proteoliposomes.** E. coli polar lipid extract (Avanti) and phosphatidylcholine (Avanti) were mixed in a 3:1 ratio (w/v) by dissolving in chloroform. Chloroform was removed under a stream of nitrogen gas to obtain a thin layer of dry lipids. Lipids were resuspended in lysis buffer containing 100 mM HEPES, pH 7.5 and 1.5% (w/v) 1-O-Octyl-β-D-glucopyranoside.
(Anagrade, Affymetrix) and the detergent was removed by dialysis against 11 of 100 mM HEPES, pH 7.5. The resulting liposomes were divided into aliquotes, frozen in liquid nitrogen and stored at —80 °C. For protein incorporation into liposomes, the protocol used was adapted from Rigaud et al.22. The concentration of thawed liposomes was adjusted to 10 mg ml⁻¹ with 100 mM HEPES, pH 7.5; 0.11% (w/v) Triton X-100 was added to the liposome-containing solution and mixed by vortexing. Protein, purified as previously described, was then added in a ratio of 1:80 (0.125 mg protein to 10 mg lipid). The mixture was incubated at room temperature with agitation for 15 min. A unit of 60 mg of pretreated and equilibrated Bio-Beads SM-2 (BioRad) were added to the mixture and incubated at room temperature for 1 h under constant agitation. An additional 60 mg of Bio-Beads SM-2 was added to the mixture and incubated at room temperature for 1 h under constant agitation. Then, 120 mg of Bio-Beads were added to the mixture and incubated overnight at 4 °C under constant agitation, after which the proteoliposomes were separated and removed from the Bio-Beads by careful pipetting. The concentration of proteoliposomes was adjusted by ultracentrifugation (148,000g for 30 min at 4 °C) and resuspension in the correct volume of HEPES, pH 7.5. Proteoliposomes were divided into aliquotes, flash frozen in liquid nitrogen and stored at —80 °C.

Preparation of cell-free homogenates for functional assays. Frozen recombinant E. coli expressing Pip, syn sort constructs (r = 2 g), grown as described above, were suspended in 5 ml buffer A (50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂ and 5 mM β-mercaptoethanol) and disrupted by sonication (5 × 60 s pulses with 60-s cooling intervals between pulses). Cell debris and unbroken cells were separated by centrifugation (10,000g, 10 min, 4 °C), and membrane fractions were obtained by centrifugation of the supernatant at 100,000g for 2 h at 4 °C. The membrane fractions were suspended in 0.5 ml of buffer A and frozen at —20 °C until use.

Preparation of l-myo-[¹⁴C]inositol-1-phosphate. l-myo-¹⁴C]inositol-1-phosphate was prepared from [¹⁴C(U)]glucose (Perkin Elmer Life Sciences) using hexokinase of Thermoproteus tenax and l-myo-inositol-1-phosphate synthase (IPS) of Archaeoglobus fulgidus. E. coli cells harbouring the hexokinase or the ip gene were grown in Luria–Bertani medium at 37 °C supplemented with 100 μg ml⁻¹ ampicillin to an optical density of 0.5 at 600 nm, and protein expression was induced for 4 h with 1 mM isopropropyl-β-D-1-thiogalactopyranoside. Partial purification of recombinant hexokinase and IPS was performed by heating the cell extracts for 30 min at 90 and 60 °C, respectively, followed by centrifugation to remove denatured proteins. The production of [¹⁴C(U)]glucose-6-phosphate was carried out in a reaction mixture containing the recombinant hexokinase, [¹⁴C(U)]glucose (3.7 MBq per 336 nmol), 10 mM glucose, 50 mM Tris-HCl (pH 7.6). After incubation at 85.5 °C (10,000g), 10 mM MgCl₂, 40 mM β-mercaptoethanol, 1% (w/v) CHAPS, 50 mM Bicine buffer (pH 8.0), the radiolabelled product was assessed as mentioned above. These functional assays were performed in duplicate.

Assessment of Kₘ of MIPIPS for inositol phosphate. The Kₘ of MIPIPS (WT, R195C and P153V) was assessed in reaction mixtures (final volume, 200 μl) containing 1 mM of inositol phosphate (from which 9 μM was l-myo-¹⁴C]inositol-1-phosphate), 10 mM MgCl₂, 10 mM β-mercaptoethanol, 1% (w/v) CHAPS, 50 mM Bicine buffer (pH 8.0), 9 μM 1–myo-¹⁴C]inositol-1-phosphate plus different concentrations of cold inositol phosphate (final concentrations of 38 μM–1 mM for WT and P153V, and 38 μM–4 mM for R195Q). The mixtures were pre-incubated for 3 min at 37 °C and the reactions initiated by the addition of thawed liposomes (pre-incubated with 0.1 M MgCl₂, 0.4 mM CDP-dioleoylglycerol, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 1% (w/v) CHAPS, 50 mM Bicine buffer (pH 8.0), 9 μM 1–myo-¹⁴C]inositol-1-phosphate) and stopped at different time points by the addition of 1 ml of 0.1 M HCl in methanol. The extraction and quantification of the radiolabelled product was performed as described above. These experiments were performed in duplicate.

Binding assays with l-myo-[¹⁴C]inositol-1-phosphate. l-myo-[¹⁴C]inositol-1-phosphate-binding assays were carried out in reaction mixtures (final volume, 100 μl) containing MIPIPS (WT, D93N or R195Q) reconstituted in proteoliposomes (9 μg of protein), 2 mM MgCl₂, 40 mM inositol phosphate (of which 16 μM was l-myo-¹⁴C]inositol-1-phosphate) and 50 mM Bicine buffer (pH 8.0). The mixtures were pre-incubated for 3 min at 37 °C absent ligand, the reactions initiated by the addition of inositol phosphate and stopped after 10 min. The binding assay mixtures were passed over HAWP 02500 filters (Millipore), and unbound l-myo-[¹⁴C]inositol-1-phosphate was separated from the bound by washing three times with 600 μl of 50 mM Bicine buffer (pH 8.0). Bound l-myo-[¹⁴C]inositol-1-phosphate was quantified by liquid scintillation counting. The effect of CDP-DAG on the binding of inositol phosphate to MIPIPS was investigated by pre-incorporating 200 mM CDP-DAG in the proteoliposomes used in each reaction mixture. Assays and quantification of bound l-myo-[¹⁴C]inositol-1-phosphate were performed as described above. Assays on empty liposomes to calculate background were also performed as described above for proteoliposomes. These binding assays were performed in triplicate.

Crystallization. Crystals were grown at room temperature (22 °C) in lipidic cubic phase, using as host lipid either monoolein alone (NuChek Prep) or a mixture of 2% CDP-dioleoylglycerol (Avanti Polar Lipids) and 98% monoolein by mass. The mixture of CDP-dioleoylglycerol and monoolein was prepared the day before it was needed, and involved dissolving CDP-dioleoylglycerol in chloroform, adding it to molten monoolein in the appropriate amount to generate a 2:98 ratio by mass, vortexing, and then evaporating the chloroform with argon gas first and then overnight in a vacuum desiccator. Protein from peaks of SEC was concentrated to 35–40 mg ml⁻¹ (estimated by A₂₈₀nm) for crystallization using a centrifugal concentrator (Millipore) with a 100 kDa molecular weight cutoff. Concentrated protein was mixed with molten lipid in (w/v) ratio of 4:1 with molar lipid in (w/v) ratio of 1:1. A Mosquito LCP (TTP Labtech) robot was used to dispense a typical volume of 50–75 nl of protein/lipid mixture onto a 96-well glass sandwich plate, which was covered with 750 nl precipitant solution and sealed with a glass coverslip. Glass sandwich plates were stored in a 22 °C incubator. Crystals appeared after 1–2 days and grew to full size in about 1 week. Crystals grew in (a) 20% (v/v) polyethylene glycol 400, 0.1 M MES (pH 6.7) and 0.2 M lithium sulfate (RiPIPS-ΔN), and (b) 30% (v/v) polyethylene glycol 300, 0.1 M MES pH 6.0, 0.1 M sodium chloride and 0.1 M magnesium chloride (RiPIPS-FL in 2% CDP-DAG/98% monoolein). A tungsten carbide glass cutter (Hampton Research) was used to cut and remove the glass coverslip, and crystals were collected using 20–100 μm MicroLoops and MicroMounts (MiTeGen). Crystals were flash cooled directly in liquid nitrogen without additional cryoprotection. RiPIPS-ΔN crystals stabilize in space group P 2₁ 2₁ 2₁, with unit cell parameters (A) = a = 48.63, b = 94.07, c = 130.92, with one protomer in the asymmetric unit, and diffraction to 3.6 Å.

Data collection and structure determination. Diffraction data were collected on beamlines 24-ID-C and 24-ID-E at the Advanced Photon Source (Argonne, IL). The data were indexed, integrated, scaled and merged using XDS26 and AIMLESS27. The structure of RiPIPS-ΔN was solved by molecular replacement using Phaser28, searching for the tetrameric form of the extracellular domains of A2299 (PDB ID 406M). The final data set includes data collected from four isomorphous crystals. After density modification using PARROT29, the model
was manually corrected and completed using Coot, refined using the PHENIX crystallographic software package, alternating between cycles of manual building in Coot and refinement in PHENIX. The final R cryst/Rfree model has an R cryst/Rfree of 0.2284/0.2520. The structure of RsPIPS-FL was solved by molecular replacement using Phaser, searching separately for four copies each of the extramembrane and transmembrane domains from the structure of RsPIPS-ΔΔN. Density modification, including non-crystallographic averaging, was performed using PARROT, and the model was completed following the same protocol as for the structure of RsPIPS-ΔΔN, using the structure of RsPIPS-ΔΔN as a reference model for the generation of restraints, in addition to the application of non-crystallographic symmetry based torsion angle restraints and secondary structure restraints, giving a model with a final R cryst/Rfree of 0.280/0.2997. All protein structure figures were prepared using UCSF Chimera. In the structure of RsPIPS-ΔΔN, many partially ordered lipid molecules were readily apparent in the electron density map (Supplementary Fig. 8). As no head groups or identifying features were discernable in the density, all lipids were modelled as isolated alkyl chains and assigned the residue code UNL, the PDB-recommended code for all unidentified ligands.

**References**

1. Umessi, F. E., Sanki, A. K., Boucou, J., Ronning, D. R. & Suckeh, S. J. Recent advances toward the inhibition of mAG and LAM synthesis in Mycobacterium tuberculosis. J. Med. Res. 30, 290–326 (2010).

2. Anglade, S. K., Belardini, J. M., Huc-Claustré, E., Wheat, W. H. & Jackson, M. The cell envelope glycosconjugates of Mycobacterium tuberculosis. Crit. Rev. Biochem. Mol. Biol. 49, 1–39 (2014).

3. Fischl, A. S. & Carman, G. M. Phosphatidylinositol biosynthesis in Saccharomyces cerevisiae: purification and properties of microsomal-associated phosphatidylinositol synthase. J. Bacteriol. 154, 304–311 (1983).

4. Morii, H., Oga, M., Fukuda, K., Taniguchi, H. & Koga, Y. A revised biosynthetic pathway for phosphatidylinositol in Mycobacteria. J. Biochem. 148, 593–602 (2010).

5. Morii, H., Kiyonari, S., Ishino, Y. & Koga, Y. A novel biosynthetic pathway of archaeatidyl-myo-inositol via archaeatidyl-myo-inositol phosphate from CDP-archaeol and D-glucose 6-phosphate in methanoarchaeon Methanothermobacter thermautotrophicus cells. J. Biol. Chem. 284, 30766–30774 (2009).

6. Carman, G. M. & Belunis, C. J. Phosphatidylglycerophosphate synthase activity in Saccharomyces cerevisiae. Can. J. Microbiol. 29, 1452–1457 (1983).

7. Bae-Lee, M. S. & Carman, G. M. Phosphatidylserine kinase in Saccharomyces cerevisiae. Purification and characterization of membrane-associated phosphatidylserine synthase. J. Biol. Chem. 259, 10857–10862 (1984).

8. Sciarri, G. et al. Structural basis for catalysis in a CDP-alcohol phosphotransferase. Nat. Commun. 5, 4068 (2014).

9. Nogly, P. et al. X-ray structure of a CDP-alcohol phosphotransferase membrane enzyme and insights into its catalytic mechanism. Nat. Commun. 5, 4169 (2014).

10. Gonçalves, L. G. et al. Evolution of the biosynthesis of di-myoinositol phosphate, a marker of adaptation to hot marine environments. Environ. Microbiol. 14, 691–701 (2011).

11. Jackson, M., Crick, D. C. & Brennan, P. J. Phosphatidylinositol is an essential phospholipid of mycobacteria. J. Biol. Chem. 275, 30092–30099 (2000).

12. Salman, M., Lonsdale, J. T., Besra, G. S. & Brennan, P. J. Phosphatidylinositol synthesis in mycobacteria. Biochim. Biophys. Acta 1436, 437–450 (1999).

13. Morii, H. et al. Studies of inositol 1-phosphate analogues as inhibitors of the phosphatidylinositol phosphate synthase in mycobacteria. J. Biochem. 153, 257–266 (2013).

14. Brito, J. A., Borges, N., Vonrhein, C., Santos, H. & Archer, M. Crystal structure of Archaeoglobus fulgidus CTP:inositol-1-phosphate cytidyltransferase, a key enzyme for di-myoinositol-phosphate synthesis in (hyper)thermophiles. J. Bacteriol. 193, 2177–2185 (2011).

15. Rodrigues, M. V. et al. Bifunctional CTP:Inositol-1-phosphate cytidyltransferase/CDP-archaetidyl-myo-inositol phosphate synthase, the key enzyme for di-myoinositol-phosphate synthesis in several (hyper)thermophiles. J. Bacteriol. 189, 5405–5412 (2007).

16. Caffrey, M. Crystallizing membrane proteins for structure determination: use of lipidic mesophases. Annu. Rev. Biophys. 38, 29–51 (2009).

17. Fryer, J. L. & Sanders, J. E. Bacterial kidney disease of salmonid fish. Annu. Rev. Microbiol. 35, 273–298 (1981).

18. Williams, J. G. & McMaster, C. R. Scanning alanine mutagenesis of the CDP-alcohol phosphotransferase motif of Saccharomyces cerevisiae cholinephosphotransferase. J. Biol. Chem. 273, 13482–13487 (1998).
How to cite this article: Clarke, O. B. et al. Structural basis for phosphatidylinositol-phosphate biosynthesis. Nat. Commun. 6:8505 doi: 10.1038/ncomms9505 (2015).