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A mitochondrial phosphatase required for cardiolipin biosynthesis: the PGP phosphatase Gep4

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Cardiolipin (CL), a unique dimeric phosphoglycerolipid predominantly present in mitochondrial membranes, has pivotal functions for the cellular energy metabolism, mitochondrial dynamics and the initiation of apoptotic pathways. Perturbations in the mitochondrial CL metabolism cause cardiomyopathy in Barth syndrome. Here, we identify a novel phosphatase in the mitochondrial matrix space, Gep4, and demonstrate that it dephosphorylates phosphatidylglycerolphosphate to generate phosphatidylglycerol, an essential step during CL biosynthesis. Expression of a mitochondrially targeted variant of *Escherichia coli* phosphatase PgpA restores CL levels in Gep4-deficient cells, indicating functional conservation. A genetic epistasis analysis combined with the identification of intermediates of CL biosynthesis allowed us to integrate Gep4 in the CL-biosynthetic pathway and assign an essential function during early steps of CL synthesis to Tam41, which has previously been shown to be essential for the maintenance of normal CL levels. Our experiments provide the framework for the further dissection of mechanisms that are required for accumulation and maintenance of CL levels in mitochondria.

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Introduction

Mitochondria are dynamic organelles with essential functions for the survival of eukaryotic cells. They ensure the production of cellular energy, maintain the cellular Ca^{2+} homeostasis and are integrated in diverse cellular signalling cascades, including cell death pathways (McBride *et al*, 2006). In addition, the synthesis of a variety of metabolites, cofactors like iron sulphur clusters, but also phospholipids critically depend on mitochondria (Voelker, 2004; Lill, 2009). The majority of phosphatidylethanolamine (PE), a major phospholipid of all cellular membranes, is generated by decarboxylation of phosphatidylserine (PS) within mitochondria (Schuiki and Daum, 2009). Similarly, the synthesis of the dimeric glycerophospholipid cardiolipin (CL) occurs in mitochondria (Schlame, 2008). Increasing evidence suggests that the lipid composition of mitochondrial membranes and the regulation of these phospholipid biosynthesis pathways are crucial for the plasticity of the mitochondrial network and the maintenance of mitochondrial activities in response to different physiological demands (Joshi *et al*, 2008).

CL is ubiquitously distributed in bacteria and mitochondria of eukaryotic cells, where it is predominantly localized in the mitochondrial inner membrane. Recent studies, however, established the presence of CL also in the outer membrane of mitochondria (Gebert *et al*, 2009). CL interacts with many mitochondrial proteins and is required for their optimal activities (Hoffmann *et al*, 1994; Lange *et al*, 2001; Shinzawa-Itoh *et al*, 2007; Claypool *et al*, 2008). Accordingly, the loss of CL impacts on a variety of mitochondrial functions including the activity of respiratory chain complexes and of the F_1F_O-ATP synthase, the stability of protein translocases and protein import, mitochondrial genome stability, mitochondrial fusion and the activation of apoptotic pathways (Jiang *et al*, 2000; Zhong *et al*, 2005; Choi *et al*, 2006, 2007; Gonzalvez *et al*, 2008; Kutik *et al*, 2008; Gebert *et al*, 2009). Perturbations in the CL metabolism are associated with Barth syndrome, an X-linked recessive cardiomyopathic disorder. Barth syndrome is caused by mutations in tafazzin encoding a transacylase that is involved in remodelling of the CL acyl chains (Bione *et al*, 1996; Schlame and Ren, 2006; Houtkooper *et al*, 2009).

CL is a unique dimeric phospholipid as it is composed of two phosphatidic acid (PA) moieties that are linked through glycerol and thus contains four acyl chains. The synthesis of CL starts with the conversion of PA to CDP-diacylglycerol (CDP-DAG), which is catalysed by the enzyme Cds1 (Shen *et al*, 1996). Although apparently mainly localized in the endoplasmic reticulum (ER), Cds1 activity has also been detected within mitochondria (Kuchler *et al*, 1986). CDP-DAG is converted by mitochondrial Pgs1 to phosphatidylglycerolphosphate (PGP) (Dzugasova *et al*, 1998; Chang *et al*, 1998a), which is subsequently dephosphorylated to phosphatidylglycerol (PG). Although, enzymatic activity of a PGP phosphatase has been detected in mitochondria (Kelly and Greenberg, 1990), the encoding gene has not yet been identified. PG is either degraded to DAG by Pgc1 or reacts with CDP-DAG to form CL in a reaction catalysed by the CL-synthase Crd1 (Jiang *et al*, 1997; Chang *et al*, 1998b; Simockova *et al*, 2008). CL synthesis is completed by remodelling of the acyl chains by the successive action of the lipase Cld1 and the transacylase Taz1, which produce in most

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cases a CL species with four identical acyl chains (Gu *et al*, 2004; Beranek *et al*, 2009).

Although the general steps of CL biosynthesis have been recognized, the function of a number of proteins affecting the accumulation of CL in mitochondria remains to be determined. Tam41, for instance, was identified as a protein required for mitochondrial protein import by the TIM23 and TIM22 protein translocases in the inner membrane (Gallas *et al*, 2006; Tamura *et al*, 2006; Kutik *et al*, 2008). Loss of Tam41 additionally resulted in destabilized respiratory supercomplexes and reduced mitochondrial membrane potential, which is reminiscent of cells lacking CL. Indeed, CL was strongly reduced in Δ*tam41* mitochondria, suggesting that Tam41 is required for the formation of CL (Kutik *et al*, 2008). The accumulation of PA, but no other CL intermediates, in Δ*tam41* mitochondria suggested a function of Tam41 early during the CL biosynthesis. Similarly, in a recent genetic screen for synthetic lethal interactors of prohibitins, we have identified several genes whose loss is associated with decreased CL levels in mitochondria (Osman *et al*, 2009). However, it remained enigmatic in many cases how they affect the accumulation of CL. Here, we show that one of these genes, *GEP4*, encodes the PGP phosphatase, a missing biosynthetic enzyme of the CL biosynthesis pathway in mitochondria. Moreover, the detection of intermediates of the CL biosynthesis combined with an epistasis analysis provides new insight into the topology of CL biosynthesis and the function of Tam41.

Results

A phosphatase regulating accumulation of CL

Gep4 was identified in a genome wide genetic array for genes that are essential for the survival of yeast cells in the absence of prohibitins (Osman *et al*, 2009). A blast search identified homologous proteins in fungal and plant species but not in mammals (Figure 1A). Gep4 homologues are characterized by the presence of a hydrolase domain, containing a conserved inverted DXDX(T/V) motif that was proposed to serve as an intermediate phosphoryl acceptor in various phosphotransferases (Collet *et al*, 1998). Replacement of either one of the aspartate residues in this motif impairs the phosphotransferase activity of these enzymes (Collet *et al*, 1998).

To assess the importance of the phosphotransferase motif for the function of Gep4, we generated mutant forms of Gep4, in which these aspartate residues were replaced by asparagines ($Gep4^{D45N}$ and $Gep4^{D47N}$). Cells lacking a genomic copy of *GEP4* were complemented with a control plasmid or plasmids encoding either wild-type or mutant forms of C-terminally myc-tagged Gep4, which were expressed under the control of the endogenous promoter. Loss of Gep4 impaired cell growth on glucose- or glycerol-containing medium at 30°C and abolished cell growth completely at 37°C (Figure 1B; Supplementary Figure S1). Moreover, we observed a strongly retarded growth of Δ*gep4* cells on media containing ethidium bromide, which leads to the loss of mitochondrial DNA (Figure 1B). Expression of myc-tagged Gep4 restored normal growth of Δ*gep4* cells, demonstrating that the tag does not compromise Gep4 function. In contrast, $Gep4^{D45N}$ and $Gep4^{D47N}$ harbouring mutations in the predicted phosphoryl acceptor motif failed to restore growth of Δ*gep4* cells at 37°C or in the presence of ethidium bromide (Figure 1B). We therefore conclude that the integrity of the phosphotransferase motif is essential for Gep4 activity.

Next, we examined the assembly of respiratory chain complexes in Gep4-deficient cells, as several genes genetically interacting with prohibitins function during assembly of the respiratory chain (Osman *et al*, 2007, 2009). Mitochondria were isolated from Δ*gep4* cells or from Δ*gep4* cells expressing wild-type Gep4 or mutant variants thereof. Mitochondrial membranes were solubilized with digitonin and respiratory complexes were separated by blue-native gel electrophoresis (BN-PAGE) (Figure 1C). The F_1F_O-ATP synthase migrates in two distinct forms under these conditions, which correspond to dimeric and monomeric F_1F_O-ATP synthase complexes (Arnold *et al*, 1998). Respiratory complexes III and IV form two distinct supercomplexes composed of two copies of complex III and either one ($III_2 + IV$) or two ($III_2 + IV_2$) copies of complex IV (Wittig *et al*, 2006). These supercomplexes were less abundant in mitochondria lacking a functional Gep4, whereas the assembly status of the F_1F_O-ATP synthase was unaltered (Figure 1C). An additional complex that appeared in the absence of functional Gep4 most likely represents a dimeric form of complex III. Mass spectrometric analysis revealed the presence of various complex III subunits, whereas no subunit of complex IV was detected (data not shown). The reduced amounts of supercomplexes $III_2 + IV_2$ and $III_2 + IV$ in mitochondria lacking functional Gep4 were also confirmed by immunoblotting using antisera specific for the complex III subunit Cor1 or the complex IV subunit Cox2 (Figure 1C). Furthermore, Cor1 and Cox2 antisera detected complexes migrating at ~500 and ~400 kDa, respectively, which were absent in wild-type mitochondria, and most likely represent dimeric forms of complex III or IV.

Taken together, these results show that the loss of Gep4 or mutations in the phosphotransferase motif of Gep4 destabilizes respiratory chain supercomplexes and impairs cell growth at elevated temperature or in the presence of ethidium bromide. Notably, cells that lack the CL-biosynthetic enzymes Pgs1 or Crd1 (and concomitantly CL) show similar deficiencies (Janitor and Subik, 1993; Jiang *et al*, 2000; Pfeiffer *et al*, 2003). Moreover, reminiscent of Δ*pgs1* cells, the temperature sensitivity of cells lacking wild-type Gep4 was entirely suppressed in the presence of 1M sorbitol (Figure 1B) (Zhong *et al*, 2005). Phenotypes of Δ*gep4* cells are therefore consistent with the reduced CL levels that we have observed recently in Δ*gep4* mitochondria when analysing the genetic interactome of prohibitins (Osman *et al*, 2009).

To substantiate the function of Gep4 for the maintenance of normal CL levels in mitochondria, we examined a genetic interaction between *GEP4* and *UPS2*. Ups2, also termed Gep1, was identified as a putative regulator of mitochondrial phospholipid composition (Osman *et al*, 2009; Tamura *et al*, 2009). Loss of Ups2 results in decreased stability of mitochondrial PE and concomitantly decreased PE levels within mitochondria. CL-deficient yeast cells cannot tolerate a reduction of mitochondrial PE levels, which likely reflects essential and overlapping functions of the non-bilayer forming lipids CL and PE for mitochondrial function and cell viability (Janitor *et al*, 1996; Gohil *et al*, 2005). After tetrad dissection of a diploid strain heterozygous for deletions of *GEP4* and *UPS2*, no double-mutant spores lacking *UPS2* and *GEP4* were obtained indicating synthetic lethality (Figure 1D).

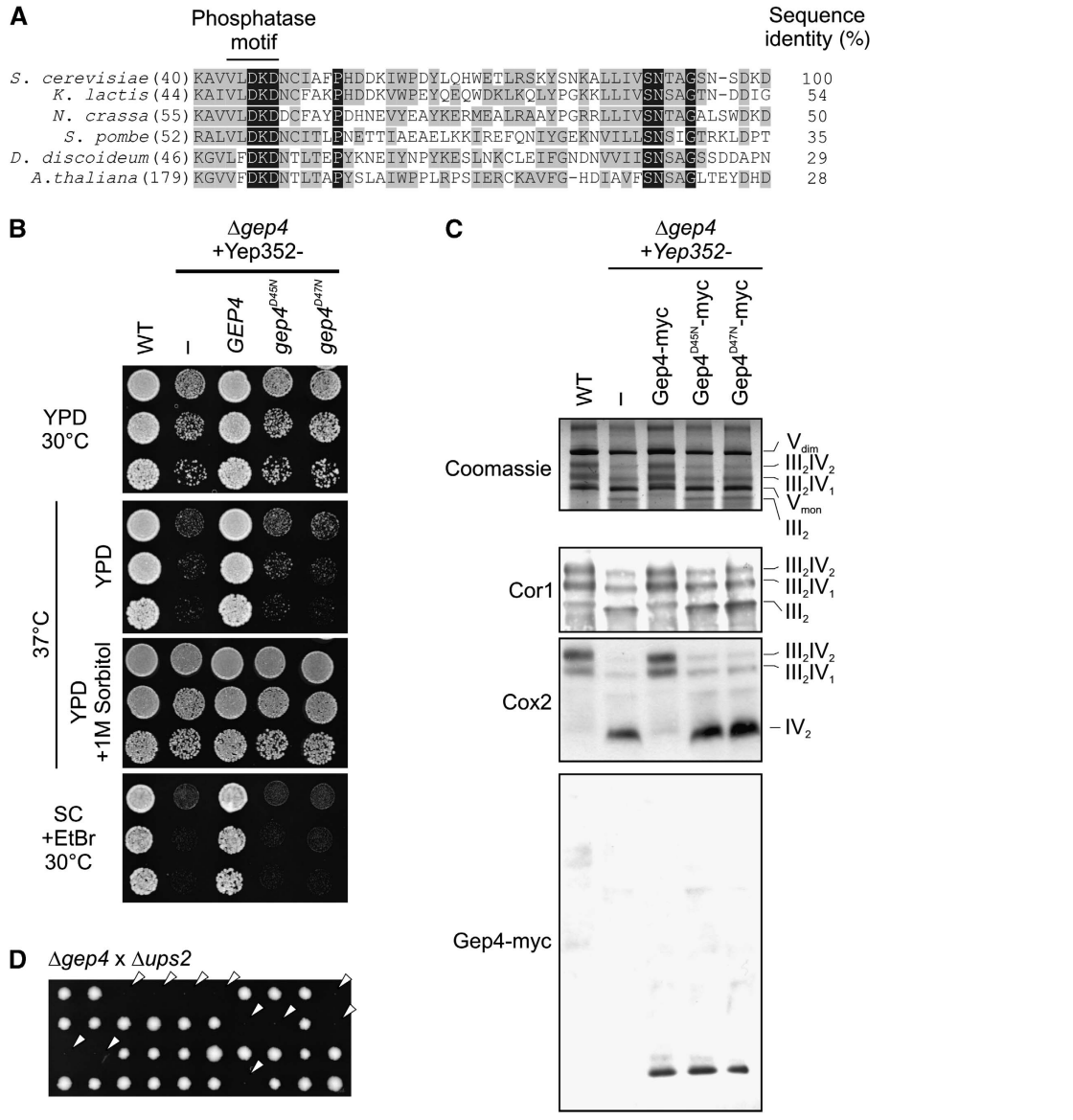

Figure 1 Δ*gep4* cells phenocopy CL-deficient cells. (**A**) Alignment of proteins homologous to Gep4. The region containing the inverted phosphatase motif DXDX(V/T) is shown. (**B**) Growth phenotypes of cells lacking functional Gep4. Serial dilutions of the indicated strains were spotted on YPD or synthetic growth (SC) media, which contained 1 M sorbitol or 25 μg/μl ethidium bromide (EtBr) when indicated, and incubated at 30 or 37°C. (**C**) Gep4 is required for the stability of respiratory chain supercomplexes. Mitochondria were isolated from cells indicated and solubilized with digitonin (4 g/g) and analysed by BN-PAGE and immunoblotting. Immunoblotting with myc-specific antibodies revealed equal expression of Gep4 and Gep4 variants thereof. III, complex III; IV, complex IV; V, F_1F_O-ATP synthase, mon, monomer, dim, dimer. (**D**) Synthetic lethal interaction of Δ*gep4* and Δ*ups2*. A diploid strain heterozygous for deletions of *GEP4* and *UPS2* was subjected to sporulation and tetrad dissection. Arrowheads indicate inviable double-mutant progeny.

This is consistent with the observed phenotypes of Δ*gep4* cells and further supports a function of Gep4 in maintaining normal CL levels in mitochondria.

Gep4 is a mitochondrial matrix protein attached to the inner membrane

Our results characterize Gep4 as a phosphatase affecting CL in mitochondria. It is conceivable that Gep4 controls directly the accumulation of CL within mitochondria. Alternatively, it may act in a cellular signalling cascade regulating the mitochondrial phospholipid metabolism. We therefore determined the subcellular localization of Gep4. Gep4 was highly enriched in sucrose-gradient purified mitochondria, which is in line with earlier findings (Figure 2A) (Kumar *et al*, 2002). We currently cannot rule out that minor amounts of Gep4 are present also in other cellular compartments, as our antibodies only poorly detect Gep4 in total cell lysates (Figure 2A). Treatment of mitochondria or mitoplasts, which were generated by hypotonic disruption of the outer membrane, by proteinase K did not result in the degradation of Gep4 or the matrix-localized α-subunit of the F_1-ATPase, which depended on solubilization of mitochondrial membranes by Triton X-100 (Figure 2B). In contrast, the intermembrane space protein Yme1 was degraded by proteinase K in mitoplasts (Figure 2B).

Gep4 lacks putative transmembrane segments and, similar to the soluble matrix protein Aco1, was recovered in the soluble fraction after alkaline extraction of mitochondrial

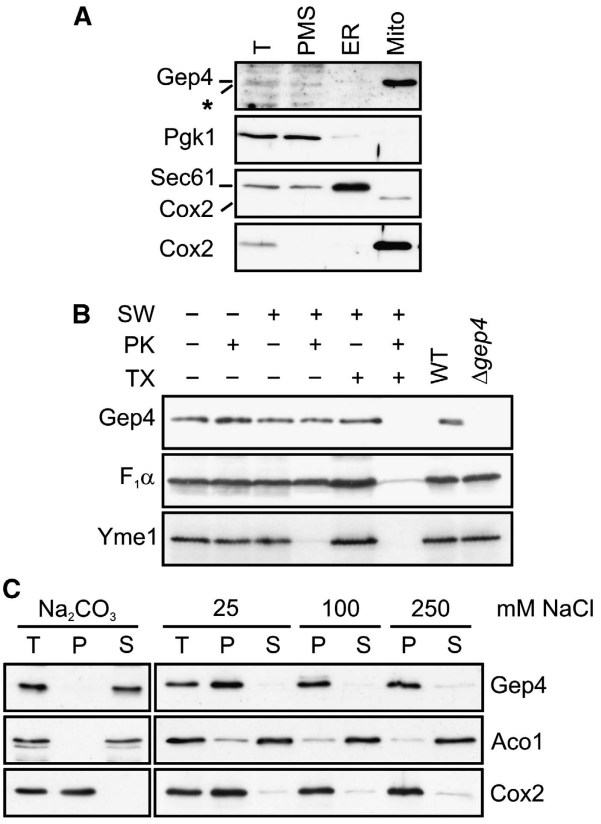

Figure 2 Gep4 is a mitochondrial matrix protein attached to the inner membrane. (**A**) Subcellular fractionation. Whole-cell lysates (T) were centrifuged at 10 000 *g* to obtain a crude mitochondrial pellet and a postmitochondrial supernatant (PMS). The crude mitochondrial fraction was further centrifuged in a sucrose density gradient at 100 000 *g* for 1 h to remove ER membranes (Mito). The PMS was subjected to a clarifying spin, to remove residual mitochondrial membranes, at 30 000 *g* for 30 min and further centrifugation at 40 000 *g* for 30 min to obtain a microsomal membrane pellet (ER) (Gaigg *et al*, 1995). A measure of 40 μg of the cell lysate and the PMS and 20 μg of the ER and mitochondrial fraction were analysed by SDS–PAGE and immunoblotting with the indicated antisera. Sec61, Cox2 and Pgk1 served as marker proteins for ER, mitochondria and cytosol, respectively. The asterisk indicates an unspecific cross-reaction of the Gep4 antiserum. (**B**) Subfractionation of mitochondria. Mitochondria or mitoplasts generated by hypotonic disruption of the OM (+SW) were incubated with or without proteinase K (PK, 50 μg/ml) and analysed by SDS–PAGE and immunoblotting. The IMS protein Yme1 and the matrix protein $F_1\alpha$ served as controls. Mitochondrial membranes were solubilized with Triton X-100 (TX) (0.02%) when indicated. For control, mitochondria isolated from wild-type (WT) and Δ*gep4* cells were analysed in parallel. (**C**) Mitochondria (50 μg each sample) isolated from wild-type cells were treated with Na_2CO_3 (pH 11.5) or disrupted by sonication in the presence of 25, 100 or 250 mM NaCl. Extracts were separated into membrane (P) and soluble (S) fractions by ultracentrifugation (100 000 *g*, 30 min) and analysed by SDS–PAGE and immunoblotting. The integral inner membrane protein Cox2 and the soluble matrix protein Aco1 served as controls. T, input.

membranes (Figure 2C). It remained in the membrane fraction, however, if mitochondria were disrupted by sonication (Figure 2C). This is characteristic of peripheral membrane proteins, whereas soluble proteins, like matrix-localized Aco1, are recovered from the soluble fraction (Figure 2C). We conclude that Gep4 is a novel phosphatase, which is localized in the mitochondrial matrix space, where it is peripherally attached to the mitochondrial inner membrane, suggesting that it may directly affect accumulation of CL within mitochondria.

Gep4 with a functional phosphatase motif is required for the synthesis of CL

To further define the function of Gep4 for the mitochondrial phospholipid metabolism, we assessed the accumulation of a variety of phospholipids by thin layer chromatography and mass spectrometry. In agreement with our earlier findings (Osman *et al*, 2009), no CL was detected by TLC in mitochondria isolated from cells lacking *GEP4* (Figure 3A). Similarly, mutations in the phosphoryl acceptor motif of *GEP4* impaired the accumulation of CL (Figure 3A). Although CL was severely reduced in the absence of functional Gep4, PE accumulated at slightly increased levels in these mitochondria (Figure 3A). This is consistent with a compensatory accumulation of PE in response to reduced CL, as it has been described previously for Δ*crd1* and Δ*ups1* mitochondria (Gohil *et al*, 2005; Osman *et al*, 2009). Levels of other phospholipids remained unchanged in the absence of Gep4.

A quantification of mitochondrial phospholipids by mass spectrometry confirmed these results (Figure 3B). CL was present at ~14% of total phospholipids in wild-type mitochondria but was lowered to background levels in Δ*gep4* and Δ*crd1* mitochondria (Figure 3B). In contrast, PE levels were increased to ~42 and ~39% in Δ*gep4* and Δ*crd1* mitochondria, respectively, when compared with wild-type mitochondria, where PE represented ~35% of total phospholipids (Figure 3B). PG accumulated in Δ*crd1* mitochondria to ~11% of total phospholipids, which is in line with earlier findings (Jiang *et al*, 2000). No accumulation of PG was observed in Δ*gep4* mitochondria. The mass spectrometric analysis did not reveal any significant changes in the levels of other phospholipids. These data clearly show that Gep4 is required for normal CL levels in mitochondria. This function relies on the integrity of the phosphoryl acceptor motif of Gep4, suggesting that Gep4 acts as a phosphatase.

A reduction of CL in mitochondria of cells lacking Gep4 could be the result of either a decreased synthesis or an increased turnover of CL. To distinguish between these possibilities, we incubated wild-type or Δ*gep4* cells in the presence of $[^{32}P]_i$ and analysed the incorporation of $[^{32}P]_i$ into phospholipids at different time points. CL was synthesized in wild-type cells but did not accumulate in cells lacking Gep4 or Crd1 (Figure 3C). The synthesis of other phospholipid species was not affected significantly in Δ*gep4* or Δ*crd1* cells. These results suggest that Gep4 is required for synthesis rather than stability of CL.

Gep4 acts after Pgs1 during the CL biosynthesis

We performed an epistasis analysis to determine which step of CL synthesis depends on Gep4. A *GEP4* deletion was combined with deletions of other genes encoding enzymes involved in CL synthesis (Figure 4A) and mitochondrial lipids were analysed by TLC. Δ*pgs1* mitochondria lack CL and displayed slightly reduced levels of PE, whereas levels of phosphatidylinositol (PI), PA and PS appeared increased in our analysis (Figure 4B). The same profile was observed for lipids obtained for Δ*gep4*Δ*pgs1* mitochondria, indicating that *PGS1* is epistatic to *GEP4*. In contrast, *GEP4* was epistatic to all other known genes involved in CL synthesis. Deletion of

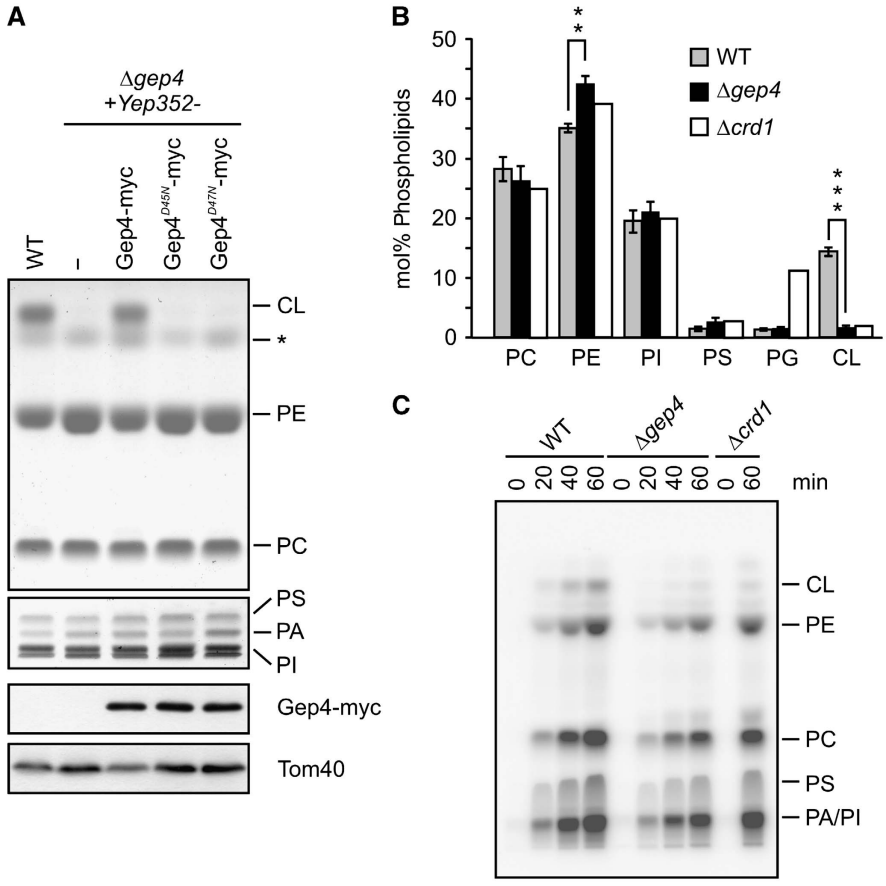

Figure 3 Gep4 is required for the maintenance of normal CL levels. (**A**) Mitochondria were isolated from cells indicated and the lipid composition was analysed by TLC (two top panels, the asterisk indicates an unidentified lipid species). Protein extracts were analysed by SDS–PAGE and immunoblotting using myc-specific antibodies and, as a loading control, Tom40 (two lower panels). (**B**) Mass spectrometric analysis of the phospholipid composition of mitochondria isolated from wild-type (WT), Δ*gep4* and Δ*crd1* cells. Data represent mean values ± s.d. of three (WT, Δ*gep4*) or two (Δ*crd1*) independent mitochondrial isolations each analysed in duplicates. $^{**}P<0.005$, $^{***}P<0.001$. (**C**) Impaired CL synthesis in Δ*gep4* cells. WT, Δ*gep4* or Δ*crd1* cells were grown in the presence of $[^{32}P]_i$ for the indicated time periods. $[^{32}P]_i$ incorporated into phospholipids was determined by TLC analysis and audioradiography (an unknown lipid species migrating at the same heights as CL is detected in all samples). PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin.

GEP4 in cells lacking *PGC1*, *CRD1*, *CLD1* or *TAZ1* resulted in mitochondrial phospholipid profiles virtually indistinguishable from those observed in Δ*gep4* mitochondria (Figure 4B). These results reveal that Gep4 acts after the production of PGP and before the conversion of PG to CL.

The PGP-synthase Pgs1 is inhibited by phosphorylation (He and Greenberg, 2004). It is therefore conceivable that Gep4 acts as a Pgs1-specific phosphatase and activates Pgs1. Alternatively, Gep4 might be the yet unidentified PGP phosphatase. Whereas no PGP phosphatase has been identified in eukaryotic cells, two PGP phosphatases, PgpA and PgpB, have been described in *Escherichia coli*, neither of which show sequence homology to Gep4. PgpA is specific for PGP, whereas PgpB has broader substrate specificity (Funk *et al*, 1992; Touze *et al*, 2008). We reasoned that if Gep4 is the PGP phosphatase in yeast mitochondria, phenotypes associated with its loss could be rescued by mitochondrially targeted PgpA. To ensure mitochondrial targeting of the bacterial enzyme in yeast, we fused the first 69 amino acids of the well-characterized mitochondrial presequence of subunit 9 of the F_O-ATPase of *Neurospora crassa* to its N-terminus (Su9-PgpA) and expressed it in cells lacking *GEP4*. Strikingly, expression Su9-PgpA completely restored growth of Δ*gep4* cells at 37°C (Figure 4C). A TLC analysis of lipids extracted from purified mitochondria from Δ*gep4* strains expressing Su9-PgpA revealed that CL accumulated in significant amounts in these mitochondria (Figure 4C). Thus, the *E. coli* PGP phosphatase can functionally substitute for Gep4 in yeast mitochondria, strongly suggesting that Gep4 exerts PGP phosphatase activity.

PGP accumulates in Gep4-deficient cells

If Gep4 is the mitochondrial PGP phosphatase, PGP cannot be converted to PG in Δ*gep4* mitochondria and should accumulate. PGP, however, could not be detected previously by TLC or mass spectrometry. We therefore examined various lipid extraction procedures and succeeded to identify PGP by mass spectrometry after an acidic lipid extraction in Δ*gep4* mitochondria. PGP accumulated in the absence of Gep4 at ~40 fold increased levels when compared with wild-type mitochondria (Figure 5A). No accumulation of PGP was observed in mitochondria lacking Crd1, where in contrast PG accumulated. For detection of PGP by TLC, we combined the acidic lipid extraction with a developing system previously

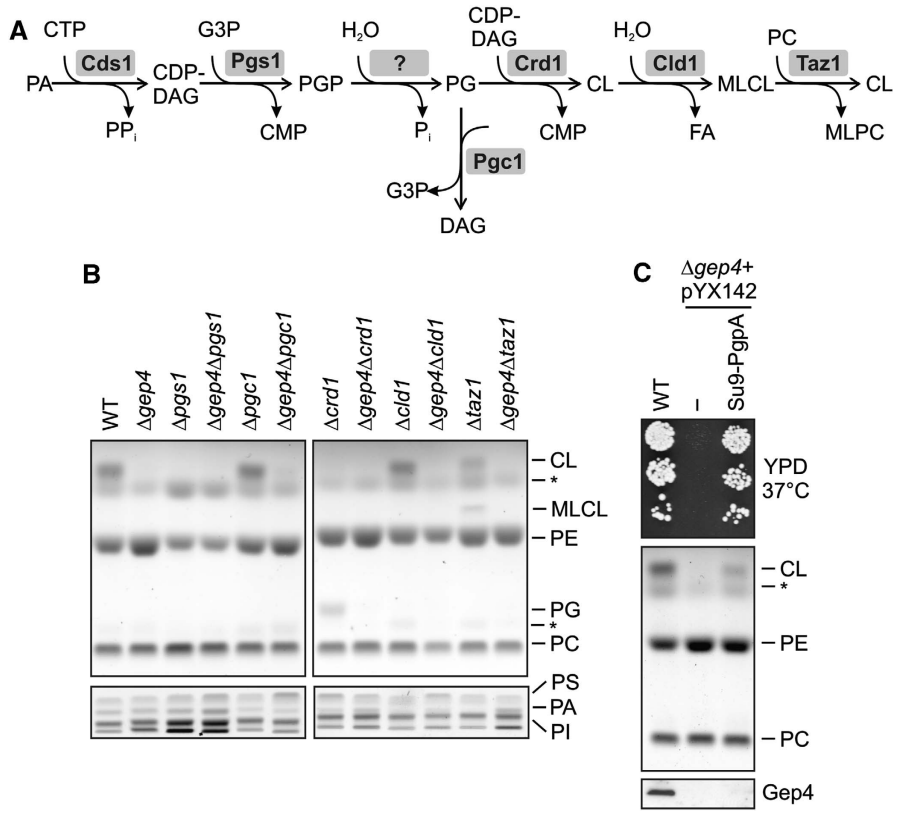

Figure 4 Gep4 acts at the step of PGP dephosphorylation in CL biosynthesis. (**A**) Schematic representation of the CL biosynthesis. PA, phosphatidic acid; CDP-DAG, cytidine diphosphate-diacylglycerol; G3P, glycerol-3-phosphate; PGP, phosphatidylglycerolphosphate; PG, phosphatidylglycerol; DAG, diacylglycerol; FA, fatty acid; MLCL, monolysocardiolipin; MLPC, monolysophosphatidylcholine. (**B**) Epistasis analysis of the CL biosynthesis pathway. The mitochondrial phospholipid composition of the indicated cells was analysed by TLC (the asterisks indicate unidentified lipid species). (**C**) The *E. coli* PGP phosphatase PgpA can complement deficiencies of Δ*gep4* cells. Serial dilutions of indicated strains were spotted on YPD plates and incubated at 37°C (upper panel). Mitochondria were isolated from cells indicated and levels of CL, PE and PC were determined by TLC (middle panel, the asterisk indicates an unidentified lipid species). Extracts of Δ*gep4* and Δ*gep4* + pYX142-*Su9-PgpA* mitochondria were analysed by SDS–PAGE and immunoblotting with antiserum specific for Gep4 (bottom panel).

described for the separation of phosphatidylinositolphosphates, which share structural similarities to PGP (Hegewald, 1996). The TLC analysis of lipid extracts revealed the presence of a lipid species specifically in Δ*gep4* but not in WT or Δ*crd1* mitochondria (Figure 5B). The band was scraped off the TLC plate and was identified as PGP by mass spectrometry (Figure 5C). To further document the phospholipid profile of Δ*gep4* mitochondria, we performed two-dimensional TLC analyses, where we combined developing solvents used for separation of PGP and other phospholipid species (Figure 5D). These experiments confirmed the reduction of CL and accumulation of PGP in Δ*gep4* mitochondria, but did not reveal further significant changes in the phospholipid profile (Figure 5D).

Gep4 dephosphorylates PGP in vitro

We developed an *in vitro* assay to directly demonstrate that Gep4 is capable of dephosphorylating PGP. N-terminally hexahistidine-tagged Gep4 or Gep4^{D45N} harbouring a mutation in its phosphatase motif were expressed in *E. coli* and purified by Ni-NTA affinity purification (Figure 6A). Gep4 or Gep4^{D45N} were added to liposomes generated from lipid extracts of Δ*gep4* mitochondria, and PGP and PG levels were monitored on further incubation of the samples. PGP was dephosphorylated and PG accumulated in the presence of Gep4, whereas all other mitochondrial phospholipids remained stable (Figure 6B). Neither phosphatidylinositol-4-phosphate (PI4P) nor phosphatidylinositol-4,5-bisphosphate (PI4,5P) were dephosphorylated under these conditions by Gep4, substantiating the specificity of Gep4 for PGP (Supplementary Figure S2). PGP remained stable and no PG was detected when Gep4^{D45N} was added to the lipid extract (Figure 6B). These results show that Gep4 exerts PGP phosphatase activity, which depends on the integrity of the phosphatase motif of Gep4.

Integration of Tam41 in the CL biosynthesis pathway

The identification of the PGP phosphatase Gep4 and the possibility to monitor PGP levels allowed us to further dissect the CL biosynthesis pathway. The protein Tam41 has previously been shown to be essential for accumulation of CL and proposed to act early during the CL biosynthesis (Kutik *et al*, 2008). To determine the step at which Tam41 acts, we extended our epistasis analysis and compared the mitochondrial phospholipid profiles of double-mutant cells lacking *TAM41* and *GEP4* or *PGS1* to those of the respective single-mutant cells. The phospholipid levels of Δ*gep4*Δ*tam41* mitochondria were virtually identical to the levels observed in Δ*tam41* mitochondria (Figure 6C). PGP accumulated in Δ*gep4* mitochondria but was undetectable in mitochondria from Δ*tam41* and Δ*gep4*Δ*tam41* cells, indicating that *TAM41* is epistatic to *GEP4*. This is further supported by a strong

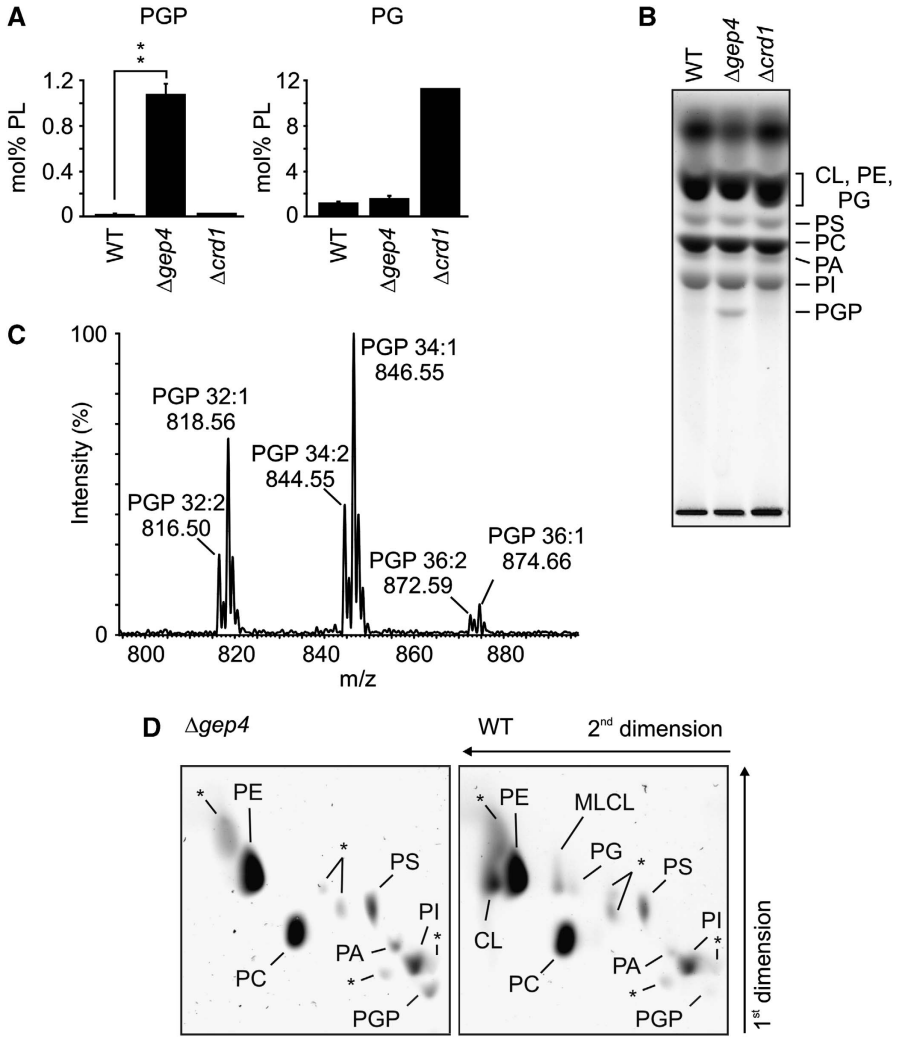

Figure 5 PGP accumulates in Δ*gep4* mitochondria. (**A**) Mass spectrometric analysis of PGP and PG in wild-type, Δ*gep4* and Δ*crd1* mitochondria. Data represent mean values ± s.d. of three (WT, Δ*gep4*) or two (Δ*crd1*) independent mitochondrial isolations each analysed in duplicates. PL, phospholipids. $^{**}P<0.005$. (**B**) Detection of PGP in Δ*gep4* mitochondria by TLC. (**C**) Mass spectrometric profile of PGP. The lipid species are indicated by their fatty acid chain length and saturation state (e.g. PGP 34:1). The *m/z* values represent positively charged ammonium adducts of PGP. Selective scanning for PGP was done as described in Materials and methods. (**D**) Two-dimensional TLC of mitochondrial lipids from wild-type (WT) and Δ*gep4* cells. The identity of the spots was determined using synthetic phospholipid standards and, for control, mitochondrial phospholipids from Δ*taz1* and Δ*crd1* cells (the asterisks indicate unidentified lipid species).

growth defect of a Δ*gep4*Δ*tam41* strain that is also observed for Δ*tam41*, but not for Δ*gep4* cells (Figure 6D). When we compared the mitochondrial phospholipid profiles of Δ*pgs1*, Δ*tam41* and Δ*tam41*Δ*pgs1* cells, we noted an accumulation of an additional lipid species in Δ*pgs1* mitochondria in our TLC analysis that we used for the detection of PGP (Figure 6C). As Pgs1 converts CDP-DAG to PGP, we hypothesized that CDP-DAG could accumulate in the absence of Pgs1. Indeed, a CDP-DAG standard migrated at the same heights in our TLC analysis, suggesting that the additional lipid species in Δ*pgs1* mitochondria most likely represents CDP-DAG. Elevated levels of CDP-DAG were detected in mitochondria from Δ*pgs1* cells but not in Δ*tam41* or Δ*tam41*Δ*pgs1* mitochondria, revealing that *TAM41* is epistatic to *PGS1*. Consistently, Δ*tam41*Δ*pgs1* like Δ*tam41* cells showed residual growth on galactose-containing medium (Figure 6D). We conclude that Tam41 acts before Pgs1 and Gep4 during the CL biosynthesis.

Discussion

Although the physiological importance of CL has been recognized, the nature of a key enzyme for CL biosynthesis, the PGP phosphatase that converts PGP to PG, remained enigmatic. Our experiments provide compelling evidence that Gep4 exerts this function within mitochondria: (1) Gep4 is required for the synthesis of CL; (2) the function of Gep4 depends on the integrity of the conserved phosphoryl acceptor motif, suggesting that Gep4 exerts phosphatase activity; (3) our epistasis analysis reveals that Gep4 acts downstream of the PGP-synthase Pgs1 but before Crd1 that converts PG to CL; (4) the *E. coli* PGP phophatase PgpA, targeted to mitochondria by a heterologous sorting signal, can functionally complement a yeast strain lacking Gep4; (5) PGP accumulates ~40-fold in Δ*gep4* mitochondria and (6) purified Gep4 is capable and sufficient to dephosphorylate PGP *in vitro*.

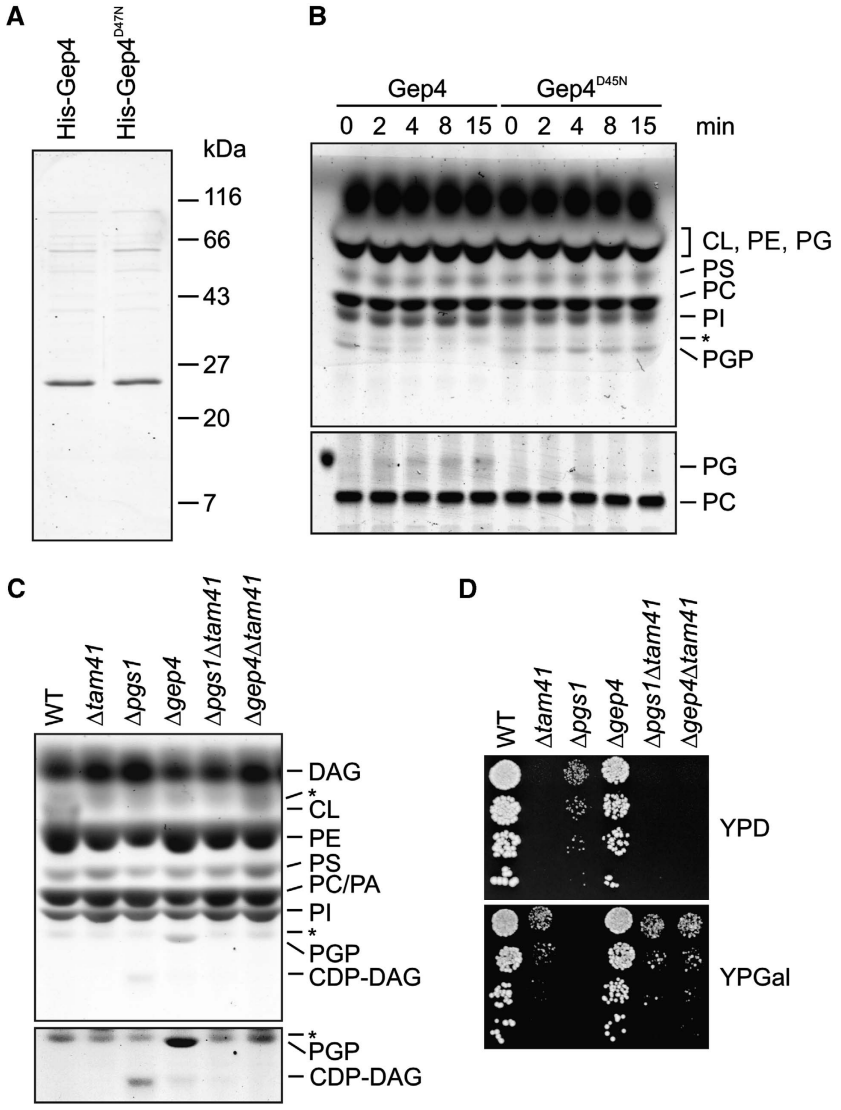

Figure 6 Gep4 dephosphorylates PGP *in vitro*. (**A**) Gep4 and Gep4^{D45N} harbouring N-terminal hexahistidine peptides were expressed in *E. coli* and purified by Ni-NTA chromatography. Eluate fractions used for the *in vitro* experiments are shown. (**B**) *In vitro* PGP dephosphorylation assay. Purified Gep4 or Gep4^{D45N} were mixed with lipids extracted from Δ*gep4* mitochondria in assay buffer and incubated at 25°C for time periods indicated. The dephosphorylation of PGP (upper panel) and the formation of PG (bottom panel) were monitored (the asterisk indicates an unidentified lipid species). (**C**) *TAM41* is epistatic to *PGS1* and *GEP4*. Mitochondrial phospholipids isolated from the indicated strains were analysed by TLC. The bottom panel shows a section of the TLC in the upper panel using higher contrast settings (the asterisks indicate unidentified lipid species). (**D**) Growth of various CL-deficient strains on YPD or YPGal medium at 30°C.

Our experiments offer new genetic and biochemical approaches, which allow to determine the functional relevance of CL intermediates like PG and PGP and to further dissect the function of individual proteins during CL synthesis. Earlier experiments were largely restricted to cells lacking the CL-synthase Crd1. These cells accumulate PG, which is thought to partially substitute for CL in mitochondrial processes. Although Δ*gep4* cells lack CL as do Δ*crd1* cells, PGP and not PG accumulates in the absence of Gep4. We observed an inability of Δ*gep4* cells to grow in the presence of ethidium bromide. This phenotype has also been observed for cells lacking Pgs1 and is likely caused by cell wall defects (Zhong *et al*, 2005). Interestingly, cells lacking Crd1 do not display petite lethality (Chen *et al*, 2010). It therefore appears that PG but not PGP is able to functionally substitute for CL to support cell wall biogenesis and growth on ethidium bromide. On the other hand, the stability of respiratory chain supercomplexes and the ability to grow at elevated temperatures depends on CL, because neither PG in Δ*crd1* cells nor PGP in Δ*gep4* cells can substitute for this function of CL (Zhang *et al*, 2002; Pfeiffer *et al*, 2003). We also demonstrate that Δ*pgs1* cells accumulate CDP-DAG but lack CL, PG or PGP in mitochondria. It is noteworthy in this context that PE levels did not appear increased in Pgs1-deficient mitochondria in our analysis, but in cells lacking Crd1 or Gep4. Although a more detailed quantification of PE levels in Δ*pgs1* mitochondria is required, these findings suggest that the simultaneous reduction in CL and PE levels might explain the severe growth defect of Δ*pgs1* cells. Moreover, Pgs1 might participate in compensatory regulatory processes that result in the accumulation of PE in CL-deficient mitochondria. It is conceivable that CDP-DAG accumulating in the absence of Pgs1 affects the biosynthesis of PE.

The ability to monitor biochemically the accumulation of CDP-DAG or PGP also allowed us to further define the function of Tam41, which has been identified as a maintenance protein for the TIM23 complex in the inner membrane (Gallas *et al*, 2006; Tamura *et al*, 2006) and was shown to be essential for the accumulation of CL in mitochondria (Kutik *et al*, 2008). Our epistasis analysis demonstrates that Tam41 acts before Pgs1 and Gep4 during the synthesis of CL. Neither CDP-DAG nor PGP accumulate, when a *TAM41* deletion is combined with a *PGS1* or *GEP4* deletion, respectively. An early function of Tam41 during the CL biosynthesis is in line with the previously reported accumulation of PA in Δ*tam41* mitochondria (Kutik *et al*, 2008). How Tam41 affects CL levels on the molecular level remains to be determined. Tam41, which localizes to the mitochondrial matrix, may exert a regulatory function ensuring the accumulation of CL, for example by determining the activity of Cds1 or other yet to be identified components that make PA available for Cds1 (Figure 7). Given the fact that Tam41 has initially been implicated in the maintenance of the TIM23 complex, it cannot be excluded that Tam41 is specifically required for the import of proteins required for the CL biosynthesis.

Uncertainty exists about the topology of the CL biosynthesis. Tam41 and Gep4 are localized in the matrix (Gallas *et al*, 2006; Tamura *et al*, 2006), whereas Crd1 is an inner membrane protein that exerts its activity on the matrix side (Figure 7) (Schlame and Haldar, 1993). Enzymatic activities of Pgs1 and Cds1 have been detected in preparations of the mitochondrial inner membrane (Kuchler *et al*, 1986). It remains unknown, however, if early CL intermediates, like PA or CDP-DAG, are synthesized in a subfraction of the ER that is closely associated with mitochondria (mitochondria-associated membrane (MAM)) and harbours many enzymes required for phospholipid biosynthesis (Vance, 1991; Gaigg *et al*, 1995; Achleitner *et al*, 1999). Interestingly, loss of Mdm12, Mmm1 or Mdm10, which were recently identified to participate in mitochondria-ER tethering, results in decreased CL levels (Kornmann *et al*, 2009; Osman *et al*, 2009). The recently characterized Ups1 localizes to the IMS and is required for the accumulation of CL in mitochondria (Osman *et al*, 2009; Tamura *et al*, 2009). It appears to act early during the CL biosynthesis, as Δ*ups1* cells show strong growth defects on glucose-containing media (Sesaki *et al*, 2006) and do not accumulate CDP-DAG nor PGP or PG (CO and TL, unpublished observations). It is therefore an intriguing possibility that CL intermediates like PA or CDP-DAG may require protein-mediated transport from the outer to the inner mitochondrial membrane or between leaflets of the inner membrane to be accessible for matrix-localized enzymes. Ups1 and Tam41 may participate in such transport events, which at the same time could serve as important regulatory steps for the CL biosynthesis.

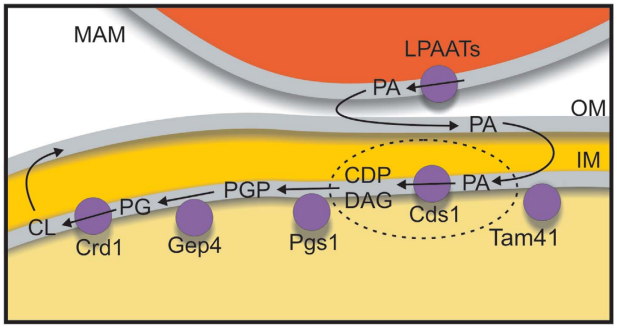

Figure 7 The synthesis of CL in mitochondria. Gep4 and Tam41 are localized to the matrix space, peripherally attached to the inner membrane. Crd1 is an inner mitochondrial membrane protein catalytically active on the matrix side. Pgs1 lacks transmembrane regions and is predicted to be localized in the matrix. Cds1 is mainly present in the ER but Cds1 activity was also detected in mitochondrial inner membrane fractions. Whether Cds1 exerts its activity on the matrix side or the IMS side of the inner membrane remains unknown. Several lysophosphatidic acid acyltransferases (LPAATs) involved in PA biosynthesis are localized to the ER in yeast, the contribution of each of those to phospholipid biosynthesis remains to be determined. Whether PA is also provided by phospholipases D within or outside mitochondria remains unknown. CL is synthesized in the mitochondrial inner membrane but is also present in the mitochondrial outer membrane (Gebert *et al*, 2009). Therefore, mechanisms must exist that ensure transport from the inner to the outer membrane. Steps that might be affected by Tam41 are marked with a dashed circle. IM, inner mitochondrial membrane; OM, outer mitochondria membrane; MAM, mitochondria-associated membrane; LPAATs, lysophosphatidic acid acyltransferases.

The suppression of growth phenotypes and the CL deficiency of Δ*gep4* cells by the unrelated *E. coli* PGP phosphatase PgpA demonstrate functional conservation. However, our bioinformatic analysis did not reveal Gep4 homologues in higher eukaryotes. This is surprising as Pgs1 and Crd1 that act before or after Gep4 in CL synthesis, respectively, are conserved. Notably, we observed low levels of PG in Δ*gep4* mitochondria (Figure 5A), pointing to the existence of an additional enzyme capable of dephosphorylating PGP. It is tempting to speculate that this enzyme, although playing only a minor function for PGP dephosphorylation in yeast, might be homologous to the PGP phosphatase in higher eukaryotes. The functional complementation assay described here combined with the newly established procedure for PGP detection should provide the means to identify this enzyme in higher eukaryotes.

Materials and methods

Yeast strains and growth conditions

Yeast strains used in this study are derivatives of S288c (Brachmann *et al*, 1998) and are listed in the Supplementary Table 1. Yeast cells were grown according to the standard procedures (Sherman, 2002). Mitochondria were purified from yeast cells grown in YP (1% yeast extract, 2% bacto peptone) supplemented with 2% galactose and 0.5% lactate.

Lipid analysis

Lipid extraction from gradient purified mitochondria and determination of the phosphate concentration of extracted lipids was performed as described earlier (Rouser *et al*, 1970; Osman *et al*, 2009). For separation of phosphatidylcholine (PC), PE and CL, 7 nmol phospholipids were spotted on TLC plates (HPTLC; Merck & Co., Inc.) and were developed with chloroform/methanol/25% ammonia (50:50:3 vol/vol/vol). For separation of PS, PA and PI, 12 nmol phospholipids were spotted on TLC plates and were developed with chloroform/methanol/25% ammonia (65:35:5 vol/vol/vol). Phospholipids on TLC plates were stained with 470 mM $CuSO_4$ in 8.5% *o*-phosphoric acid and subsequent incubation at 180°C for 10 min.

For analysis of PGP levels, 500 μg gradient purified mitochondria were pelleted, resuspended in 750 μl chloroform/methanol/37% HCl (40:80:1 vol/vol/vol) and vigorously shaken for 15 min at 25°C in a polypropylene microfuge tube. Samples were transferred to ice, 250 μl cold chloroform and 450 μl cold 0.1 M HCl were added and samples were vortexed for 1 min at 25°C. After centrifugation at 7500 *g* for 2 min at 4°C, the organic phase was transferred to a clean

polypropylene microfuge tube and samples were dried under a constant stream of air.

For one-dimensional separation of PGP, 12 nmol phospholipids were spotted on TLC plates and were developed with ethylacetate/2-propanol/ethanol/6% ammonia (3:9:3:9 vol/vol/vol/vol) (Hegewald, 1996). The same developing solvent was used for the first dimension of two-dimensional TLC analysis. Chloroform/methanol/25% ammonia (65:35:5 vol/vol/vol) was used for the second dimension.

Mass spectrometric lipid analysis

Mitochondrial fractions were pelleted (13 800 *g* at 4°C for 20 min) and resuspended in 150 mM ammonium hydrogen carbonate. The amount of phospholipids was determined according to Rouser *et al* (1970). For mass spectrometry, 1.5 or 3 nmol phospholipids per sample was extracted in the presence of internal standards providing spike amounts of 100 pmol PC (26:0, 28:0, 40:0 and 42:0), 80 pmol PE (28:2, 40:2 and 44:2), 80 pmol PI (32:0), 50 pmol PS (28:2, 40:2 and 44:2), 25–50 pmol PG (28:2, 40:2 and 44:2) and 50 pmol CL (56:0). Unsaturated standards were synthesized and purified through HPLC as described (Koivusalo *et al*, 2001). Saturated PC, PI and CL standards were purchased from Avanti Polar Lipids (Alabaster).

Lipid extractions were performed according to the method of Bligh and Dyer (1959). After solvent evaporation, the samples were resuspended in 10 mM ammonium acetate in methanol and further processed by mass spectrometry as described below and elsewhere (Brügger *et al*, 1997, 2000, 2004). For CL analysis, the solvent system was adjusted to a final concentration of 5 mM ammonium acetate and 0.05% piperidine in methanol. For the recovery of PGP, an acidic extraction procedure was applied as described above. The dried lipids were dissolved in 25 mM ammonium acetate in chloroform:methanol:H_2O (1:1:0.05) and subjected to mass spectrometric analysis.

Quantification of PC, PE, PI, PS, PG and qualitative analysis of PGP was performed in positive ion mode on a triple-stage quadrupole tandem mass spectrometer (QII, Micromass) equipped with a nano-ESI source (Z spray). Argon was used as collision gas at a nominal pressure of 2.5×10^{-3} millibar. The cone voltage was set to 30 V (50 V for PC analysis). The quadrupoles Q1 and Q3 were operated at unit resolution. Detection of PC was achieved by precursor ion scanning for the fragment ion *m/z* 184 at a collision energy (CE) of 32 eV. PE, PI, PS, PG and PGP measurements were carried out by scanning for neutral losses of *m/z* 141 (CE: 20 eV), *m/z* 277 (CE: 30 eV), *m/z* 185 (CE: 20 eV), *m/z* 189 (CE: 20 eV) and *m/z* 269 (CE: 25 eV), respectively.

Quantification of CL and PGP was performed on a quadrupole time-of-flight (TOF) mass spectrometer (QStar Elite, Applied Biosystems) controlled by the Analyst QS 2.0 software. The lipid extracts were automatically infused by the Triversa Nanomate system (Advion Biosciences) operated with the Chipsoft software (V.7.1.1.60703). Ionization voltage was set to −1.1 kV (negative ion mode) and 1.6 kV (positive ion mode), the gas pressure was set to 0.5 psi.

CLs were detected in negative ion mode as single-charged molecules $[M\text{-}H]^-$. CL species (all combinations of fatty acids 16:0, 16:1, 16:2, 18:0, 18:1 and 18:2) were analysed by product ion analysis at a CE of −85 eV. The quadrupole Q1 was operated at unit resolution. The TOF mass analyzer was set to *m/z* 200–350 to monitor fatty acid fragment ions of the corresponding CL precursors. Trapping of ions in Q2 (peak enhancement) was applied to a mass of *m/z* 275. For quantification, the peak areas of CL-derived fatty acid fragments were extracted from the respective product ion spectra through the 'Extract Fragments' script (Analyst QS 2.0). Isotope correction for M + 2 ions was done manually, and values were corrected for response factors of standards.

Quantification of PGP was achieved by product ion analysis of PGP species as identified in lipid extracts of *gep4* mitochondria (Figure 5C). As a PGP standard is commercially not available, we performed quantitations of PGP using the PG standard mixture, assuming that PG and PGP show the same mass spectrometric response behaviour. Product ion analyses were conducted at a CE of 25 eV. The TOF mass analyzer was set to *m/z* 450–750 for monitoring fragments derived from neutral losses of *m/z* 189 (PG) and *m/z* 269 (PGP) of the corresponding PG and PGP precursors. Peak enhancement in Q2 was applied to a mass of *m/z* 600. Quantification of the parent molecules was done as described for CL.

$[^{32}P]_i$ in vivo labelling

In all, 10 OD_{600} cells of a logarithmically growing culture were harvested per time point and resuspended in 1.7 ml YP medium, which contained 2% (w/w) of galactose and 0.5% (w/v) lactate and was supplemented with 10 μCi $[^{32}P]_i$. After an incubation for 0, 20, 40 or 60 min, samples were snap frozen and crude membrane fractions were prepared. Then, phospholipids were extracted and analysed by TLC and audioradiography.

In vitro dephosphorylation assay

For heterologous expression in *E. coli*, Gep4 and mutant variants were fused to an amino-terminal hexahistidine tag. Protein expression was performed according to the manufacturer's protocol using the vector pQE30 (Qiagen) and Arctic Expression cells (Stratagene) harbouring an additional plasmid that encodes the lac repressor to prevent leaky expression. Expressed proteins were purified by metal chelating chromatography (Qiagen) according to the manufacturer's instructions. The copurifying chaperonin Cpn60 was removed from the resin in an additional washing step (20 mM HEPES/KOH pH 7.0, 150 mM KCl, 10 mM Mg_2Cl, 5 mM ATP, 20 mM Imidazole) (Joseph and Andreotti, 2008). Bound proteins were eluted with 250 mM imidazole and used for the *in vitro* PGP dephosphorylation assay.

Total lipids were extracted from gradient purified Δ*gep4* mitochondria (120 μg per time point). Dried lipids were solubilized in assay buffer (50 mM MES/KOH pH 5.5, 10% glycerol, 0.2% Triton X-100, 80 mM KCl, 10 mM Mg_2Cl, 5 mM ATP, 1 μg/μl BSA) (100 μl per time point) by brief sonification (5 × 5 bursts, Output level 2, Interval 2, Branson Sonifier 250). Gep4 or mutant variants were added to a final concentration of 350 nM. After incubation at 25°C for different time periods, reactions were terminated by the addition of 750 μl chloroform/methanol/37% HCl (40:80:1 vol/vol/vol) and snap freezing in liquid nitrogen. PGP and PG were extracted and analysed as described above.

Miscellaneous

BN-PAGE analysis, cellular fractionation, localization studies and growth analyses were performed as described earlier (Gaigg *et al*, 1995; Osman *et al*, 2007).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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