Phosphatidylcholine-protein interactions and remodeling of cardiolipin in yeast mitochondria

Fosfatidylcholine-eiwit interacties en remodeling van cardiolipine in mitochondriën van gist

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
woensdag 9 juni 2010 des middags te 12.45 uur

door

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geboren op 4 november 1980 te Hedel

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Dit proefschrift werd mede mogelijk gemaakt met financiële steun van het gebied Aard- en Levenswetenschappen (ALW) van de Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO).

Het drukken van dit proefschrift werd mede mogelijk gemaakt met financiële steun van Crucell Holland BV.

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Reproductie: Ridderprint BV, Ridderkerk

ISBN: 978-90-5335-284-7

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Abbreviations

CDP-DAG	Cytidine diphosphate-diacylglycerol
CL	Cardiolipin
DHAP	Dihydroxyacetonephosphate
ER	Endoplasmic reticulum
G3P	Glycerol-3-phosphate
IM	Inner membrane (mitochondrial)
IMS	Intermembrane space (mitochondrial)
LPL	Lysophospholipid
MLCL	Monolysocardiolipin
NFCS	Non-fermentable carbon source
OM	Outer membrane (mitochondrial)
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGP	Phosphatidylglycerolphosphate
PI	Phosphatidylinositol
PL	Phospholipid
PPrN	Phosphatidylpropanolamine
PS	Phosphatidylserine

CHAPTER 1

General introduction

1.1 Structure and function of biological membranes

Cells and intracellular compartments (called organelles) are separated from their environment through an almost impermeable barrier with a thickness of only ~35 Å (about 1/10,000 of a human hair). These barriers, hereafter referred to as membranes, are composed of lipid molecules that are arranged in two layers or leaflets. The hydrophobic part of the lipids in each layer are directed inward, while the hydrophilic headgroups in both layers are exposed to the aqueous environment (Figure 1). The hydrophobic core renders membranes virtually impermeable for polar compounds. Protein channels and pumps are embedded in the membrane to facilitate selective transport across the membrane, *e.g.*, of nutrients and waste products. In addition, each membrane harbors its own unique set of proteins which are required for the functions of the enclosed compartment.

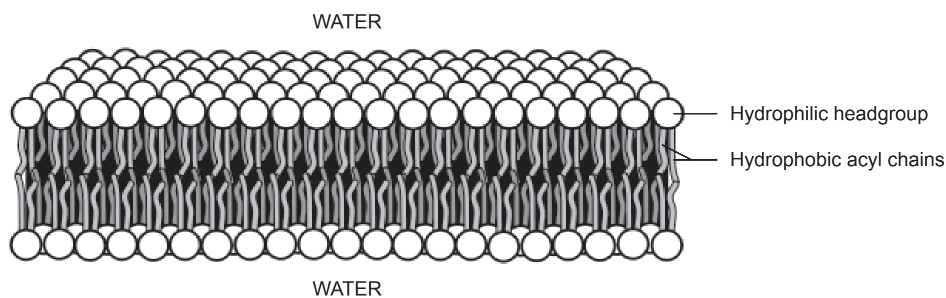

Figure 1 Schematic representation of a cross section of a lipid bilayer. The circles represent the hydrophilic headgroups of the lipids and the wavy lines represent the hydrophobic acyl chains. Adapted from [411].

Membranes not only vary in the proteins associated with them, but also in their lipid composition. Hundreds of different lipids have been identified, each with its own unique properties indicating that lipids play more roles besides being mere membrane building blocks. Indeed, they also create the appropriate environment for the optimal functioning and/or stability of various membrane proteins and complexes thereof. Therefore, knowledge of the synthesis, properties and functions of membrane lipids is of paramount importance for understanding cellular and organellar processes.

In the research described in this thesis, the focus was on two different lipids found in mitochondria, the 'power plants' in cells. First, the role of the abundant phosphatidylcholine (PC) in the functioning of two mitochondrial proteins (Gut2 and Put1) was addressed. Second, it was studied how the unique mitochondrial lipid cardiolipin (CL) gets its acyl chains.
The studies were carried out in *S. cerevisiae*. This organism is more commonly known as baker's yeast with longstanding and widely appreciated applications in

bakeries, wineries and breweries. However, yeast is also very valuable to science. Yeast cells grow fast, they are amenable and tolerant to genetic modifications, they can be cultured under different conditions, and ethical concerns are not an issue. More importantly, cellular processes in yeast are highly similar to those in higher eukaryotes, including humans. Hence, conclusions based on yeast experiments can often be extrapolated to other organisms.
To ensure that everybody is on the same page, some topics need to be introduced. First, some background will be provided on mitochondria, including the biological context of Gut2 and Put1, and on phospholipids. Second, the two lipids that are in the spotlight in this thesis will extensively be discussed. At the end, the other chapters of the thesis will be introduced.

1.2 Mitochondria

Mitochondria are organelles that contain two distinct membranes: an outer membrane that is relatively permeable due to pore-forming proteins and an inner membrane which contains many invaginations called cristae. The space between the inner and outer membranes is usually referred to as intermembrane space (IMS), while the inner membrane encloses the mitochondrial matrix where among others the mitochondrial genome is found. Although mitochondria are often pictured as bean-

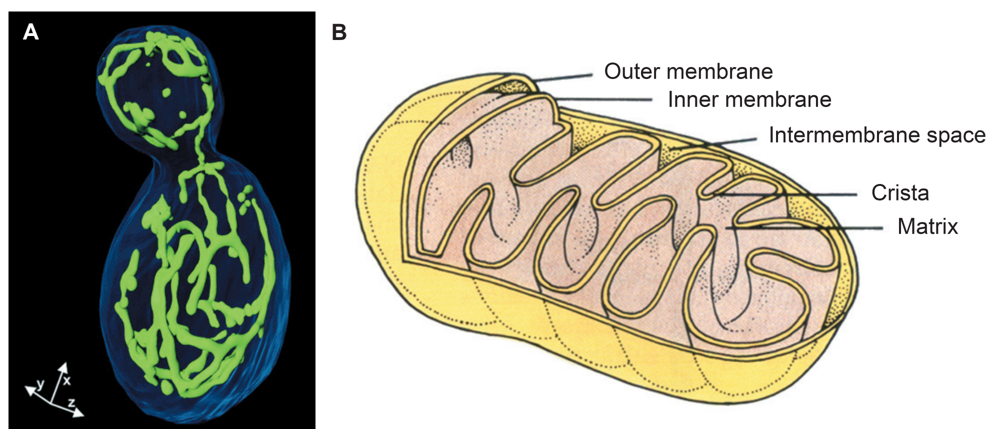

Figure 2 (A) Branched mitochondrial network in a budding yeast cell. A yeast strain expressing mitochondria-targeted green fluorescent protein was grown on the non-fermentable carbon source glycerol to logarithmic growth phase. The cell wall was stained with calcofluor white. A surface-rendered three-dimensional stack recorded with an MMM-4Pi confocal fluorescence microscope is shown. The length of each arrow corresponds to 1 μm. Taken from [412] with permission from Nature Publishing Group. Reproduced from [413] with permission from National Academy of Sciences. Copyright 2002 National Academy of Sciences, USA. (B) Schematic representation of the mitochondrial structure. Adapted from [414].

shaped particles, they actually form branched tubular structures below the cell cortex (Figure 2) that are subject to frequent fusion and fission events [1].

The biogenesis of mitochondria relies to a large extent on the import of proteins and lipids as its structural components. In *S. cerevisiae*, only about 1-2% of the mitochondrial proteins is encoded by the mitochondrial DNA and is synthesized by the organelle itself; the other proteins are imported through complex machineries [2-4]. Mitochondria are also able to produce a limited number of lipids, but the vast majority of lipids required for the expansion of the mitochondrial membranes is sourced from outside. Although it is currently thought that many lipids are imported via the mitochondria-associated membrane, a subdomain of the endoplasmic reticulum (ER), understanding of the mechanisms is still rudimentary [5-8].
The primary function of mitochondria is to generate the universal energy carrier ATP. When yeast cells are cultured in the presence of glucose as carbon source, the glycolysis pathway results in the net formation of two pyruvate, two ATP and two NADH molecules per glucose molecule. The subsequent complete oxidation of pyruvate (after decarboxylation) in the mitochondria via the well-known tricarboxylic acid or Krebs cycle results in the reduction of several FAD and NAD^+ molecules. It should be noted that the NADH generated in the cytosol during glycolysis cannot enter the mitochondrial matrix. Several systems transfer the reducing equivalents across the mitochondrial inner membrane [9,10]. The reducing power stored in $FADH_2$ and NADH is harnessed by large respiratory protein complexes in the mitochondrial inner membrane to pump protons from the matrix to the IMS, generating a proton gradient over the membrane. The protons drive ATP synthesis when they flow back to the mitochondrial matrix through the ATP synthase. Oxidative phosphorylation thus increases the ATP yield per glucose molecule from 2 (generated during glycolysis) to about 30 ATP molecules [11], assuming that yeast and mammalian mitochondria are comparably efficient. In case of non-fermentable carbon sources (NFCS), like ethanol, glycerol and lactate, yeast cells completely rely on oxidative phosphorylation for their net ATP production.
As touched upon in the previous paragraph, *S. cerevisiae* has different mechanisms to regenerate cytosolic NADH which is formed from NAD^+ in oxidative catabolic reactions outside mitochondria. One of these mechanisms is the glycerol-3-phosphate (G3P) shuttle, which comprises two reactions (Figure 3). First, electrons from NADH are used to reduce dihydroxyacetonephosphate (DHAP) to G3P in a reaction catalyzed by the cytosolic G3P dehydrogenase Gpd1 (and possibly its isoform Gpd2) [9,12-14]. In the second reaction, the mitochondrial dehydrogenase Gut2, one of the main players in this thesis, converts G3P back to DHAP and passes the electrons on to the respiratory chain via its cofactor FAD. By cycling back and forth between G3P and DHAP, the reducing power of the electrons in cytosolic NADH is transferred to

the mitochondrial matrix where they contribute to ATP generation.
Apart from its role in the G3P shuttle, Gut2 operates in mitochondria at the crossroad between carbon/energy and lipid metabolism. The name Gut2 is derived from Glycerol UTilization, and refers to the role the enzyme plays in the use of glycerol as carbon source. Glycerol is phosphorylated by Gut1 and subsequently oxidized by Gut2 before it is processed further to pyruvate in the glycolysis pathway [15-18]. The substrate of Gut2 is also the precursor of glycerophospholipids as will be discussed in the next section.

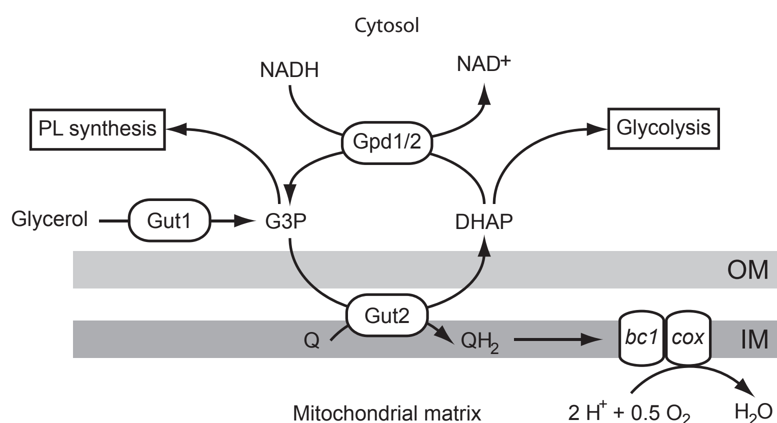

Figure 3 Scheme of the G3P shuttle in its cellular context. See main text for details. Abbreviations: *bc1* – cytochrome *bc1* complex; *cox* – cytochrome *c* oxidase complex; IM – mitochondrial inner membrane; OM – mitochondrial outer membrane; PL - phospholipid; Q(H_2) – (reduced) ubiquinone.

S. cerevisiae mitochondria are also involved in the biosynthesis of heme [19-22] and iron-sulfur clusters [23,24], breakdown of β-oxidation products of fatty acids [25], apoptosis [26], cell signaling [27], and phospholipid synthesis (see Section 1.3). Furthermore, several steps in amino acid metabolism are localized to mitochondria [28-32], including the degradation of proline (Figure 4) [33-37]. The ability to degrade proline is very important, considering that this amino acid is the most abundant nitrogen source in grapes and grape must, the natural habitat of *S. cerevisiae* [38]. The mitochondrial matrix proteins Put1 and Put2 convert proline to glutamate, but only in the presence of a functional respiratory chain [35,39]. Put1, another main player in this thesis, oxidizes proline to Δ^1-pyrroline-5-carboxylate (P5C). Subsequently, the ring structure of P5C is broken in a spontaneous hydrolysis reaction. The formed semialdehyde is finally oxidized to glutamate by Put2. The produced glutamate serves as an intracellular nitrogen donor in biosynthetic reactions [40-42], or is further processed by the Central Nitrogen Metabolism (CNM) to glutamine, another intracellular nitrogen donor [43-45].

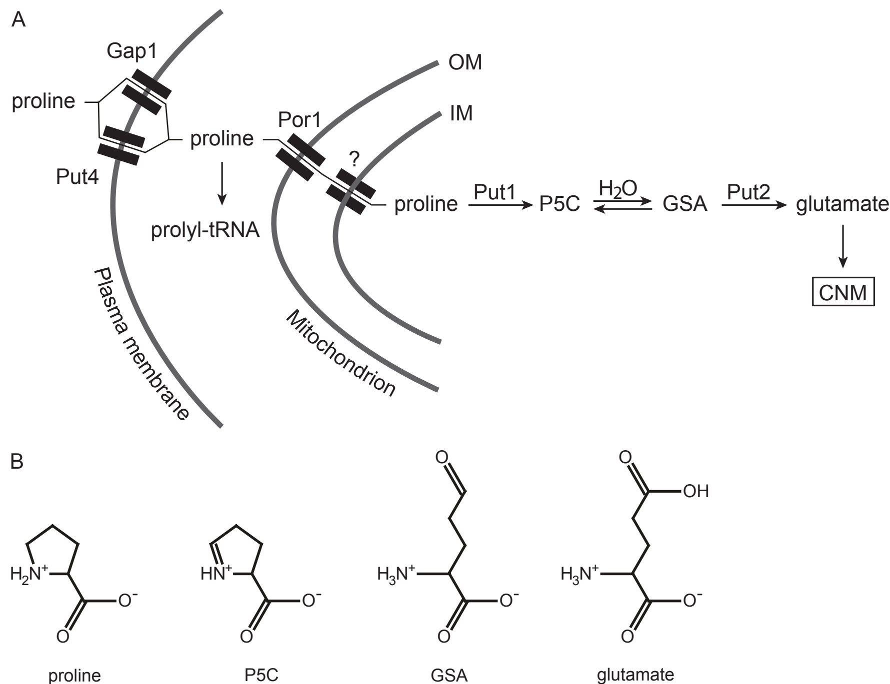

Figure 4 Proline utilization in *S. cerevisiae*. (A) Scheme depicting the import and intracellular fates of proline. See main text for details. (B) Chemical structures of the intermediates in proline degradation. Abbrevations: GSA – glutamate semialdehyde; IM – mitochondrial inner membrane; OM – mitochondrial outer membrane.

1.3 Phospholipids

The three major types of lipids found in *S. cerevisiae* membranes are glycerophospholipids, sphingolipids and sterols (see [46] for a classification system of lipids). Considering the scope of this thesis, only glycerophospholipids (hereafter referred to as phospholipids) will be briefly introduced in this section.

Phospholipids are characterized by a glycerol backbone with fatty acids esterified to the *sn*-1 and *sn*-2 positions and a polar headgroup attached via phosphate to the *sn*-3 position. An exception is CL, in which a single headgroup is shared by two glycerol backbones with in total 4 acyl chains.

Phospholipids are subdivided into different classes according to their headgroup (Figure 5). Since phospholipids can also differ with regard to their acyl chains, each class comprises different molecular species, *i.e.*, lipids that share a particular headgroup and an identical combination of acyl chains. In yeast, the majority of

phospholipid molecules is built with saturated and mono-unsaturated acyl chains that are 16 or 18 carbon atoms long [47-50]. Typically, the saturated and unsaturated acyl chains are enriched at the *sn*-1 and *sn*-2 positions of the glycerol backbone, respectively [48,51].

Phospholipid synthesis is a complex interplay of different enzymes in different organelles including the mitochondria [52]. In *S. cerevisiae*, phospholipid synthesis (Figure 6; reviewed in [53-55]) starts with the acylation of G3P, and to a lesser extent DHAP [56], to form phosphatidic acid (PA). From PA, phospholipid synthesis can proceed in two directions. Dephosphorylation of PA results in diacylglycerol (DAG), which is used in the Kennedy pathway for the synthesis of phosphatidylethanolamine

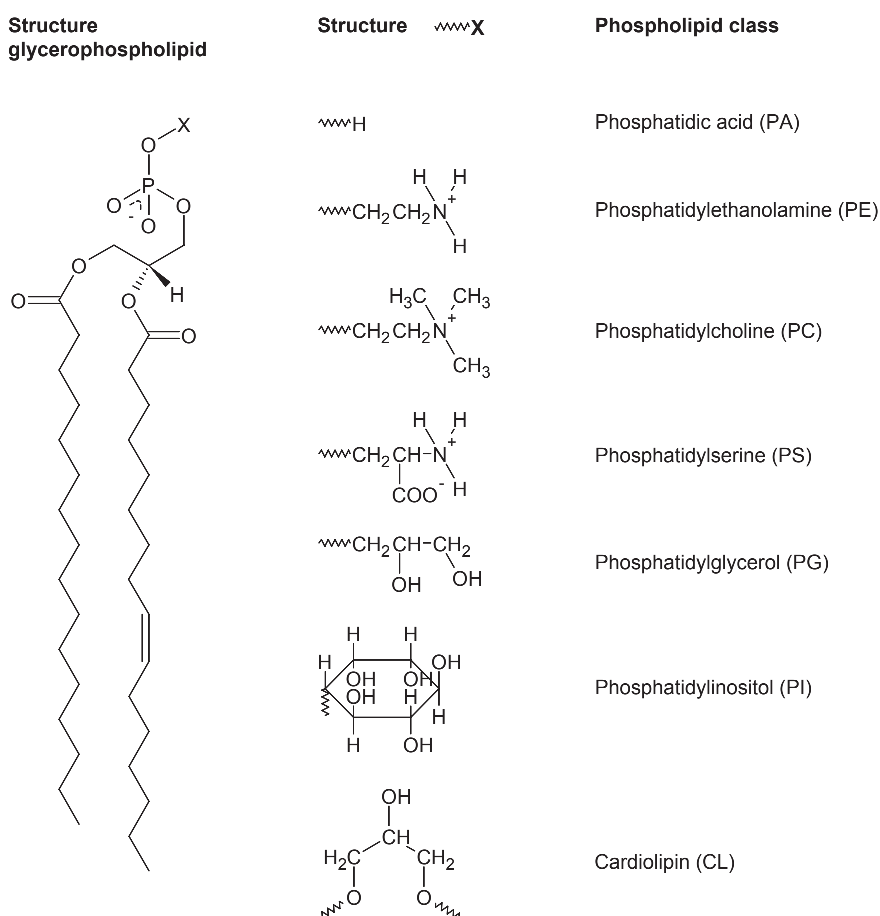

Figure 5 Structures of the major glycerophospholipids in yeast. The glycerophospholipids are subdivided into different classes, based on moiety X attached to the phosphate. In the case of CL, a glycerol group is shared by two phosphatidate moieties. The two acyl chains depicted are abundant in yeast.

(PE) and phosphatidylcholine (PC). PA can also be converted to cytidine diphosphate-diacylglycerol (CDP-DAG), which is a precursor of phosphatidylinositol (PI), phosphatidylserine (PS), and also of CL via phosphatidylglycerol (PG). PS can be decarboxylated to form PE, and PE can be converted to PC via triple methylation. A difference between yeast and higher eukaryotes is that PS in the latter is synthesized by an exchange reaction between PE or PC and serine and not from CDP-DAG and serine.

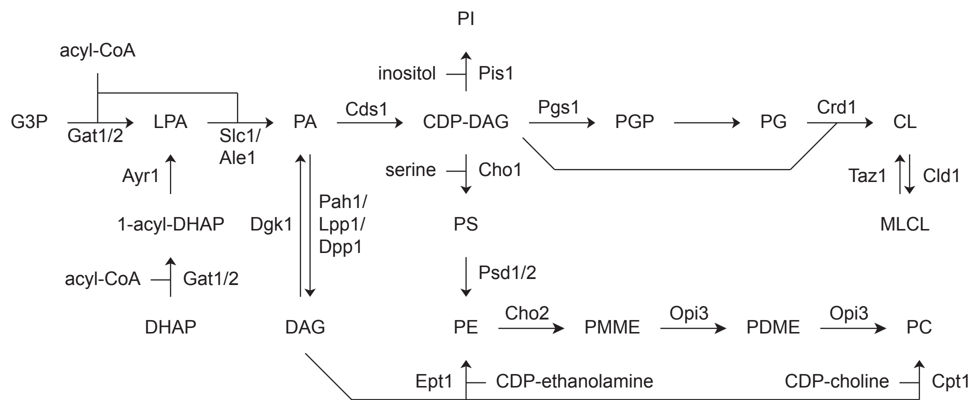

Figure 6 Overview of phospholipid biosynthesis in yeast. The enzymes that have been identified at the gene level are indicated. Abbreviations: CDP – cytidine diphosphate; CoA – Coenzyme A; LPA – lyso-phosphatidic acid; MLCL – monolysocardiolipin; PDME – phosphatidyldimethylethanolamine; PGP – phosphatidylglycerolphosphate; PMME – phosphatidylmonomethylethanolamine.

Table I Phospholipid composition of mitochondrial membranes in lactate-grown wild type yeast cells [57]

Phospholipid class	*% of total phospholipid content*		
	Mitochondria	*Inner membrane*	*Outer membrane*
PC	40.2	38.4	45.6
PE	26.5	24.0	32.6
PI	14.6	16.2	10.2
PS	3.0	3.8	1.2
CL	13.3	16.1	5.9
PA	2.4	1.5	4.4

Obviously, not all phospholipids are equally abundant. In *S. cerevisiae*, PC accounts for almost half of the total phospholipid content [57,58]. Also in mitochondria, PC is by far the most abundant phospholipid (Table I).
Characteristic for mitochondria is the presence of CL which constitutes a substantial

part of especially the mitochondrial inner membrane. Since PC and CL will be focused on in this thesis, the following two sections will provide a more detailed discussion of their structure, properties, synthesis and functions.

1.4 Phosphatidylcholine

After its initial extraction from egg-yolk and brain tissue by Gobley in the mid 19th century [59], PC was termed lecithin after the Greek word for egg-yolk, *lekithos*. Soon thereafter, it was demonstrated that PC contains the base choline [60-62], which had also been found in bile (in Greek: *cholè*) [63,64]. The molecular structure of PC including its stereochemistry was finally resolved in 1950 [65].
PC is typically found as a major constituent of eukaryotic membranes where it has multiple functions. In this section, the current knowledge on the location, structure, synthesis and functions of PC will be summarized, with the focus on *S. cerevisiae*. For a review of PC in prokaryotes, refer to [66,67].

1.4.1 Cellular localization of PC

In eukaryotic cells, PC is the most ubiquitous phospholipid. For instance, 40-50% of the phospholipids in *S. cerevisiae* membranes consists of PC [57,58] and in other eukaryotes comparable levels of PC have been found (see *e.g.*, [68] and references therein). An exception is formed by flight muscle cells, where PE is more abundant than PC [69,70].
The relative contribution of PC to the total phospholipid pool is comparable between the plasma membrane and most organellar membranes [57,71,72] (see also Membrane Protein Lipid Composition Atlas (MPLCA), http://opm.phar.umich.edu/atlas.php; accessed 29OCT09), although in the plasma membrane of *S. cerevisiae* PC accounts for only 10-20% of the total phospholipid content, depending on the carbon source [57,71]. It has been stated that the PC content gradually decreases from ER to plasma membrane via the Golgi [73], but this decrease is not obvious in all cells (see aforementioned MPLCA). Within mitochondria, the outer membrane contains slightly more PC than the inner membrane (Table I; [57,69]). The mechanisms involved in intermembrane lipid transport to establish the lipid composition in individual membranes will not be discussed here; recent excellent reviews can be found in [6,72].
PC is often asymmetrically distributed over the two leaflets of a membrane. This phenomenon is probably not a remnant of the ‘sidedness’ of lipid synthesis in the ER, since so-called ‘scramblase’ proteins in the ER are thought to facilitate bi-directional transport of lipids resulting in comparable lipid compositions for both ER membrane leaflets [72,74]. Rather, the transbilayer asymmetry in other membranes

is established through so-called 'flippases' and 'floppases', enzymes that facilitate intramembrane transport of lipids toward or away from the cytoplasmic-facing leaflet, respectively [74,75].

Not all (intra)cellular membranes have been studied extensively and results have often been conflicting [76]. Nevertheless, it is thought that PC is predominantly present in the membrane leaflets facing the interior of the organelles and in the external leaflet of the plasma membrane [72,74]. In case of the latter membrane, it has been demonstrated that the asymmetry in PC distribution is accomplished by energy-dependent transporters [72,77]. For mitochondria, the results are less clear cut. According to Hovius *et al.* and Dolis *et al.*, PC is symmetrically distributed over both leaflets of the mitochondrial outer membrane in rat liver cells [78,79] in agreement with data obtained earlier for *S. cerevisiae* [80]. However, results obtained later with yeast mitochondrial outer membrane preparations suggested that the inner leaflet contained a somewhat larger share of PC [81]. Seemingly inconsistent results have also been reported for the mitochondrial inner membrane (*cf.* [82-85]).

It is beyond the purview of this introduction to discuss the discrepancies, but it should be noted that both experimental methods and sample preparation can lead to artifacts [76]. Furthermore, it is conceivable that the transmembrane distribution of a particular lipid is dynamic and/or organism-dependent.

Although disturbances in lipid (a)symmetry can have severe consequences as illustrated by diseases resulting from defective flippases and floppases [86], it is unknown whether the distribution of PC itself is biologically relevant.

1.4.2 Structure and properties of PC

PC belongs to the glycerolipids, because of its glycerol backbone with acyl chains esterified at the *sn*-1 and *sn*-2 positions. The presence of phosphate in the headgroup places PC more specifically in the family of the (glycero)phospholipids (see also Section 1.3 and Figure 5). In case of PC, the hydroxyl group at the *sn*-3 position on glycerol is esterified to a phosphorylcholine moiety.

PC is structurally related to PE, since they both carry an aminoalcohol in their headgroups. As a result, these lipids are zwitterionic, having a positive and negative charge on the amine and phosphate, respectively. However, PC has three methyl groups attached to the amine (while PE has none), which has the following implications [87,88]:

1) The cross-sectional area occupied by the PC headgroup is comparable to that of the acyl chains, *i.e.*, PC is a typical bilayer lipid with a 'cylindrical' shape (*cf.* PE, a 'conical-shaped' non-bilayer lipid which has intrinsic curvature due to its relatively small headgroup; see Figure 7);
2) The PC headgroup is not capable of forming H-bonds, *e.g.*, with neighboring

lipids;

3) The hydration level of PC is higher than of PE.

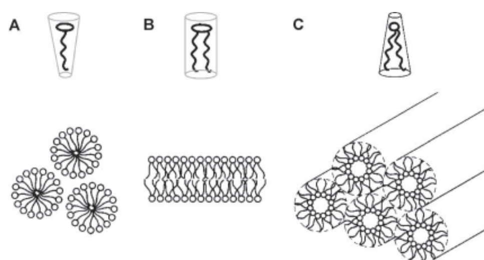

Figure 7 Schematic representation of the molecular shapes of lipids. (A) Molecules that have an overall inverted conical shape, such as various lysophospholipids, form structures with a positive curvature, like micelles. (B) Cylindrical-shaped lipid molecules preferentially form bilayer structures. (C) When lipid molecules have an overall conical shape, structures with a negative curvature are preferred, such as the hexagonal (H_{II}) phase. Taken from [162] with permission from Elsevier.

All three effects contribute to weaker lipid-lipid interactions [87,89-92], as illustrated by the fact that the gel-to-liquid transition temperature of PC is about 20 °C lower than that of PE with similar acyl chains, an important determinant of the fluidity of biological membranes. The properties of PC are also dependent on its acyl chains. Long and saturated acyl chains promote the packing of PC molecules, thereby increasing the aforementioned transition temperature. Short and unsaturated acyl chains have the opposite effect (see [92] and references therein).

Probably the most important property of PC is its ability to spontaneously form a bilayer in an aqueous environment, rendering PC ideally suited to function as a bulk structural component of biological membranes.

1.4.1 Biosynthesis and remodeling of PC

S. cerevisiae produces the majority of its PC via methylation of PE. The alternative route, *i.e.*, the Kennedy or CDP-choline pathway, is involved in the recycling of choline obtained from PC turnover by phospholipase D and only contributes to net PC synthesis when exogenous choline is present [53,54,93-96]. Nevertheless, either route is able to meet the cellular demand for PC. Normal PC levels are observed in cells with an impaired PE methylation pathway (provided that exogenous choline is present) [97,98] and in cells with a blocked Kennedy pathway [99,100].

In mammalian cells, the Kennedy pathway is the main source of PC and the PE methylation pathway contributes little, if at all [94,101,102]. An exception is found in hepatic cells, where 5-40% PC can be derived from PE [103-105] – methylation of PE possibly provides liver cells with extra PC to aid the production of serum lipoproteins and bile (recall the Greek word *cholè* for bile) [94].

A recent publication presented evidence for the existence of a third route, at least in *S. cerevisiae*, which might be involved in choline recycling via glycerophosphocholine [106]. The biological relevance of this route and its positioning with respect to the other pathways remains to be investigated. Below, all three routes will briefly be

discussed.

PE methylation route

Eukaryotes contain *N*-methyltransferases which are able to catalyze the methylation of PE to produce PC. In *S. cerevisiae*, Cho2 converts PE to phosphatidyl-*N*-monomethylethanolamine (PMME) and Opi3 is required for sequential attachment of the other two methyl groups with phosphatidyl-*N*-dimethylethanolamine (PDME) as intermediate and PC as end product. *S*-adenosyl-L-methionine serves as a methyl donor in these reactions [97,98,107]. Remarkably, in mammalian cells all three steps are facilitated by only one enzyme (reviewed in [108]), although it should be noted that also the yeast Opi3 enzyme is able to do this to some extent: *cho2* mutants still have appreciable levels of PC (~11% of total PL) when cultured in the absence of exogenous choline [97,98], and a transformation of a *cho2* strain with a high copy number plasmid containing the *OPI3* gene was found to suppress the *cho2* phenotype [109].

Using mass spectrometry, the substrate specificities of the two methyltransferases in *S. cerevisiae* were investigated. Both enzymes (especially Cho2) were found to have a preference for substrates with two C16:1 acyl chains [110]. Because of the available PE substrates, the majority of PE-derived PC contained either two C16:1 chains or a C16:1 and a C18:1 acyl chain [111]. Considering the importance of the PE methylation pathway for PC synthesis and the role of PC as a major membrane building block, this specificity maximizes the fluidity of the biological membrane within the restraints posed by the available substrate species. In other eukaryotes, the substrate preference of the methyltransferases is probably less pronounced [112].

Both *N*-methyltransferases in yeast were predicted to be integral membrane proteins [113] and reside in the ER membrane [52,57,114,115], although it has also been suggested that PE methylation occurs in the Golgi [50].

Kennedy or CDP-choline route

The Kennedy pathway also consists of three steps. The first reaction comprises the phosphorylation of choline in the cytosol by the choline kinase Cki1 [116]. The formed phosphocholine is subsequently converted to CDP-choline in the rate-limiting step catalyzed by the phosphocholine cytidylyltransferase Pct1 [95,117,118], a membrane-associated enzyme localized to ER and nuclear membranes [119]. In the final step, the cholinephosphotransferase Cpt1 produces PC from CDP-choline and DAG. The subcellular localization of Cpt1 is uncertain at present; most likely the enzyme resides in the ER and/or Golgi [115,120,121]. The aforementioned first and last step can also be catalyzed by enzymes of the related Kennedy pathway for PE synthesis, *i.e.*, Eki1 and Ept1, which show some cross-pathway reactivity [122-124].

Like the PE methylation route, the Kennedy pathway primarily produces 32:2 PC [111,124]. The main difference between the two routes is that the latter also yields appreciable amounts of the mono-unsaturated species. It is unknown whether this can be attributed to enzyme specificity or whether it merely reflects the species distribution in the substrate pool [124].

Glycerophosphocholine route
Recently, a possible third route for PC synthesis in *S. cerevisiae* was uncovered [106]: glycerophosphocholine (GPC) was shown to be acylated by an unknown acyltransferase in yeast microsomal membranes. The formed lyso-PC was efficiently acylated to PC by Ale1. Although GPC can be sourced from the medium, it is likely that this route is involved in the recycling of GPC generated via phospholipase B-mediated deacylation of lyso-PC.

It has been argued that steady state species profile of PC cannot be accounted for by the PC synthetic routes, indicating species selective turnover or remodeling of PC [111]. Indeed, remodeling of PC has been observed (see *e.g.*, [125]), but at present, little is known about the mechanism(s) responsible for bulk PC remodeling.

1.4.2 Regulation of PC synthesis

The biosynthesis of PC is regulated via a complex system involving multiple factors, both at the levels of gene expression and enzyme activity. In this section, an overview of the main factors will be presented; for details refer to the references provided.

Inositol and choline
Many enzymes involved in phospholipid synthesis are subject to transcriptional regulation via a UAS_{INO} element in the promoter region of the encoding genes (reviewed in [53,54,126,127]). As a result, the transcription of those genes is repressed when inositol is present in the medium. The repression is stronger if choline is present too, while choline itself only has a limited effect [53,128]. In the absence of inositol, PA accumulates in the ER membrane and the transcription factor Opi1 is firmly bound to this PA pool and the ER protein Scs2. Under these conditions, Ino2-Ino4 heterodimers bind to the UAS_{INO} elements of different genes, promoting their transcription. However, when inositol is added to the medium, the PA levels in the ER decline due to the increase in PI synthesis. As a result, Opi1 is released from the ER membrane and transported to the nucleus. Here, Opi1 binds Ino2 and thus represses gene transcription [126,129,130]. Interestingly, ongoing PC synthesis is a prerequisite for this regulatory mechanism to function [54,126,130].
Most enzymes of the PE methylation and Kennedy pathways contain UAS_{INO}

element(s) in the promoter regions of the genes encoding them, and their transcription is hence subject to the availability of inositol and choline in the medium [131-134]. The only exception is Pct1, which raises the question as to what extent the flux through the Kennedy pathway is affected at the level of gene transcription by the presence or absence of inositol and choline, since Pct1 catalyzes the rate-limiting step of this pathway [95,117].

In addition to its ability to regulate PC synthesis through gene expression, inositol influences PC synthesis indirectly through inhibition of the PS synthase Cho1. In the presence of inositol, less PS is produced, resulting in a reduced availability of PS-derived PE for the PE methylation pathway [135].

Nutrient starvation and growth phase

The aforementioned Opi1-mediated repression of UAS_{INO}-controlled genes is also invoked when cells enter the stationary phase and/or when nutrients become scarce [54,126,130].

Griac and Henry convincingly demonstrated that nitrogen depletion caused repression of the *INO1* gene through UAS_{INO}. This repression was shown to depend on the presence of Opi1 and on ongoing PC synthesis [136]. It is therefore conceivable that nitrogen starvation induces the translocation of Opi1 to the nucleus, but it remains to be elucidated how this induction is established mechanistically.

In case of zinc depletion, expression of the *PIS1*-encoded PI synthase is increased via the interaction of the zinc-sensing transcription factor Zap1 with the UAS_{ZRE} element in the *PIS1* promoter region. The elevated PI synthesis draws upon the PA pool in the ER, triggering the Opi1 release from the ER membrane with consequences discussed above [137].

Remarkably, PC levels are relatively unaffected by zinc depletion, despite the repression of *CHO2* and *OPI3* [138]. It has been claimed that this is due to the activation of Cki1 expression by Zap1 (overruling *CKI1* repression via the UAS_{INO} element) [139], but this is probably not the complete picture considering that Cki1 is not the bottleneck in PC synthesis via the Kennedy pathway.

The transcription levels of UAS_{INO}-regulated genes are also dependent on the growth phase: high during exponential growth and low during the stationary growth phase, reflecting the cellular need for net lipid biosynthesis to drive membrane expansion. This also applies to the PC biosynthetic genes, which are indeed repressed in stationary phase [54,116,126,130].

Transcription factors Ume6, Sin3 and Rpd3

Ume6, Sin3 and Rpd3 are transcription factors that are needed for normal PC levels, through a complicated regulatory system (see for a detailed review [126]). Sin3 negatively regulates *CHO2* and *OPI3* through the UAS_{INO} element, which is reflected

by the elevated PC levels in *sin3* cells. Rpd3 is thought to repress UAS_{INO}-regulated genes too, but, curiously, PC levels are decreased in the absence of this protein. An *ume6* strain only had a higher PC content when cultured in the presence of inositol and choline; in the absence of these lipid precursors, the PC levels were normal [140]. Part of the explanation might be that Ume6, although being a negative regulator of *INO1*, is a positive regulator of the other UAS_{INO}-regulated genes through stimulation of *INO2* expression [140]. The increased Ino2 level might oppose Opi1-mediated repression in the presence of inositol and choline, allowing the extra choline to be used for PC synthesis.

Posttranslational modification of PC biosynthetic enzymes

The K_M of Pct1 for its substrate cytidine triphosphate (CTP) is higher than the actual intracellular CTP concentration [141-143]. Since the production of CDP-choline from CTP and phosphocholine is rate-limiting for PC synthesis via the Kennedy pathway, changes in the CTP concentration are immediately reflected in the PC production rate. In *S. cerevisiae*, most CTP is produced by the *URA7*-encoded CTP synthase. Stimulation of this enzyme through phosphorylation by the protein kinases A and C resulted in an increased PC synthesis [144]. Elevated CTP levels also boost the rate of PA synthesis, and thus indirectly phospholipid synthesis [142,144-146].
Cki1 is stimulated through protein kinase A-mediated phosphorylation [147,148].

Sec14

In 1989, Sec14 was implicated in the transport of secretory proteins from the Golgi complex [149]. Soon thereafter, Cleves *et al.* uncovered a link between Sec14 and PC synthesis via the CDP-choline pathway [150], and it was demonstrated that Sec14 bound to PC inhibits the activity of Pct1 and hence the CDP-choline pathway [151]. It is currently thought that Sec14 is involved in regulating the activity of the CDP-choline route in order to create a favorable membrane environment for Golgi-derived vesicle transport, although the molecular details of this regulation are poorly understood yet (see [152,153] and references therein).

Other factors

S-Adenosylhomocysteine (AdoHcy) is the byproduct of the methylation reactions catalyzed by Cho2 and Opi3. AdoHcy is also a potent inhibitor of both enzymes [154], which suggested that regulation of its removal by the AdoHcy hydrolase Sah1 could have an impact on PC synthesis. Indeed, recently it was reported that downregulation of *SAH1* expression led to increased AdoHcy levels and a decrease in *de novo* PC synthesis [155]. However, it is unknown whether cells actively employ modulation of AdoHcy levels as a tool to regulate phospholipid synthesis.
The choline kinase Cki1 apparently is heavily regulated. Apart from the factors

mentioned earlier, Cki1 is regulated by its substrate ATP and by its byproduct ADP. ATP promotes oligomerization of Cki1, and possibly as a consequence its activity, whereas ADP inhibits the enzyme by altering its catalytic properties and affinity for the substrates choline and ADP [156].
The activity of Pct1 is strongly dependent on the presence of anionic lipids (CL, PG and PI; PS was not tested), probably via membrane-association, like the mammalian homologue [157-159]. Furthermore, the enzyme was stimulated by oleic acid (C18:1) [157]. A recent study led to the identification of two new regulators of Pct1: α-importin Kap60 and β-importin Kap95 [119]. Both proteins are involved in the nuclear import of Pct1, but in addition, Kap95 (and to a lesser extent Kap60) was found to be essential for full Pct1 activity *in vivo* via an as of yet unknown mechanism [119].
It is not surprising that synthesis of the most abundant phospholipid is subject to different regulatory mechanisms. However, the significance of the factors and their interactions in the regulation of PC synthesis awaits further research.

1.4.3 Functions of PC

S. cerevisiae cells that are depleted of PC rapidly cease growth and loose their viability [97,160,161], indicating that PC is involved in essential cellular processes.
An important function of PC is to form a stable matrix for (intra)cellular membranes, considering its ubiquitous presence in essentially all eukaryotes. This is facilitated by the geometrical shape and biophysical properties of PC as discussed earlier. The role of PC in ensuring an overall ‘bilayer propensity’ in membranes is illustrated by the response observed when *S. cerevisiae* cells were depleted of PC: the acyl chains in PE, another major phospholipid, became shorter and more saturated, presumably to decrease the non-bilayer propensity of PE and, consequently, to keep the intrinsic membrane curvature within an acceptable range [160].
Together with other membrane constituents, PC shapes the lateral pressure profile within membranes, *i.e.*, the repulsive/attractive forces between lipids as a function of the depth in the membrane [162]. By changing the forces at the appropriate depths in the membrane, the stability and/or activity of membrane proteins and complexes thereof might be influenced. An example might be a mechanosensitive Ca^{2+}-influx mechanism in the yeast plasma membrane. Although the protein(s) involved are unknown, PC depletion was shown to sensitize them, resulting in an increased Ca^{2+} influx after osmotic down shock [163].
A completely different function of PC is to serve as a reservoir of lipid messengers. Many signaling cascades involve a step in which PC is hydrolyzed to yield PA, DAG, lyso-PC and/or free fatty acids [164-167]. A well-known enzyme in this regard is the yeast phospholipase D Spo14; see for a review [168].
Furthermore, PC is thought to influence the activity and stability of various proteins.

The activity of many enzymes is stimulated by phospholipids, with little regard for a particular headgroup. However, a few proteins have been identified that have a strong or absolute preference for PC. Examples are soybean β-mannosyltransferase [169], rat progesterone 5-α-reductase [170], rabbit lysosomal acid lipase [171], *L. donovani* acid phosphatase ACP-P_3 [172], bovine heart *bc*1 complex [173], bovine heart β-hydroxybutyrate dehydrogenase [174] and *B. thuringiensis* PI-specific phospholipase C [175].

Recently, the remarkable observation was made that cells with an inactivated PE methylation pathway could be cultured in the presence of propanolamine instead of choline, which was incorporated in the new phospholipid phosphatidylpropanolamine (PPrN) [176]. The authors claimed that PC is not essential for yeast cells. However, it should be taken into account, that i) propanolamine does not occur in the natural habitat of *S. cerevisiae* [177,178], and that ii) PPrN is apparently not able to take over all the functions of PC: replacement of PC by PPrN was accompanied by changes in the levels of the other membrane lipids, and cells with PPrN instead of PC were not able to survive without mitochondrially synthesized PE, in contrast to cells with PC [176]. The latter observation indicates that PC is especially important for mitochondrial functioning, consistent with an earlier finding that yeast cells with a defective PC biosynthesis have problems in maintaining their respiratory competence [179].

It is conceivable that optimal mitochondrial functioning in yeast depends on specific PC-protein interactions, as found in the crystal structure of the cytochrome bc_1 complex [180]. However, at present insight into such interactions is very limited. In fact, the RCSB Protein Data Bank (http://www.rcsb.org/pdb/; accessed 05NOV09) contains only one PC-containing structure from *S. cerevisiae*: the aforementioned cytochrome *bc*1 complex.
To search for additional interaction partners of PC, two approaches have recently been followed. Mitochondrial preparations were incubated with a PC analogue containing the photoactivatable 3-trifluoromethyl-3-aryldiazirine (TID) moiety in one of its acyl chains. Upon exposure to UV light, the diazirine is converted to a carbene which is so reactive that it attaches to anything in its vicinity. One of the most prominently labeled proteins was identified as the mitochondrial protein Gut2 [181]. As described in Section 1.3, this enzyme is an important enzyme responsible for the conversion of G3P to DHAP at the intersection between lipid synthesis and carbon/energy metabolism. It was suggested that Gut2 activity is dependent on phospholipids, or more specifically PC [181]. A lower phospholipid content would suppress Gut2 activity, resulting in an increased allocation of available G3P to phospholipid synthesis.

Another approach to find proteins that rely on PC for their function or stability is to monitor how yeast cells *in vivo* respond to changes in the PC content. Therefore, mRNA levels of all genes were investigated as a function the PC content. The underlying assumption was that cells would attempt to restore processes affected in PC-depleted cells by increasing the expression of the relevant genes. Interestingly, the mRNA levels of two genes encoding proteins involved in proline utilization as nitrogen source were found to be upregulated, namely *GAP1* and *PUT1* (M.C. Koorengevel *et al.*, unpublished data). Both genes encode proteins involved in proline utilization as nitrogen source. Especially *PUT1* is interesting: the encoded Put1 is a proline oxidase, which is found in the mitochondrial matrix and which requires a functional respiratory chain.

1.4.4 Diseases resulting from defects in PC homeostasis

A disturbed PC homeostasis has been found in several pathological conditions, like hyperlipoproteinemia (type I) [182], Gaucher disease [183] and Alzheimer's disease [184], but not as a primary cause. However, two diseases might be caused by an affected PC metabolism. High red cell phosphatidylcholine hemolytic anemia or leaky red cell syndrome has been speculated to be caused by a defective enzyme involved in the transfer of fatty acids from PC to PE (OMIM database, http://www.ncbi.nlm.nih/gov/omim; entry %179700, accessed 06NOV09). However, this has not been backed up by publications in peer-reviewed journals and does not explain the increased PC levels found in patients' red blood cells [185].
Infant respiratory distress syndrome is often found in premature babies and can be caused by insufficient production of pulmonary surfactant. This surfactant lowers the surface tension in alveoli, preventing their collapse and thus facilitating breathing. One of the most important components is PC with fully saturated acyl chains. Apparently, one of the causes of this disease is a low rate of *de novo* synthesis of this PC [186].

1.5 Cardiolipin

In the beginning of the 20th century, the first effective diagnostic test for syphilis was developed by Von Wassermann, based on the phenomenon that mixing human serum and an alcoholic beef heart extract under suitable conditions results in the formation of precipitates/coagulates [187]. One of the essential ingredients of the beef heart extracts turned out to be an unknown phospholipid, which was isolated by Pangborn in 1942. She designated the lipid 'cardiolipin', alluding to the tissue it was harvested from [188].
Since its discovery, it has become clear that CL is a unique phospholipid in terms

of cellular localization, structure (and hence properties) and functions, as will be expounded hereafter.

1.5.1 Cellular localization of CL

CL is found in both eukaryotic and prokaryotic cells [189-194]. In eukaryotes, CL resides virtually exclusively in the mitochondria, primarily in the mitochondrial inner membrane, accounting for approximately 10-15% of the total mitochondrial phospholipid content [57,69,71]. There is experimental evidence that CL is also a component of peroxisomal membranes in some organisms including *S. cerevisiae* [57,71,195], while not in others [196,197]. It has been speculated that any peroxisomal CL might have been delivered by the recently discovered mitochondria-derived vesicles [198,199]. Since this area is largely unexplored yet, it will not be discussed further in this introduction.

The presence of CL in the mitochondrial outer membrane is a matter of controversy. Relatively small amounts of this lipid have been found in mitochondrial outer membrane fractions of various organisms [69], but it has been argued that the preparations were contaminated with inner membranes [200-202]. Although this might be true to some extent, the measured CL levels are in certain cases too high to be brushed aside as an artifact. For instance, CL was reported to account for about 6% of the total phospholipid content in the mitochondrial outer membrane of *S. cerevisiae* (*cf*. 16-19% in the inner membrane) [57,69]. Furthermore, also in highly purified outer membrane preparations from different organisms significant amounts of CL have been detected [78,203-205], albeit not in outer membranes of for instance rat liver mitochondria [205]. A possible route by which CL could end up in the outer membrane is via contact sites between the inner and outer membrane. Contact sites were reported to be enriched in CL and could therefore function as a gate for CL to the outer membrane [206-208].

The distribution of CL over the inner and outer leaflet of the mitochondrial inner membrane has also been a subject of debate. In the 1970s and 1980s, several studies indicated that 70-90% of CL in the inner membrane was present in the matrix-facing leaflet [83-85], consistent with CL being produced here (see below). A few years later, those results were challenged by Cheneval and co-workers who reported that the majority of CL is actually in the *outer* leaflet, pointing to flaws in earlier experimental approaches [209]. However, it is conceivable that the CL distribution over both leaflets of the inner membrane is not the same in all cell types and, maybe more importantly, depends on the mitochondrial activity. In 1997, it was elegantly demonstrated that in *S. cerevisiae* about 80% of CL resided in the outer

leaflet under fermentative growth – when the mitochondrial activity is low. When the cells started to use the NFCS ethanol as a carbon source, the share of CL in the inner leaflet transiently increased from 20% to 70% (due to the boosted CL synthesis at this side of the inner membrane) and then stabilized at 37% [210]. In a recent study, it was unintentionally illustrated how different human cell lines have different CL distributions, despite being cultured and assayed according to the same procedure [211].

1.5.2 Structure and properties of CL

CL is a phospholipid that is composed of two 1,2-diacylphosphatidate moieties that are connected to the 1- and 3-hydroxyl groups of a single glycerol headgroup (see also Section 1.3 and Figure 8). Thus, CL is essentially a fusion of two regular phospholipids, which results in unique properties.
Due to the two phosphate groups, CL could have a net charge of -2, which was experimentally supported by a ^{2}H-NMR study on POPC:CL and POPC:PG bilayers [212] and by a study in which the lamellar/inverted hexagonal phase transition temperature of CL:cationic lipid mixtures was determined as a function of the membrane surface charge [213]. However, in a titration experiment CL appeared to have a pK_a of 2.8 and a second pK_a of 7.5-9.5, implying that CL is a monovalent anion under physiological conditions. In line with this, a recent analysis of CL-vesicles by IR spectroscopy resulted in pK_a values of 4.7 and 7.9 [214]. Since the second pK_a was much lower in the absence of the hydroxyl group of the central glycerol group, it was postulated that the second proton is trapped in a resonance structure involving this hydroxyl group and the two phosphate groups [215]. The possible significance of proton trapping in oxidative phosphorylation will be discussed below. It is important to realize, that destabilization of the resonance structure by *e.g.*, protein binding will lead to unpredictable shifts in the pK_a values of CL [214]. Likewise, the shift in the second pK_a as the titration proceeds could be due to changes in the interactions between adjacent CL molecules [216].
Although to my knowledge the conflicting conclusions on the charge of CL have never been reconciled, it could be that the resonance structure is destabilized in the presence of positive charges, like in the headgroups of PC or PE, thereby lowering the threshold for CL to loose the second proton. A similar effect has been proposed for PA in the vicinity of positively charged amino acids and PE [217,218].

The fact that the glycerol headgroup is shared by two phosphatidate moieties has several implications, besides the ability to trap protons. First, the cross-sectional area of the CL headgroup is relatively small compared to that of the acyl chains, rendering CL inclined to form inverted hexagonal membrane structures, especially

when the repulsive forces between the phosphates are decreased in the presence of cations, including protons (*i.e.*, low pH) [219-222]. An interesting and yet not understood exception is Ba^{2+} (and to a lesser extent Sr^{2+}) which does not promote the inverted hexagonal phase of CL [223-225]. The ability to switch between lamellar and inverted hexagonal structures might play a role in membrane fusion and fission processes [222,226,227], in shaping the mitochondrial cristae [228,229] and the contact sites between the mitochondrial inner and outer membranes [207,208], but it might also facilitate the presence of CL in membrane domains with different curvatures. Second, the flexibility and mobility of the glycerol headgroup is impaired, since it is anchored at both ends to the membrane. This hinders the formation of glycerol-to-phosphate hydrogen bonds, intramolecular or with neighboring lipids, leaving the negatively charged phosphate groups relatively open to interactions with solvents, solutes and not in the least proteins [230]. Third, the two phosphatidyl moieties are diastereotopically inequivalent (Figure 8): one is in the *pro-S* and the other in the *pro-R* position with respect to the prochiral center at the central carbon atom of the glycerol headgroup [231] and they will behave differently in chemical and enzymatic reactions and possibly in interactions with proteins [230,231]. Since the two obligate chiral centers in the outer glycerols are both in the *R* configuration in naturally occurring CL [231,232], there is only one CL stereoisomer if i) the two *sn-1* acyl chains are equal, and if ii) the two *sn-2* fatty acids are equal; in other words, if the acyl chain distribution in CL is symmetrical. In contrast, if the acyl chain distribution is asymmetrical, then the prochiral center has become chiral, leading to the presence of two stereoisomers.

Whereas regular phospholipids contain a broad range of acyl chains that differ in length and in degree of unsaturation, the repertoire of acyl chains found in CL is very limited. The majority of CL contains only one or two types of acyl chains, like C16:1 and/or C18:1 in *S. cerevisiae*, with the preferred acyl chain(s) varying between organisms and even between tissues of the same organism [232]. It has been pointed out that restricting the variety of acyl chains strongly decreases the number of possible CL species, especially since those acyl chains are not randomly

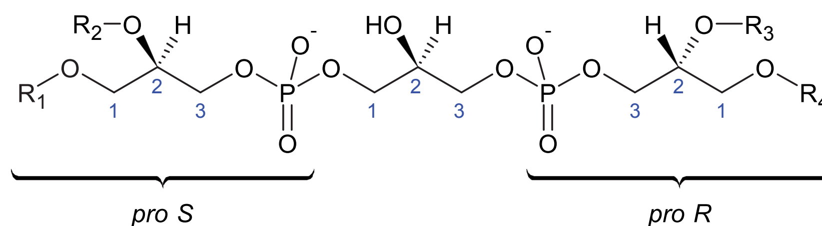

Figure 8 Stereochemical structure of CL. The two phosphatidate moieties carry their chiral center C2 in the *R* conformation. It is indicated which moiety is in the *pro-S* and which one is in the *pro-R* position with respect to the prochiral center, *i.e.*, carbon atom C2'. The four acyl chains of CL are denoted R_{1-4}.

distributed over the four acyl chain positions in CL [232]. The most striking example is CL from human heart in which 80% of the CL molecules contains four C18:2 acyl chains [233]. Also in yeast the proportion of symmetrical CL $[(sn\text{-}1\text{-}sn\text{-}2)_2]$ is very high: 40% of the CL pool was found to consist of $(C18{:}1\text{-}C18{:}1)_2$, $(C18{:}1\text{-}C16{:}1)_2$ and $(C16{:}1\text{-}C18{:}1)_2$ CL [232].

It is not known whether symmetrical CL offers any biological advantage, although some suggestions have been put forward by Schlame and co-workers [232]. The two most conceivable ideas are that i) symmetrical CL might be sterically less constrained to adopt the optimal configuration with respect to the central carbon atom in the glycerol headgroup for binding with other molecules (like proteins), and that ii) the aforementioned resonance structure responsible for proton trapping might be less stable if the resonance structure contains two inequivalent phosphatidyl moieties [232].

An alternative explanation for the high degree of symmetry might be that it is just a side-effect of the limited acyl chain repertoire in CL, which in itself reduces the number of possible CL species and hence increases the chance to find CL molecules that are each other's mirror image – something that could be beneficiary for protein-protein interactions mediated by CL [232].

Hypotheses as to how the characteristic acyl chain composition for CL is established will be discussed in the next section.

1.5.3 Biosynthesis and remodeling of CL

The synthesis of CL from the common phospholipid precursor PA is a four-step process (see also Figure 6; reviewed in [194]). In the first step in yeast, the CDP-DAG synthase Cds1 incorporates a cytidine monophosphate (CMP) moiety from CTP into PA, resulting in CDP-DAG. The freshly attached CMP group is subsequently exchanged for G3P in a reaction catalyzed by Pgs1, yielding phosphatidylglycerolphosphate (PGP). The third step is the formation of PG through dephosphorylation of PGP by a phosphatase that has not been identified yet. So far, the prokaryotic and eukaryotic synthetic routes for CL synthesis are identical. In the last step, prokaryotes use two PG molecules to make a single CL (with glycerol as a side product) in an equilibrium reaction, whereas the eukaryotic CL synthases (Crd1 in yeast) couple PG to CDP-DAG, resulting in CL and CMP. The latter reaction is irreversible, since it is driven by the cleavage of an energy-rich phosphate bond in CDP-DAG.

Where is CL synthesized? For CL synthase in rat liver mitochondria, it has been shown extensively (although indirectly) that CL is synthesized in the matrix-facing leaflet of the mitochondrial inner membrane:

1) The rat CL synthase was protected against inactivation by proteases by an intact mitochondrial inner membrane;

2) Blocking the supply of the cofactor Mn^{2+} (CL synthases require divalent cations as cofactor; Crd1 prefers Mg^{2+} [234,235]) across the mitochondrial inner membrane by ruthenium red impaired CL synthesis;
3) A membrane-impermeable inhibitor of the CL synthase had no effect when added to the cytoplasmic side of the mitochondrial inner membrane [236].

Also the first enzyme of the CL biosynthetic pathway, CDP-DAG synthase, was protected by the mitochondrial inner membrane against degradation by externally added proteases, suggesting that all four reactions from PA to CL occur in the matrix-facing leaflet of the mitochondrial inner membrane [236]. Experiments with rat liver mitochondria indicated that both PG and CL are primarily if not exclusively produced in the inner membrane [237]. Most likely, the findings in rat mitochondria apply to other organisms: the application of a CL-specific dye in *S. cerevisiae* revealed that newly synthesized CL appears first in the inner leaflet of the inner membrane [210]. Another study with *S. cerevisiae* showed that Pgs1 activity was localized to the mitochondrial inner membrane, as was Cds1, although the latter was also found to be present in the outer membrane derived from a crude mitochondrial fraction and in the ER [115].

As mentioned in the previous section, CL has a unique acyl chain composition, which cannot be explained by the acyl chain composition of its precursors (at least in rat liver mitochondria [238]), nor by the substrate specificity of the enzymes involved in CL synthesis [239-241]. Consistent with this, the incorporation of a major acyl chain in human CL (linoleic acid, C18:2) was not impaired in H9c2 cells when *de novo* phospholipid synthesis was reduced by cyclopentenylcytosinetriphosphate, an inhibitor of CTP synthase [242].

Already in 1990, evidence for remodeling of CL was found in rat liver mitochondria [243]. Since then, the existence of a remodeling system has been demonstrated in various organisms [244-248]. Although remodeling is now widely believed to be responsible for ‘correcting’ the acyl chain composition of newly synthesized CL molecules, it is not fully understood how this process occurs.

According to the oldest hypothesis, CL is remodeled through the Lands’ cycle like other phospholipids: an acyl chain is cleaved off by a phospholipase A_2 and an acylCoA:lysophospholipid acyltransferase subsequently attaches a new chain [194,243,249]. Support for this hypothesis came among others from the identification of potential acylCoA-lysocardiolipin acyltransferases in pigs [250] and mice [251], albeit not very convincingly: the murine protein was found to reside in the ER and the *in vitro* acyl chain specificity of both enzymes was not as would have been expected based on the CL species profile [194]. Furthermore, the murine protein was found to be rather promiscuous in accepting different kinds of lysophospholipids as substrate [252,253]. Recently, Taylor and Hatch reported the identification and characterization

of a human monolysocardiolipin (MLCL) acyltransferase, which uses preferentially linoleoylCoA as substrate [254].
In 2007, Mancuso and co-workers demonstrated that the mitochondrial calcium-independent phospholipase A_2 (iPLA$_2$) was required for a regular CL species profile in murine cells [255], suggesting that this lipase is responsible for deacylation of CL in the Lands' cycle [256]. It has been questioned whether iPLA$_2$ contributes significantly to CL remodeling, since the absence of iPLA$_2$ activity was said to have only a minor impact on the levels of 'remodeled' CL [257]. However, the profile of newly synthesized CL before remodeling, *i.e.*, the starting point, is not known: not all new CL molecules need to be remodeled. Furthermore, the extent of remodeling is probably dependent on mitochondrial activity [47,258].
CL remodeling *in*dependent of acylCoA or CoA has been firmly established [259], and occurs by a mechanism involving the transacylase Taz1 and its homologues, such as the human tafazzin. The absence of an active Taz1 homologue caused profound derangements of the CL species profile, as well as an accumulation of MLCL [244,246,247,259-265]. Taz1 is an average-sized protein of about 44 kDa, which was initially found to be embedded in the mitochondrial outer membrane, facing the IMS [265]. This would imply that CL for its remodeling has to be transported across the inner membrane and probably even to the outer membrane (see also [82]). However, more recent data suggest that Taz1 is associated with both the mitochondrial outer and inner membranes, through an interfacial membrane anchor inserted in the IMS-facing leaflets of the membranes [266]. As mentioned above, the extent of remodeling might be dependent on mitochondrial activity. In this context, it is interesting that Taz1 was shown to interact with the respiratory components (AAC2 and ATP synthase) [267], possibly allowing the alignment of CL remodeling with mitochondrial activity.

There are currently two hypotheses as to how Taz1 is involved in CL remodeling, both implicating phospholipids (PL) as acyl chain donor. The first postulates that Taz1 catalyzes two reactions, using catalytic amounts of lysophospholipid (LPL) [194,257]:

A. LPL + original CL → PL + MLCL
B. MLCL + donor PL → remodeled CL + LPL

There is some discussion as to how the specific acyl chain composition for CL is attained via this remodeling mechanism. According to some studies, CL remodeling displays a rather strong acyl chain specificity [259,260], but this is at odds with other findings that there is no such specificity [257] and with the concept of reversibility of the transacylation process [268]. It has been proposed by Schlame, that CL

remodeling strives for a CL species profile with the lowest total free energy [268]. At some point, the gain in enthalpy from more extensive remodeling can not compensate for a further decrease in entropy caused by the non-random acyl chain distribution. The position of this equilibrium is dependent on the environment: the membrane structure, the pool of acyl chains available for CL remodeling, and other processes affecting CL and its acyl chain donors and acceptors.

The CL remodeling mechanism according to the second Taz1-based hypothesis is a combination of the Lands' cycle and the other hypothesis starring Taz1: CL is first deacylated by phospholipase A_2 and the produced MLCL is subsequently reacylated by Taz1 via the aforementioned reaction B.
Recently, a CL-specific lipase was identified in *S. cerevisiae* mitochondria, *i.e.*, Cld1 [269]. The results indicated that the lipase preferentially removes C16:0 from CL, providing an elegant mechanism to limit the number of different acyl chains in CL and to allow flexibility in CL acyl chain composition, depending on the pool of acyl chains available for remodeling.
The notion of distinct deacylation and reacylation steps gives a credible explanation for the accumulation of MLCL in case reacylation is impaired in the absence of Taz1. Consistent with this, no MLCL accumulation was observed in cells lacking both Taz1 and Cld1 [269]. Since remodeling through deacylation/reacylation is supported by data described in Chapter 4, this mechanism will be discussed more extensively in Chapter 4 and 5.

1.5.4 Regulation of CL synthesis

Considering the central role CL plays in mitochondrial functioning, it is not surprising that CL synthesis is subject to a complex interplay of various regulatory factors at the levels of gene expression and enzyme activity. Despite more than four decades of research, the regulatory mechanisms are still poorly understood. A complicating factor is that regulation of CL synthesis might to some extent be organism-specific. For instance, Pgs1 expression in *Schizosaccharomyces pombe* is stimulated by inositol starvation [270], whereas inositol does not affect Pgs1 expression, but rather its enzymatic activity in *S. cerevisiae* (see below). In this section, a summary of the current knowledge on the regulation of CL synthesis is presented, focused on *S. cerevisiae*.

Mitochondrial development
Already in 1971, Jakovcic and co-workers reported that the level of CL correlates with the 'state of development of mitochondrial membranes', as expected: the highest CL levels were found in cells growing on NFCS, in the presence of oxygen,

and in *rho*^{+} cells (*i.e.*, cells without mutations in chromosomal or mitochondrial DNA affecting respiratory competence) [271]. Later studies confirmed that CL synthesis is promoted by conditions stimulating mitochondrial development at the level of Pgs1 and Crd1 expression and activity [272-274]. PGP phosphatase is probably differently regulated, since its activity level is independent of the carbon source [275]. Worth mentioning in this context is the rapid and abundant increase in CL synthesis as visualized by the CL-specific dye 10-*N*-nonyl-3,6-bis(dimethylamino)acridine (NAO) during the diauxic shift from fermentative to gluconeogenic growth [210].

The underlying mechanisms responsible for the alignment of CL synthesis and mitochondrial development are poorly understood. Some mechanisms regulate gene expression [272,273], but through unknown transcription factors. A plausible hypothesis regarding regulation at the enzymatic level is that the CL synthase Crd1 is regulated by the transmembrane pH gradient, based on the high pH optimum (pH ~9) of this enzyme [235,276]. A decreased transmembrane pH gradient (either in respiratory complex mutants [277] or in the presence of the oxidative phosphorylation inhibitor carbonylcyanide-3-chlorophenylhydrazone (CCCP) [278]) indeed resulted in a reduced CL synthesis. It has been ruled out that CL synthesis requires a membrane potential or ongoing ATP synthesis [278].
It was recently found that the mitochondrial translocator assembly and maintenance protein 41 (Tam41) might play a role in attuning mitochondrial structure and function, too. This protein is required for the integrity and activity of the mitochondrial TIM23 complex (involved in the import of all precursors of matrix proteins, most inner membrane proteins and many IMS proteins [4]) and for the assembly of carrier proteins that are imported via the TIM22 complex [279,280]. Kutik and co-workers noticed that the phenotype of cells lacking Tam41 resembled that of *crd1* cells by exhibiting among others impaired AAC oligomerization and supercomplex assembly. Lipid analysis revealed that yeast cells accumulate PA in the absence of Tam41, whereas PG and CL were barely detectable. Since Pgs1 and Crd1 levels in *tam41* mitochondria were comparable to those in wild type mitochondria, it was suggested that Tam41 is required at an early stage of CL synthesis [281], *i.e.*, at the level of Cds1. Elucidation of the mechanism by which Tam41 influences CL synthesis awaits further investigation.

A complicated issue is the influence of the growth phase on CL synthesis. Several studies have shown higher steady state CL levels and/or elevated transcription and expression levels of *PGS1* and *CRD1* in cells that have reached stationary growth [244,271-274,278,282], but the results are not always consistent. For example, in one study, a substantial increase in CL content was observed in stationary *vs* log phase cells cultured on glycerol-ethanol [244], whereas in another study no such difference

was observed on the same carbon source [274]. Furthermore, several experiments have been performed using cells cultured on glucose, confounding the effect of the growth phase with that of the switch to ethanol as carbon source (the diauxic shift; after glucose has been exhausted during exponential growth). Nevertheless, labeling of newly synthesized CL using ^{32}P in cells cultured on glycerol-ethanol indicated that most CL is actually produced during logarithmic growth [244] and similar results have been obtained for the fermentable carbon sources dextrose and galactose [278]. Speculatively, elevating the transcription/expression of *PGS1* and *CRD1* is a way of stationary phase cells to cope with a possibly reduced efficiency of the CL synthetic enzymes in those cells.

Since cells cultured on NFCS contain more CL than other cells [244,274,278], it would stand reason that the portion of newly synthesized CL in the total pool of new phospholipids is higher in the former cells. However, data from a pulse labeling experiment suggest otherwise. Regardless of the type of carbon source, 1.3-1.4% of ^{32}P incorporated in newly synthesized phospholipids in logarithmically growing cells ended up in CL. This suggests that the turnover of CL is relatively low compared to other phospholipid classes (CL accounts for significantly more than this percentage of the total phospholipid pool), but more importantly, it also suggests that the turnover rate of CL is higher on glucose than on glycerol-ethanol. If this is indeed the case, CL degradation would play an important role in establishing CL levels. However, apparently conflicting data have been reported regarding the influence of the carbon source on the rate of CL synthesis: the specific activity of Crd1 (and also Pgs1; based on total mitochondrial protein content) in isolated mitochondria was reportedly up to 7-fold higher on NFCS compared to glucose [283], although it is unknown to what extent this is attributable to differences in Crd1 levels in the mitochondrial preparations.

Inositol
The synthesis of many phospholipids is controlled by the Ino2-Ino4-Opi1 circuit which lowers the expression of phospholipid biosynthetic enzymes in the presence of inositol [54]. In 1988, it was reported by Greenberg and co-workers that also Pgs1 was regulated by inositol, but independent of Opi1 [284]. Since Pgs1 activity dropped significantly within minutes upon inositol supplementation, it was suspected that inositol did not affect the expression of the enzyme, but rather triggered its inhibition or degradation. In later studies, it was confirmed that Pgs1 is not under transcriptional control by inositol [285], but that the protein is phosphorylated in response to inositol [286]. The conclusion that Pgs1 activity is dependent on phosphorylation is in line with earlier observations in human H9c2 cells that stimulation of PGP synthase by ceramides is attenuated by protein phosphatase inhibitors [287]. Results from two

studies which suggested that Pgs1 activity is yet regulated on a transcriptional level by Ino2-Ino4-Opi1 [272,288] have been attributed to artifacts [285]. This is supported by the fact that the Pgs1 activity is the highest during stationary growth [274], whereas the activities of Ino2-Ino4-Opi1 regulated proteins peak during exponential growth [54,136,289]. Concerning other CL synthetic enzymes, the PGP phosphatase and Crd1 activities have been shown to be not responsive to inositol [235,275,282]. However, Su and co-workers reported also that Crd1 expression is elevated in *ino4* and not in *ino2* mutants [282], stressing the need for further investigation.

Coordination with PE synthesis
Recently, it has been discovered that the synthesis of CL is regulated in concert with the synthesis of mitochondrial PE by Psd1 [290]. Synthetic genetic arrays identified 35 genes which were essential for the viability of the yeast cells lacking the mitochondrial protein prohibitin Phb1. The fact that *CRD1* and *PSD1* were among these genes (confirming earlier results for the latter [291]) suggested some link between prohibitin and phospholipids. Further experiments revealed that five genes were important for CL homeostasis (*UPS1*, *MDM34*, *GEP4*, *MDM32* and *CRD1*), three genes for PE levels (*MDM35*, *GEP1* and *PSD1*) and a group of nine genes influenced the levels of both lipids (*MMM1*, *MDM31*, *YTA10*, *YTA12*, *MDM10*, *COX6*, *GEP3*, *GEP5* and *GEP6*). Although the roles played by most of these genes remains to be identified, it has been observed that Gep1 is essential for the stability of PE synthesized by Psd1, and that Ups1 is needed for the accumulation of CL. Interestingly, overexpression of Gep1 resulted in a reduced CL level and deletion of *GEP1* restored CL levels in *ups1* cells, suggesting that Gep1 and Ups1 antagonize one another to modulate the PE/CL ratio [290]. It was recently reported that Ups1 is also antagonized by Ups2 [292].
What could be the reason that an impaired PE or CL synthesis results in cell death in the absence of Phb1? It has been speculated that PE and CL can be accumulated within prohibitin-encircled membrane domains when scarce in the bulk membrane, to preserve membrane integrity and to provide PE/CL-rich scaffolds for essential processes relying on adequate levels of these lipids [290].

Other factors
As discussed above, the CL biosynthetic enzymes reside in the mitochondrial inner membrane. This membrane is characterized by a high protein/lipid ratio and a high degree of protein conglomeration. It is therefore not surprising, that also Crd1 was found to be part of a protein complex [277]. Although the identity of the interaction partners has not yet been elucidated, it is not unlikely that they are able to modulate Crd1 activity. Another mystery described by Zhao and co-workers is the dependence of Crd1 activity on the *assembly* rather than the activity of Complex IV [277]. Later,

it was demonstrated that this dependence is not at the level of *CRD1* transcription [273].
As mentioned earlier, also Taz1 was found to interact with other proteins, namely the respiratory components AAC2 and ATP synthase [267]. The functional significance of this interaction is also unknown, but it is tempting to speculate that it plays a role in aligning CL remodeling and mitochondrial activity.
Apart from the proteins, lipids might also function as regulators of CL synthesis. Purified Crd1 was shown to be stimulated by its own product, *i.e.*, CL [234]. Remarkably, ten years later, it was suggested that the lower CL content in *taz1* cells triggers the increased Crd1 activity in those cells, without reference to the older, conflicting data [234] and unsupported by experimental evidence [244]. Pgs1 from *Schizosaccharomyces pombe* was shown to be inhibited by PI (possibly to control the total content of anionic lipids in the membrane) and CL apparently had a stimulatory effect on the enzyme [293]; however, it is unknown whether these findings also apply to *S. cerevisiae*.
Finally, the CL synthases need either Mg^{2+}, Mn^{2+} or Co^{2+} as cofactor [241]. Crd1 prefers Mg^{2+} (and is inactive when supplied with Mn^{2+}) [235], whereas the mammalian CL synthases display the highest activity in the presence of Co^{2+} [241].

1.5.5 Functions of CL

The fact that CL is found virtually exclusively in mitochondria and not throughout the cell like other phospholipids implies that CL is not a mere membrane building block. A lot of knowledge has been gained by studying CL metabolic mutants, although this approach is like opening the box of Pandora: CL plays a role in so many structures and processes, that it is often hard to tell whether a phenotype is a specific and primary effect or rather an indirect and pleiotropic effect of the altered CL homeostasis. Loss of CL has been reported to result among others in impaired growth (especially on NFCS at elevated temperatures) [273,283,294-297], loss of mitochondrial DNA [296], reduced viability [296], reduced mitochondrial membrane potential [265,296,298] and a decreased osmotic stability [264,298]. Zhang and co-workers stated that phenotypes observed in *crd1* strains with the *hisΔ200* allele are exaggerated through an impaired expression of *PET56* [299]. A year later, this conclusion was contradicted by new experimental evidence which demonstrated that the loss of CL impacts mitochondrial functioning, cell viability and mitochondrial DNA stability independent of *PET56* expression levels [300]. It should also be kept in mind that cells devoid of CL only survive because PE and PG can at least partially substitute for CL as will be discussed below. Nevertheless, it has been demonstrated that CL supports mitochondrial structure and function in various ways.

CL and proteins
Already in 1974, it was suggested by Mikel'saar that CL might be involved in physically linking components of the respiratory chain in mitochondria [301]. Since then, an astounding body of evidence has been collected in especially yeast and bovine mitochondria demonstrating that CL is important for the stability (or assembly) and activity of the respiratory complexes I [302-306] (not present in *S. cerevisiae* [9,307]), III [173,180,214,302,308-315], IV [314,316-321], V [322] and for supercomplex III_2IV_2 [265,297,314,315,323]. A few years ago, indications have been found that CL is also important for the stability of a supercomplex comprising Complex I, III and IV, and for the biogenesis of Complex I [324]. Furthermore, the activity, stability and oligomerization of the ADP/ATP carrier have been shown to be CL-dependent [325-331], as is the activity of the phosphate carrier [332,333]. Recently, some other functional CL-protein interactions have been reported: CL apparently recruits the mitochondrial creatine kinase and the nucleoside diphosphate kinase to the mitochondrial inner membrane bringing them in close contact with the adenylate translocator [334]. The membrane binding of the kinases has been claimed to facilitate several other processes, including lipid transfer between the outer and inner membrane [334]. Another recent paper revealed that CL facilitates the assembly and/or promotes the stability of two interesting protein complexes: Taz1-Complex V and Taz1-ADP/ATP carrier AAC2 [267]. Although it remains to be investigated what the functional significance of those complexes is, it is tempting to speculate that they play a role in aligning ATP synthesis with CL remodeling, as indicated above. In another article, Claypool *et al.* reported several other CL-dependent interaction partners of AAC2, mostly other members of the mitochondrial carrier family and components of supercomplex III_2IV_2 [328].
It is highly likely, considering that new complexes are still being discovered, that more CL-protein interactions will be identified in the future.

It is beyond the purview of this introduction to discuss the significance of CL for each protein in detail. In short, CL binds to proteins through hydrophobic and electrostatic interactions as well as through H-bonding as inferred among others from crystal structures. The CL-protein interaction can be so tight, as in the bovine ADP/ATP carrier, that CL only is released upon protein denaturation, suggesting that CL functions as a chaperone [326]. CL stabilizes protein structures and complexes by acting as a 'glue' in filling grooves [297] and in neutralizing repulsive electrostatic forces [315] at protein interfaces. In addition, the non-bilayer shape of CL might affect the lateral pressure profile in the membrane, thereby influencing the stability of protein complexes [162], although to my knowledge this has never been discussed in literature.

By bringing together proteins, CL might facilitate substrate channeling, sequestration of reactive intermediates and efficient shuttling of electrons, thereby increasing the efficiency of oxidative phosphorylation and of other processes [264,267,296,298, 323,328,335-338]. As an example, the CL-mediated interaction between respiratory supercomplexes and AAC2 has been claimed to be beneficiary for both parties: the activity of the ADP/ATP carrier might be maximized by being located immediately next to proton pumps and AAC2 in turn might stabilize the supercomplexes and boost the activity of complex IV [328,337]. Furthermore, CL can be part of or close to a catalytic center and thus play an active role in catalyzing reactions [180,214,313,339].

CL and mitochondrial biogenesis and structure
In addition to its role in stimulating protein(complex) activity, assembly and/or stability, CL is involved in regulating protein expression, protein import and in shaping the mitochondria. Regarding the first, several mitochondrial proteins require CL for optimal expression, including the nucleus-encoded Cox4 [340,341] and the mitochondrion-encoded Cox1-3 [340]. The inositol phosphosphingolipid phospholipase C Isc1 might be a key player here, since it is activated among others by PG and CL [342,343] and it was later found to be required for the massive reprogramming of gene expression during a diauxic shift [344].
The import of newly synthesized proteins is also dependent on CL. Twenty years ago, it was discovered that doxorubicin (a CL-binding compound) blocked the import of a mitochondrial precursor proteins by yeast mitochondria [345]. Later investigations hinted at a decreased mitochondrial membrane potential in CL-deficient cells as a cause for a reduced protein import [296]. Furthermore, CL seems to facilitate the integration of precursors protein into the mitochondrial inner membrane [346]. In addition, it has been demonstrated that Tim44, an essential component of the TIM23 complex, strongly binds to liposomes containing CL. This suggests that CL is involved in anchoring the protein import machinery to the mitochondrial inner membrane [347,348]. Lastly, the assembly state of the TIM23 complex was affected under conditions of decreased CL content, leading to a defective mitochondrial protein import [292].
The intrinsic curvature of CL might drive the lateral segregation of CL, resulting in the formation of curved CL-rich membrane domains, like the cristae in mitochondria [349]. The clustering of CL might be promoted further by proteins like creatine kinase and cytochrome *c* [334,350,351]. Evidence has been found that the curvature in cristae is also enforced by the geometry of so-called dimer ribbons of ATP synthase (Complex V) [352-354]. Strauss *et al.* calculated that for a constant membrane potential the higher proton concentration in the curved *vs*. planar membrane regions results in a pH drop of ~0.5 [353], reinforcing the non-bilayer behaviour of CL [228].

CL and proton buffering and leakage

As explained earlier, the second pK_a of CL is relatively high because of the second proton being firmly captured in a resonance structure involving the two phosphates and the central hydroxyl group [215,216]. Since this pK_a is higher than that of water, CL is able to serve as a proton buffer at the membrane-water interface on both sides of the mitochondrial inner membrane. Haines and Dencher proposed an interesting hypothesis in 2002. In short, protons that are pumped from the mitochondrial matrix across the mitochondrial inner membrane are trapped by CL in the outer leaflet of this membrane. Subsequently, the protons are shuttled from the oxidative phosphorylation complexes to the ATP synthase by lateral movement of the CL molecules. The protons are then transported back to the matrix side of the mitochondrial inner membrane through the ATP synthase to drive ATP synthesis. Here, the protons are again collected by CL for delivery at the proton pumps for a new cycle. In this model, the pH of the bulk phases on both sides of the membrane is relatively unaffected by the proton fluxes. As pointed out by Haines and Dencher, this implies that the ATP synthase is not driven by ΔpH, but rather by the transmembrane potential [216]. This is consistent with earlier results reported by Kaim and Dimroth, who demonstrated that the membrane potential is the fundamental driving force of ATP synthesis [355].

Connected to the ability of CL to accept and donate protons under physiological conditions is the role of CL in the selective leakage of protons from the outside of the mitochondrial inner membrane to the mitochondrial matrix. Apparently, CL functions as a so-called proton antenna that donates the collected protons to systems that facilitate the transport of protons across the hydrophobic membrane core. The physiological function is not well established. Since this field of research is dormant, with the last known article on this topic published over 10 years ago, it will not be discussed here further. Instead, refer to [356-358].

CL and apoptosis

Currently, at least three proteins of the apoptotic machinery are known to bind to CL as recently reviewed [359]: cytochrome *c*, tBid (the truncated form of Bid, a pro-apoptotic member of the Bcl-2 family that regulates permeabilization of the mitochondrial outer membrane [360]) and caspase-8, which is responsible for the truncation of Bid [361,362].

Under non-apoptotic conditions, a relatively small portion of the cytochrome *c* pool (10-15%) is bound to CL [363,364] through electrostatic interactions between the CL headgroup and lysine residues of cytochrome *c* and through stronger hydrophobic interactions involving the insertion of an acyl chain into a hydrophobic pocket of the protein [365-368]. Binding of cytochrome *c* to CL results in a distortion of the native protein structure, altering the redox properties of cytochrome *c*. As a consequence,

the peroxidase activity of cytochrome *c* is increased [359,363,367,369-372]. Worth mentioning is that the ability of CL to induce the peroxidase activity of cytochrome *c* correlated with the affinity of the protein-lipid interaction, with the highest affinity and strongest stimulus being observed for unsaturated CL [372]. If CL is indeed peroxidized, cytochrome *c* becomes detached and can induce apoptosis when released into the cytosol [371,373].

In an early phase of apoptosis, CL redistributes to the outer leaflet of the mitochondrial inner membrane [211]. It is not firmly established yet, whether this translocation precedes or follows the binding of tBid to mitochondrial contact sites [359,367,374]. Nevertheless, CL does bind to tBid through electrostatic interactions [375] and may allow tBid to insert into the membrane [376], although the latter can be mediated by other phospholipids too [377,378]. By the way, CL is also required for the insertion of Bax into the mitochondrial outer membrane [379]. tBid might have multiple functions in apoptosis: it might stimulate phospholipase A_2 activity in the mitochondrial inner membrane, resulting in a redistribution of CL over the mitochondrial membranes [374], and it might induce Bax/Bak oligomerization to promote permeabilization of the outer membrane [360,380]. Recently, a third apoptotic factor was found to interact with CL. Apparently, CL domains function as an anchor and activating platform for caspase-8, enabling the cleavage of Bid to tBid [381].

Other roles of CL

The last two roles of CL that remain to be discussed are in cell wall biogenesis and vacuolar biogenesis. Regarding the first, a spontaneous suppressor mutant of *pgs1* which did grow at elevated temperatures was found to have a loss-of-function mutation in the *KRE5* gene [382]. Further experiments with *pgs1* and the suppressor mutant showed that the β-1,3-glucan level was lower in *pgs1* [382] and that activation of the downstream effector Slt2 of the protein kinase C-activated cell integrity signaling cascade was impaired [383]. The decreased glucan level was caused by a lower glucan synthase activity which was in turn due to diminished levels of the catalytic subunit Fks1. In the suppressor mutant, increased expression of the alternate catalytic subunit Fks2 compensated for the loss of Fks1. In addition, activation of Slt2 was restored by the mutation in *KRE5* [383]. The precise role of PG and/or CL remains to be elucidated.

More recently, it was discovered that *crd1* cells exhibit swollen vacuoles and an impaired acidification of these organelles at elevated temperatures, in addition to their growth phenotype [384]. Similar results were obtained for *pgs1*, indicating that CL deficiency was the underlying cause. Although it is not clear how CL influences vacuolar processes, it is thought that CL affects ion homeostasis via the signaling protein Rtg2: deletion of *RTG2* or of *NHX1* (the latter gene encodes a Na^+/H^+ exchanger which among others regulates the vacuolar pH) suppressed the

phenotypes observed for *crd1*.

Although CL has numerous functions (and most likely, more will be identified in the future), it is not indispensable as evidenced by the viability of the *crd1* strain. At least two lipids are thought to be able to take over the essential functions of CL, namely PE and PG. Consistent with this notion, *pgs1* (in which PG and CL synthesis is blocked) has a more severe growth phenotype than *crd1* [294,382] and *crd1psd1* (which lacks CL and which is unable to produce PE in mitochondria) is not viable at all [227]. It is striking that PE synthesized by Psd2 in the Golgi and by the Kennedy pathway is not able to rescue *crd1psd1*; *crd1psd2* is viable [227].
The structural attribute shared by PG and CL is a negatively charged headgroup, suggesting that PG is able take over those functions of CL that rely on the presence of a negative charge. The common feature of PE and CL is less obvious. Both lipids are inclined to form non-bilayer structures [219,222], although for CL the strength of this tendency is dependent on the environment as discussed earlier. Several lines of evidence (apart from the inviability of *crd1psd1*) support the hypothesis that PE and CL share functions. Firstly, a decrease in CL content is accompanied by elevated PE levels in *S. cerevisiae* [244,300], although sometimes only to a small extent [227]. The other way around, a lower mitochondrial PE level in *psd1* is not compensated for by a rise in CL [227], but it is likely that mitochondrial PE levels are supplemented with PE synthesized by Psd2 and/or by the Kennedy pathway. Furthermore, it could be that PE is a backup for CL and not the other way around. Secondly, and maybe more importantly, PE and CL synthesis appear to be coordinately regulated as discussed above. In this context, it is worth mentioning that CL can also substitute for PE in *E. coli* provided that the medium is supplemented with divalent cations which promote the non-bilayer propensity of CL [223].

Despite the widely held opinion that the acyl chain composition of CL is important for its functioning, experimental evidence is scarce and open for discussion:

1) The activity of CL-depleted cytochrome *c* oxidase cannot be recovered by CL with two C6 chains [385], although this hardly can be claimed to be physiologically relevant, since the choice *in vivo* is usually between acyl chains that differ only 2 carbon atoms in length (*i.e.*, C16 and C18);
2) The activity of the ADP/ATP carrier AAC2 was shown to be dependent on the nature of the **5th** acyl chain of CL [330]. Again, the biological relevance is doubtful, also taking into account that the headgroup of CL is probably responsible for the high affinity interaction between CL and AAC2, rather than the acyl chains [386,387];
3) Peroxidation of unsaturated acyl chains results in the inactivation of mammalian ADP/ATP carrier [388] and in the release of cytochrome *c* from

mitochondria into the cytoplasm. The latter is an important stage in apoptosis, as discussed before [371,373]. However, a high degree of unsaturation is not found in all organisms nor in all tissues of the same organism to the same extent [232].

Considering that CL itself is not even essential for cell viability, why do cells bother to pursue a specific acyl chain composition through CL remodeling? This question becomes the more pressing, if one considers the differences in CL acyl chain composition, even between various tissues of the same organism [232]. In Chapter 5, this will be discussed further.

1.5.6 Diseases resulting from defects in CL homeostasis

Many diseases and pathologies are accompanied by a disturbed CL metabolism, as manifested on a cellular level by a decreased CL content, an altered CL species profile and/or an increased level of CL peroxidation: ischemia/reperfusion [303,389], hyper- and hypothyroidism [390,391], aging [305], heart failure [392-394], Barth syndrome [247,262,263,324,395-397], Tangier disease [398], diabetes [399], Alzheimer's disease [400], nonalcoholic fatty liver disease [306] and cancer [401]. In addition, antibodies directed against CL are found in patients suffering from various diseases [402-404].

Barth syndrome
It is remarkable, that of the aforementioned diseases only the Barth syndrome (MIM #302060) is brought on by a defect in CL homeostasis, although symptoms of the other diseases may be caused or aggravated by the disturbed CL metabolism. Although CL might not be strictly essential for cell viability, CL-related problems probably affect the fitness of an organism to such an extent that it interferes with procreation. Also in case of Barth syndrome, patients usually die in their infancy or childhood due to cardiac failure or sepsis, although their condition can be improved with adequate diagnosis and medical care (http://barthsyndrome.org; accessed 27OCT09). The disease is caused by a recessive mutant allele of the *TAZ* gene, which is located on the X-chromosome, and is therefore usually transferred from a carrier mother to her son [405,406]. Due to the mutation, the CL remodeling enzyme Tafazzin is not functional, resulting in CL containing relatively more saturated acyl chains and in accumulation of MLCL at the expense of CL [244,245,247,248,262,264,265,324, 395,396,407]. The (cardio)myopathy in Barth syndrome patients is probably caused by the reduced activity of the respiratory supercomplexes [405,408,409], which may in turn be attributed to destabilization of those complexes [265,324].
Other clinical symptoms that are often encountered are neutropenia (a reduction in the

level of neutrophils), growth retardation and urinary excretion of 3-methylglutaconate [405,406,408,409], but mechanisms underlying their manifestation are poorly understood.

1.6 Scope of the thesis

The focus of the research described in this thesis has been on two different phospholipids in yeast mitochondria, the ubiquitous PC and the signature lipid CL.
First, the relevance of the interaction between PC and the important mitochondrial enzyme Gut2 was investigated as described in Chapter 2. This study was based on the observation that this protein was efficiently crosslinked to the photoactivatable PC analogue TID-PC in isolated mitochondria [181]. The data showed that PC is important for the efficient functioning of Gut2.
Chapter 3 describes the investigation into the possible connection between PC and the utilization of proline as a nitrogen source, triggered by the observation that the transcription of *PUT1* and *GAP1* was upregulated in PC-depleted cells (M.C. Koorengevel *et al.*, unpublished results). The results indicated that proline utilization relies heavily on PC.
Subsequently, the focus was shifted to CL. Experimental work aiming at the identification of PC remodeling enzymes revealed an altered PC profile in cells depleted of the acylCoA-binding protein Acb1 [410]. However, the CL profile appeared to be strongly affected too. This raised our interest, since the CL acyl chain composition is thought to be important for the functioning of this lipid. Apparently, the CL remodeling system was not able or not triggered to establish the regular CL species profile. Moreover, PC had been implicated in CL remodeling before [259], suggesting that Acb1 could somehow link PC to the unique mitochondrial phospholipid CL. It was investigated how the acyl chain composition of CL is established in an *acb1* background. Chapter 4 presents the results which contribute to the understanding of CL remodeling in *S. cerevisiae*. No obvious role for PC in CL remodeling was identified.

The main findings of Chapter 2-4 are summarized and discussed in Chapter 5.

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CHAPTER 2

Phosphatidylcholine is essential for efficient functioning of the mitochondrial glycerol-3-phosphate dehydrogenase Gut2 in *S. cerevisiae*

Based on:

Pieter J. Rijken, Ben de Kruijff and Anton I.P.M. de Kroon (2007)
Mol Membr Biol 24: 269-281

Abstract

Gut2, the mitochondrial glycerol-3-phosphate (G3P) dehydrogenase, was previously shown to become preferentially labelled with photoactivatable phosphatidylcholine (PC), pointing to a functional relation between these molecules. In the present study we analyzed whether Gut2 functioning depends on the PC content of yeast cells, using PC biosynthetic mutants in which the PC content was lowered. PC depletion was found to reduce growth on glycerol and to increase glycerol excretion, both indicating that PC is needed for optimal Gut2 functioning *in vivo*. Using several *in vitro* approaches the nature of the dependence of Gut2 functioning on cellular PC contents was investigated. The results of these experiments suggest that it is unlikely that the effects observed *in vivo* are due to changes in cellular Gut2 content, in specific activity of Gut2 in isolated mitochondria, or in the membrane association of Gut2, upon lowering the PC level. The *in vivo* effects are more likely an indirect result of PC depletion-induced changes in the cellular context in which Gut2 functions, that are not manifested in the *in vitro* systems used.

Introduction

PC is a very abundant phospholipid in membranes of eukaryotes. Apart from functioning as an important building block of these membranes, PC is also involved in signal transduction as precursor for lipid second messengers, like phosphatidic acid (PA) and diacylglycerol (DAG) [1,2]. Disturbance of PC metabolism has been found in many pathological conditions, like Gaucher disease [3] and Alzheimer’s disease [4], and in apoptosis [5].

To get insight into the specific functions of PC, yeast is an attractive model organism. PC accounts for 40-50% of the total phospholipid content in yeast cells [6]. As in higher eukaryotes, there are two routes in yeast by which PC is produced. The most important one is the triple methylation of phosphatidylethanolamine (PE) by the enzymes Cho2 and Opi3 [7]. The CDP-choline or Kennedy pathway recycles choline from PC turnover [8]. This route only contributes to net PC synthesis when choline is supplemented in the growth medium [8,9].

Yeast strains that are unable to synthesize PC, have problems in maintaining their respiratory competence [10], indicating that PC is important for mitochondrial function, possibly because particular proteins depend on this lipid for proper functioning. To identify mitochondrial proteins that require PC, isolated yeast mitochondria were probed with a photoactivatable radiolabeled PC analogue (TID-PC) for PC interacting proteins. Photoactivation resulted in prominent labeling of Gut2, the FAD-dependent mitochondrial G3P dehydrogenase [11], indicating a special relationship between PC

and Gut2. In the present study we addressed the question whether PC is required for Gut2 functioning in yeast.
Gut2 is essential for growth of yeast on glycerol (hence its name Glycerol utilization), by converting G3P to dihydroxyacetonephosphate (DHAP) [12,13]. This reaction is also part of the G3P shuttle, in which the cytosolic G3P dehydrogenases Gpd1 and Gpd2 oxidize cytosolic NADH by passing the electrons on to DHAP. The G3P formed is then oxidized in the mitochondria by Gut2. The result is a stepwise transfer of electrons from the cytosol to the respiratory chain [14,15]. Gut2 was found to be a mitochondrial peripheral membrane protein [11] that based on its function, is most likely associated with the inner membrane, facing the intermembrane space [16].
To investigate the influence of the PC content on Gut2 functioning, yeast mutant strains were used, in which the PE methylation route for PC synthesis is impaired by deleting the *CHO2* and/or *OPI3* genes. These strains rely on exogenous choline to obtain wild type PC levels [17-20]. By depriving these strains of choline, the cellular PC contents can be lowered from 40% to less than 5% of total phospholipids [18,20], thus providing a useful system to analyze the influence of varying PC contents on Gut2 functioning.
The results obtained *in vivo*, *i.e.*, reduced growth on glycerol and increased glycerol excretion upon PC depletion, support the hypothesis that PC is required for Gut2 functioning. *In vitro* approaches suggest that the *in vivo* effects are not due to changes in cellular Gut2 content, in specific activity of Gut2 in isolated mitochondria, or in the membrane association of Gut2, but rather are an indirect result of PC-depletion induced changes in the biological context in which Gut2 functions.

Materials and methods

Yeast strains, media and culture conditions

The yeast strains listed in Table I were maintained on YPD agar plates (1% yeast extract, 2% bactopeptone and 2% glucose). Vitamin-defined synthetic media [10] contained per liter: 5 g of ammonium sulfate, 1 g of potassium phosphate monobasic, 0.5 g of magnesium sulfate, 0.1 g of sodium chloride, 0.1 g of calcium chloride, 0.5 mg of boric acid, 0.04 mg of cupric sulfate, 0.1 mg of potassium iodide, 0.2 mg of ferric chloride, 0.4 mg of manganese sulfate, 0.2 mg of sodium molybdate, 0.4 mg of zinc sulfate, 20 mg of adenine, 20 mg of arginine, 20 mg of histidine, 60 mg of leucine, 230 mg of lysine, 20 mg of methionine, 300 mg of threonine, 20 mg of tryptophan, 40 mg of uracil, 2 µg of biotin, 400 µg of panthothenate, 2 µg of folic acid, 400 µg of niacin, 200 µg of *p*-aminobenzoic acid, 400 µg of pyridoxine hydrochloride. As carbon source was added 30 g glucose (SD medium), 22 mL lactic acid with 1 g glucose (SL medium, adjusted to pH 5.5 using KOH), or 20 g glycerol with 1 g glucose (SG

medium). Media were supplemented with 75 µM inositol and where indicated with 1 mM choline (C^+). The CRD1 and *crd1* strains were grown to late log phase in YPLac, containing 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (v/v) lactate [21], supplemented with 0.1% (w/v) glucose and with a pH value of 5.5.
All cultures were grown aerobically at 30 °C (with shaking in the case of liquid cultures). Growth was monitored by measuring the OD at 600 nm, using a Hitachi 150-20 double beam spectrophotometer.

Table I Genotypes of strains employed in this study

Strain	*Designation*	*Genotype*	*Source*
SH921	wild type	*MATα leu2 ade2 ura3 his3*	[53]
SH922	*cho2opi3*	*MATα leu2 ade2 ura3 his3 cho2::LEU2 opi3::URA3*	[53]
SH414	*opi3*	*MAT**a** opi3::URA3 ade2 can1 his3 leu2 trp1 ura3*	[53]
SH458	*cho2*	*MATα cho2::LEU2 ura3 leu2 his3*	[53]
PR01	*gut2*	*MATα leu2 ade2 ura3 his3 gut2::KanMX*	this study
PR03	*cho2opi3*	*MATα leu2 ade2 ura3 his3 cho2::LEU2 opi3::URA3 GUT2-6H3HA-KanMX*	this study
FGY2	*crd1*	*MATα crd1Δ::URA3 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 thr⁻ arg⁻ met⁻*	[54]
FGY3	*CRD1*	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 thr⁻ arg⁻ met⁻*	[54]
BY4742	wild type	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Research Genetics, Invitrogen
14787	*cho2*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 cho2::KanMX*	Research Genetics, Invitrogen
HB103	*opi3*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 opi3::LEU2*	[55]
GP008	*gut2*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gut2::URA3*	this study

Epitope tagging of Gut2

Gut2 was epitope-tagged at its C-terminus by cloning a histidine (His_6) and a hemagglutinin (HA) tag at the end of the *GUT2* gene into the yeast genome, via homologous recombination with a PCR fragment, also containing the *kan*MX cassette. The plasmid pU6H3HA (EMBL accession number AJ132966, [22]) was used as a template for PCR amplification. The primers used were 5'-ACT TGA AAA AAC TGT GAA CTT CAT CAA GAC GTT TGG TGT CTC CCA CCA CCA TCA TCA TCA CG-3' (with the underlined fragment corresponding to nucleotides 1908-1947of the *GUT2* ORF) and 5'-TTA TAT TAT GTA TTG GAA ATA GAA TAT AAA CAC TAG GAA GAC TAT AGG GAG ACC GGC AGA TCC G-3' (with the underlined sequence corresponding to nucleotides 290-251 downstream of the *GUT2* ORF). The PCR product of 1815 bp was used to transform the yeast strain SH922 and

positive colonies were selected on G418 containing plates as described [22]. Correct integration of the PCR fragment into the yeast genome was checked by colony PCR. Expression of the hemagglutinin-tagged Gut2 was verified by Western blotting.

Deletion of GUT2

In the wild type strain SH921 the *GUT2* gene in the yeast genome was replaced with a PCR fragment containing a *kan*MX cassette, via homologous recombination. The plasmid pU6H3HA was used as a template for PCR amplification with the primers 5'-GCT ATT GCC ATC ACT GCT ACA AGA CTA AAT ACG TAC TAA TAT CCC TTA ATA TAA CTT CG-3' (with the underlined sequence corresponding to nucleotides (-42)-(-1) upstream of the *GUT2* ORF) and 5'-GTG AAT GTT ATC TTT GTC ACC CTT AAC TAT CAT GAT CGA TCA CCT AAT AAC TTC GTA TAG C-3' (with the underlined sequence corresponding to nucleotides 40-1 downstream of the *GUT2* ORF). The PCR product of 1637 bp was used to transform the yeast strain SH922. The *GUT2* gene was also deleted in the BY4742 background, by replacement with a PCR fragment containing the *URA3* gene, obtained using the pRS316 plasmid [23] as template and the primers 5'-TAT TGC CAT CAC TGC TAC AAG ACT AAA TAC GTA CTA ATA TCA TCA GAG CAG ATT GTA CTG AGA AGT GC-3' (with the underlined sequence corresponding to nucleotides (-40)-(-1) upstream of the *GUT2* ORF) and 5'-TTA TAT TAT GTA TTG GAA ATA GAA TAT AAA CAC TAG GAA GGC ATC TGT GCG GTA TTT CAC ACC-3', with the underlined sequence corresponding to nucleotides 290-250 downstream of the *GUT2* ORF). Correct replacement of the *GUT2* gene by the PCR fragments was verified by colony PCR.

Choline deprivation

In this study two methods were used to transfer the *cho2opi3* cells from choline-containing to choline-free culture medium. For the analysis of growth on different carbon sources, cells were grown in choline-containing synthetic medium to OD600 0.7-1.0. Subsequently the cells were collected by filtration, using 0.45 μm cellulose acetate filters (Nalgene, Rochester, NY, USA), washed with the corresponding choline-free medium and resuspended in fresh medium (with or without choline) to an OD600 of 0.1.

For the isolation of PC-depleted mitochondria, larger quantities of *cho2opi3* cells had to be deprived of choline. This was accomplished by collecting the cells (grown to an OD600 of more than 2.0 in SL C^+) by centrifugation. The cells were washed twice by resuspension in SL C^- followed by centrifugation. Finally the cells were resuspended in SL C^- and transferred to fresh SL medium (with or without choline) to an OD600 of 0.1-0.2.

Glycerol production

In order to have corresponding genotypes among the mutants tested, wild type, *cho2*, *opi3* and *gut2* strains with a BY4742 background (Table I) were used. The strains were grown in yeast nitrogen base without amino acids (YNB, Difco) with an initial pH value of 6.0, supplemented with 5 g/L glucose (10 g/L for pre-cultures) and 120 mg/L of each histidine, leucine, lysine and uracil, as described elsewhere [14]. The glycerol excretion in the medium was measured after 48 h using an enzymatic test kit (R-Biopharm AG, Almere, The Netherlands) according to the manufacturer's instructions.

Isolation of mitochondria

The *cho2opi3* strain PR03 or SH922 (Table I) was pre-cultured in SL C^+. The cells were deprived of choline by centrifugation and washing steps as described above and transferred to fresh medium with or without choline. The cells were grown for the indicated number of generations, harvested by centrifugation (10 min, 3500 *g*) and washed with water. Spheroplasts were prepared and homogenized as described [24,25]. Mitochondria were isolated using differential centrifugation as described elsewhere [25]. The obtained pellet of crude mitochondria was resuspended carefully in a minimal volume of H/S buffer (20 mM Hepes/KOH, 0.6 M D-sorbitol, 0.5% (w/v) BSA, pH 7.4). The mitochondria were frozen as 50 µL aliquots in liquid N_2 and stored at –80 °C. Samples were thawed only once before use.
Sucrose gradient-purified mitochondria of the CRD1 and *crd1* strains were isolated as described [25].

Measurement of Gut2 activity

The activity of Gut2 was measured by monitoring spectrophotometrically the phenazine methosulfate (PMS)-mediated reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) as described [11,26], with minor changes. The reaction mixture contained 50 mM Hepes pH 7.5, 10 mM KCN, 0.5 mM MTT, 0.2 mM PMS, 0.05% (v/v) Triton X-100, 50 mM DL-glycerol-3-phosphate and 50 µM FAD. In experiments in which the substrate concentration in the assay was varied, no significant influence of PC depletion on the K_M value of Gut2 was found (data not shown). Therefore, all Gut2 activities were measured under conditions where the substrate concentration was not rate limiting (data not shown). Where indicated, Triton X-100 was omitted from the assay mixture and 0.6 M D-sorbitol was added, to obtain an isotonic buffer. Gut2 specific activities were determined in a reaction volume of 1 mL, typically containing 100 µg of mitochondrial protein, using a Perkin Elmer Lambda 18 UV/VIS spectrophotometer. The variation of Gut2 activity with PC

content was measured in a reaction volume of 200 μL, typically containing 30 μg of mitochondrial protein, and monitored in 96-well plates, using a BioRad Ultramark Microplate Imaging System.
The increase in extinction at 562 nm (560 nm in the case of the plate reader) was recorded for 10 min after the addition of mitochondria. Activities were calculated from the slope, using an extinction coefficient for reduced MTT of 8.1 mM^{-1} cm^{-1} [11]. The numbers were corrected for background activity by repeating each measurement in the absence of the substrate G3P.

Influence of phospholipids on Gut2 activity

To study the effect of phospholipids on Gut2 activity a suspension of mitochondria from strain PR03 in H/S buffer (corresponding to 1.5 mg mitochondrial protein/ml) was mixed 1:4 (v/v) with a 400 μM solution of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) or 1,1',2,2'-tetraoleoyl-cardiolipin (TOCL) in 0.5% (w/v) Triton X-100. All lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The mixture was incubated for 15 min at room temperature and subsequently the Gut2 activity was measured in aliquots corresponding to 30 μg mitochondrial protein in 96-well plates as described above.

Association of Gut2 with mitoplasts

Mitoplasts were prepared by diluting mitochondria from the PR03 strain at least a factor of 10 in hypotonic buffer H (20 mM Hepes/KOH, pH 7.4) to a concentration of 1 mg protein/ml [27]. The mitochondria were incubated for 20 min on ice, followed by centrifugation for 12 min at 10,000 rpm (10,600 *g*) in a microfuge at 4 °C. The pellet was washed with buffer H, followed by centrifugation as above. The pellet was incubated for 5 min with buffer H, also containing 1 M NaCl, or 1, 2, 10, 20 or 75 mM Na_2CO_3 (freshly prepared) and centrifuged again. The pellet and supernatant fractions were analyzed by Western blotting.

Other methods

Protein concentrations were measured using the BCA method (Pierce) with 0.1% (w/v) SDS added and bovine serum albumin (BSA) as a standard. Total cell lysate for Western blotting was obtained according to [28]. Western blots were decorated with antibodies against the hemagglutinin tag (Boehringer), porin and the phosphate carrier Mir1 (both kindly provided by dr. R. Lill) and protein bands were visualized by ECL (Amersham) according to manufacturer's instructions. Protein bands were quantified with a BioRad Model GS-700 Imaging Densitometer. The phospholipid

compositions of mitochondria were analyzed by thin layer chromatography (TLC) as described [29].

Results

Effect of PC depletion on Gut2 function in vivo

Effect of PC depletion on the ability of yeast to grow on glycerol. In this study the hypothesis was tested, that PC is required for the function of Gut2, and hence for the ability of *S. cerevisiae* to grow on glycerol as carbon source. To vary the PC level, yeast cells of the *cho2opi3* strain SH922 were transferred to growth medium with or without choline. Cells cultured in the absence of exogenous choline cannot produce PC and therefore PC levels will drop during further growth [17,20,30]. In the presence of choline, cells have wild type PC levels. The media contained the fermentable carbon source glucose or the non-fermentable carbon sources lactate or glycerol. It was verified that a *gut2* strain with the same genetic background (Table I, PR01) did not grow on glycerol (data not shown).

In the presence of choline, regular growth curves were obtained for all conditions (Figure 1, squares). In the absence of choline, initially similar growth rates were observed on all three carbon sources (Figure 1, triangles). However, after approximately 10 h, the rate of growth decreased on all carbon sources compared to the + choline condition, demonstrating the importance of PC for the growth of yeast cells. The strongest decrease in growth rate was observed on glycerol, where cells virtually stopped growing after 13 h, whereas growth on the other carbon sources still continued at a low rate. At this

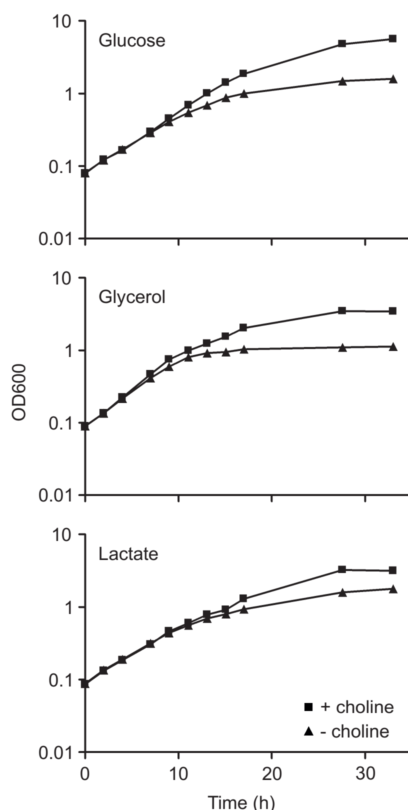

Figure 1 The effect of choline deprivation on the growth of a *cho2opi3* strain on different carbon sources. Strain SH922 was pre-cultured in SD, SG or SL C^+ to mid-log phase. Cells were collected by filtration, washed and transferred to fresh SD, SG or SL medium with (squares) or without (triangles) 1 mM choline, as described in the Materials and methods section. Growth was measured as the optical density at 600 nm at successive time points. Results from a typical experiment are shown.

time point, the PC level is estimated to be about 4% (based on the PC content being halved with each doubling of the number of cells, starting from 40% [30]). The decrease in growth rate on glycerol upon PC depletion points to a role of PC in Gut2 functioning. To further test our hypothesis, the glycerol excretion of mutants with varying PC levels was measured.

Effect of PC content on glycerol excretion. In yeast several systems exist that recycle cytosolic NADH, with the G3P shuttle generating the most ATP per oxidized NADH. The G3P dehydrogenase Gpd1 oxidizes NADH, to produce G3P. Subsequently, Gut2 converts G3P to DHAP and the reducing equivalents are passed on to the respiratory chain. It has been shown that blocking Gut2 function results in glycerol excretion, probably to get rid of accumulating G3P via dephosphorylation by Gpp1 and Gpp2 [14]. Hence, glycerol excretion is a measure of Gut2 dysfunction. It was investigated whether the glycerol production depends on the PC content of yeast cells. For this purpose *cho2* and *opi3* single deletion strains were used, because the rigorous conditions of PC depletion in the *cho2opi3* strain are not compatible with the assay of glycerol production. These strains have wild type levels of PC when grown in the presence of choline. However, in the absence of choline, the PC levels drop to about 9% and 4% of total phospholipid content in *cho2* and *opi3* strains, respectively [18,20]. The glycerol production by these strains cultured with or without choline is depicted in Figure 2, and is compared to the excretion by the wild type and a *gut2* strain with the same genetic background.

The figure shows that the deletion of the *GUT2* gene in the wild type strain results in a large increase in glycerol excretion in agreement with a previous report [14]. The excretion of glycerol by the wild type and the *gut2* strain was independent of the presence of choline in the medium, as expected because of their ability to synthesize PC via the methylation of PE. In the presence of choline the *cho2* and *opi3* strains have wild type levels of PC [18], but their glycerol excretion was somewhat less compared to that of the wild type strain. We speculate that this is due to the connection between

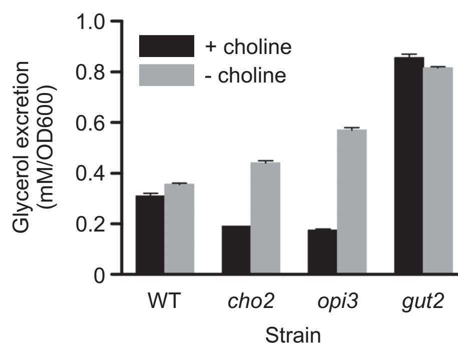

Figure 2 The effect of the PC content of yeast cells on the glycerol production. The *cho2*, *opi3* and *gut2* deletion strains and their parental wild type were grown on yeast nitrogen base, supplemented with 0.5% glucose (w/v) and 120 mg/L of each histidine, leucine, lysine and uracil in the presence (black bars) or absence (grey bars) of 1 mM choline, as described in detail in Materials and methods. The glycerol concentrations in the medium were measured after 48h and have been normalized to the OD600 values of the cultures. The error bars represent the variation in two experiments.

phospholipid and glycerol metabolism. However, in the absence of choline 2.3-fold more glycerol was excreted by the *cho2* strain than in the presence of choline and this increase was even higher (3.3-fold) for the *opi3* strain which has a lower PC content than the *cho2* strain in the absence of choline [17-20]. The observation that glycerol excretion is increased upon lowering the PC content further supports the suggestion that PC is needed for Gut2 activity *in vivo*.

Effect of PC depletion on Gut2 function in vitro

Dependence of Gut2 content and activity on PC content. The suggested reduction in Gut2 functioning in response to lower PC levels could be due to a reduction in content or to a decrease in enzyme activity. These possibilities were investigated using the *cho2opi3* strain PR03 containing a chromosomally encoded hemagglutinin tagged Gut2. PR03 cells, precultured in the presence of choline, were transferred to medium with or without choline and cultured for different periods. Analysis of cell lysates by Western blotting using an antibody against the hemagglutinin tag of Gut2, revealed that the Gut2 expression level was not affected by PC depletion. After 3 generations the Gut2 content in PC depleted mitochondria was 101% of the Gut2 content in mitochondria with a normal PC level (Figure 3A).To address the question whether the specific activity of Gut2 depends on PC, mitochondria with different PC contents were isolated from PR03 cells, cultured as above. The enzyme activity of Gut2 was measured in detergent solubilized mitochondria (Figure 3B, upper panel) and was linear in time. From the slopes a 1.8-fold reduction in enzyme activity was calculated (n = 3), after PC depletion for 3 generations. To exclude the possibility that the result of the activity assay was affected by disruption of functional interactions between Gut2 and PC, the Gut2 activity assay was carried out in the absence of this detergent and in the presence of D-sorbitol to obtain an isotonic buffer. In intact mitochondria a similar reduction of activity upon PC depletion was found (Figure 3B, lower panel), also indicating that the substrate accessibility was not affected by PC depletion.

It should be noted, that the activities were determined for Gut2 that had been tagged with His_6HA for quantification purposes. To investigate the influence of the tag, the activity of Gut2 was also measured in mitochondria from the SH922 strain containing untagged Gut2. It was found that the introduction of the His_6HA tag caused a reduction in specific activity from 50.0 ± 4.4 to 17.3 ± 2.7 nmol min^{-1} mg^{-1} (average ± SD, n = 5) in Triton X-100 solubilized mitochondria from cells grown in the presence of choline. However, the tagging of Gut2 did not affect the apparent dependence of Gut2 activity on PC. Upon PC depletion a similar reduction in Gut2 enzyme activity was found in the SH922 strain (data not shown) as in the PR03 strain (Figure 3B).

The decrease in Gut2 activity in mitochondria after PC depletion could be due to a

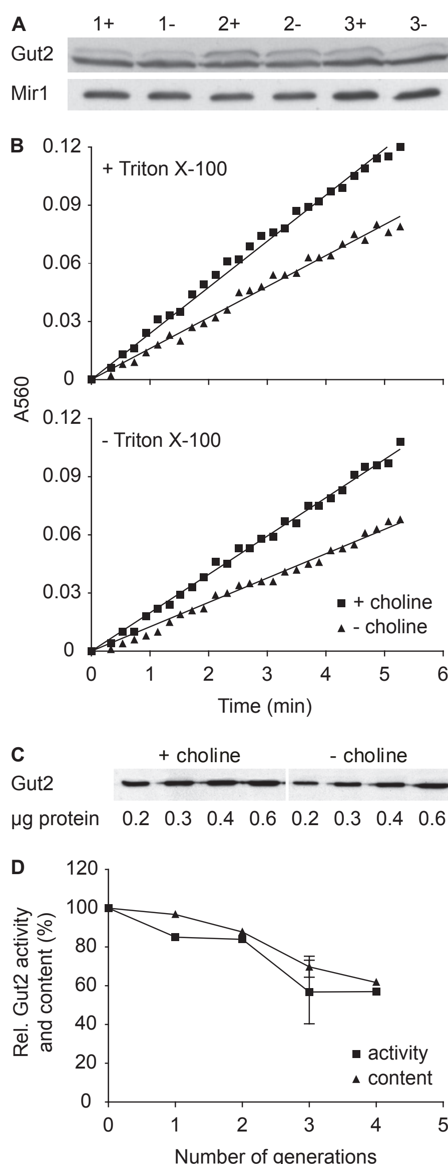

Figure 3 The effect of PC depletion on the activity and content of Gut2 in the *cho2opi3* strain PR03. (A) Gut2 content in total cell lysates. Cells were lysed after growth for the indicated number of generations (1-3) in the presence (+) or absence (-) of 1 mM choline. The Gut2 content in the cell lysate was quantified by Western blotting using an antibody against the hemagglutinin tag of Gut2, with Mir1 as a loading control (visualized using an antibody against Mir1). The faint upper band might represent precursor Gut2, based on the difference in mass and the visualization by the antibody against the hemagglutinin tag. (B) Gut2 activity in isolated mitochondria. Mitochondria were isolated from cells grown for 3 generations in the presence (squares) or absence (triangles) of 1 mM choline. The Gut2 activity was measured in detergent solubilized (upper panel) and intact (lower panel) mitochondria in 96-well plates, as described in Materials and methods. The reduction of the indicator MTT was followed spectrophotometrically at 560 nm. Results of a typical experiment are shown. (C) Quantification of the Gut2 content in mitochondria from cells harvested after growth for 3 generations in the presence or absence of 1 mM choline, using an antibody against the tag of Gut2. Different amounts of mitochondrial protein were loaded as indicated. (D) The influence of choline deprivation on the activity and content of Gut2 in isolated mitochondria from *cho2opi3* strain PR03. Mitochondria were isolated from PR03 cells grown for different numbers of generations in the presence or absence of 1 mM choline. The Gut2 activity in detergent solubilized mitochondria, corresponding to 30 µg mitochondrial protein, and the mitochondrial Gut2 content were determined as described in detail in Materials and methods. The levels of Gut2 activity and Gut2 content in PC depleted mitochondria are depicted relative to the levels in mitochondria isolated from cells grown in the presence of choline, for the corresponding number of generations. The error bars at 3 generations represent the SD ($n \geq 3$).

decrease in specific activity of the enzyme or to a decrease of the Gut2 content in the mitochondrial preparations (or a combination of both). To distinguish between these possibilities, the relative amounts of tagged Gut2 per mg mitochondrial protein were determined by Western blotting (Figure 3C). The blot suggests that there is less Gut2 in the mitochondria after PC depletion. To investigate the correlation

between the decreases in Gut2 activity and content in greater detail, mitochondria were isolated at different stages of PC depletion. The mitochondria were analyzed for Gut2 activity after solubilization in Triton X-100 and for Gut2 content (Figure 3D). Upon PC depletion for 3 generations the Gut2 activity was found to drop to 57 ± 16% of the activity found in mitochondria from cells grown in the presence of choline for the same number of generations. The decline of the amount of Gut2 per mg mitochondrial protein almost coincided with the reduction in Gut2 activity, suggesting that the specific activity of Gut2 is not affected by PC depletion, not even when the mitochondrial PC content has dropped from 42% to 2% of total phospholipids after PC depletion for 4 generations (determined by TLC, data not shown).
As mentioned above, the Gut2 expression level was constant in total cell lysates during PC depletion, which contrasts the observed decline of Gut2 content in isolated mitochondria under the same conditions. A possible explanation is that the purity of the mitochondrial preparations is influenced by PC depletion. Since mitochondria obtained by differential centrifugation are usually contaminated by endoplasmic reticulum (ER) [25,31], it was investigated whether PC depletion increases the extent of mitochondrial contamination by ER. Mitochondria were isolated from PR03 cells after 4 generations of growth in the presence or absence of choline and subjected to Western blotting, using antibodies against Sec61 and porin, a mitochondrial and an ER marker, respectively. As a control, the Sec61/porin ratios were determined in total cell lysates.

Table II Sec61/porin ratios in total cell lysates and isolated mitochondria from PR03 cells, grown in the presence or absence of 1 mM choline for 3 generations. The proteins were quantified by Western blotting using antibodies against Sec61 and porin.

	[choline] (mM)	
Sample	*1*	*0*
Cell lysate	0.91	0.96
Mitochondria	1.12	1.54

The results shown in Table II demonstrate that the Sec61/porin ratio is strongly increased in mitochondria upon PC depletion, reflecting a stronger contamination with ER of the mitochondrial preparations. The increase in ratio of about 30% is consistent with the observed decrease in Gut2 content and suggests that the mitochondrial Gut2 content is not affected by PC depletion.
In summary, depleting the mitochondria of PC does not affect the specific activity of Gut2. The possible influence of PC on Gut2 activity was further investigated by measuring the effect of PC addition on the enzyme activity.

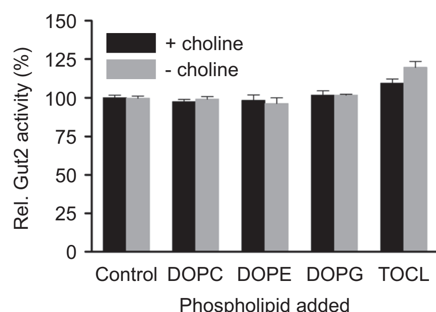

Figure 4 The influence of different phospholipids on Gut2 activity. The indicated phospholipids were added to an at least 6-fold molar excess over the corresponding mitochondrial phospholipid (based on [25]) to Triton X-100 solubilized mitochondria from *cho2opi3* cells grown in the presence (black) or absence (grey) of 1 mM choline for 4 generations. The mixture was incubated for 15 min at room temperature and subsequently the Gut2 activity was measured using the 96-well plate assay as described in the Materials and methods section. The activity in the control (no lipids added) was set at 100%. The error bars represent the SD (n = 3).

Influence of phospholipids on Gut2 activity. Detergent solubilized mitochondria were incubated with excess detergent solubilized lipids and the Gut2 activity was measured (Figure 4). The activity was not influenced by any of the phospholipids tested, irrespective of the PC content of the solubilized mitochondria, except for a small stimulation by CL. The stimulatory effect of CL on Gut2 activity may suggest a role for CL in Gut2 activity. Therefore, mitochondria were isolated from a *crd1* mutant strain (FGY2), lacking CL synthase activity and CL. The Gut2 activity in these mitochondria was found to be comparable to the activity in mitochondria of the parental wild type strain (FGY3), demonstrating that CL is not required for Gut2 activity (data not shown).
The absence of a stimulatory effect of exogenous PC on the activity of Gut2 in detergent solubilized, PC depleted mitochondria indicates that either the activity of Gut2 itself is not dependent on PC or that there is still sufficient PC left to fulfill a possible PC requirement of Gut2 activity.

Effect of PC content on binding of Gut2 to the mitochondrial membranes. To investigate whether the reduced PC content resulted in Gut2 release from the membrane and in this way affected the activity per Gut2 molecule, the binding of Gut2 to the membrane was studied by washing mitoplasts with carbonate and salt.
Mitochondria from *cho2opi3* cells PR03 cultured for 4 generations in the presence or absence of 1 mM choline were isolated, and processed to mitoplasts by disruption of the outer membrane by osmotic shock. This treatment did not induce significant release of Gut2 from the mitoplasts (data not shown). The mitoplasts were washed in hypotonic buffer, or in buffers containing NaCl or sodium carbonate, in order to determine whether PC depletion affects the membrane association of Gut2. After centrifugation the distribution of Gut2 and the integral outer membrane protein porin over the pellet and supernatant fractions was determined by Western blotting (Figure 5). As expected for an integral membrane protein, porin was recovered exclusively in the pellet fractions under all conditions tested. After washing with the

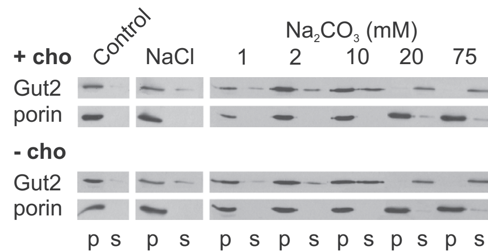

Figure 5 The effect of PC depletion on the association of Gut2 with mitoplasts. Mitoplasts were prepared by diluting mitochondria from *cho2opi3* cells (strain PR03) grown for 4 generations in the presence (+ cho) or absence (- cho) of 1 mM choline, in hypotonic buffer. After washing with buffer H the mitoplasts were incubated for 5 min in buffer H (control) or in buffer H containing 1 M NaCl or Na_2CO_3 at the concentrations indicated. The mitoplasts were spun down and the amount of Gut2 and porin in the pellet (p) and supernatant (s) fractions was determined by Western blotting, using an antibody against the hemagglutinin tag of Gut2 and against porin, respectively. Results from a typical experiment are shown.

hypotonic buffer all Gut2 was found in the pellet fraction. Washing with 1 M NaCl induced the release of a small portion of Gut2 in the supernatant, irrespective of the PC content of the mitoplasts. The gradual dissociation of Gut2 from the membrane by increasing concentrations of Na_2CO_3 was not affected by the PC content of the mitoplasts either. This suggests that PC does not regulate Gut2 enzyme activity via the association with the membrane. The complete release of Gut2 at low carbonate concentrations (20 mM) indicates that Gut2 is a peripheral membrane protein, in agreement with earlier findings [11].

Discussion

In this study the possible influence of PC on the functioning of Gut2 was investigated by *in vivo* and *in vitro* approaches, using yeast mutant strains allowing controlled depletion of the PC level.

The results obtained *in vivo*, the abrogation of growth on glycerol of *cho2opi3* cells upon choline deprivation and the glycerol production by *cho2* and *opi3* cells cultured in the absence of choline, strongly suggest that Gut2 requires PC for efficient functioning. Using different *in vitro* approaches it was tried to elucidate the underlying mechanism. It appeared that the Gut2 expression level in total cell lysate and the specific activity of Gut2 in mitochondria were not affected by PC depletion, rendering it unlikely that PC is required for the activity of the enzyme. The latter finding was confirmed by the absence of a stimulatory effect of the addition of phospholipids, including PC, on Gut2 activity in a micellar system, derived from PC depleted mitochondria. No differences in Gut2 activity were observed between detergent solubilized mitochondria and intact mitochondria, suggesting that also the supply of substrate for Gut2 is not responsible for the effects of PC depletion *in vivo*. Finally, the membrane association of Gut2 was identical in mitoplasts with normal or low PC contents, rendering a regulatory mechanism via membrane

docking unlikely. Our finding that Gut2 is a peripheral membrane protein contrasts a recent report, in which Gut was classified as an integral outer membrane protein [32], based on the partitioning of Gut2 into the detergent phase in a Triton X-114 phase separation experiment performed on isolated mitochondrial outer membrane vesicles. Previously, it was thought that Gut2 is located at the outside of the inner membrane [16], consistent with our earlier finding that Gut2 is not crosslinked upon incubating isolated outer membranes with TID-PC [11]. The apparent discrepancies in submitochondrial localization and in the nature of membrane association might be explained by a dual localization of Gut2. Alternatively, and more likely in view of the molecular weight of Gut2 reported by Burri *et al.* [32], these authors may have mistaken Gut2 for its precursor, which has recently been shown to accumulate in the outer membrane of yeast mitochondria [33].

How can we understand the effect of the PC content on Gut2 functioning *in vivo*? It should be realized that Gut2 activity *in vivo* is influenced by many factors such as the concentrations of nucleotides [34-36] and the activity of other enzymes involved in electron transfer, like the NADH dehydrogenase Nde1 [34,36]. It is conceivable that some of these factors are lost during the isolation of the mitochondria thereby masking possible effects of PC depletion in the *in vitro* Gut2 activity assay.

Another reason for the effects observed *in vivo* not being reflected in the *in vitro* experiments might be related to metabolic compartmentation. Enzymes involved in a specific process are often clustered in complexes or are enclosed by a membrane. This can avoid competition for substrates with other processes and can speed up reactions because enzymes ‘sense’ higher substrate concentrations and because reaction intermediates do not have to diffuse from one protein to the next. Compartmentation is well established for enzymes involved in bioenergetics and might apply as well to Gut2, which has been shown to be part of a supermolecular complex [37]. This complex is in tight contact with the mitochondrial inner membrane, as it is composed of proteins localized to both sites of this membrane and contains integral membrane proteins as well. Depletion of an important membrane building block, PC, might disturb the structure of this complex, hindering the transport of substrates or products. This might have been masked under the conditions of the *in vitro* assays by the ample supply of substrate and electron acceptor, and the uncoupling of the reaction from the respiratory chain used to avoid influences by other processes. An indication that the structural organization of cellular compartments after PC depletion is altered, comes from our observation that mitochondrial preparations of cells grown for 4 generations in the absence of choline, are more contaminated with ER.

These explanations for the lack of support from the *in vitro* experiments for the observations *in vivo* indicate that the apparent role of PC in Gut2 functioning results indirectly from changes induced by PC depletion, and not from a direct influence of PC on Gut2 enzyme activity.

The basis for this study was the observation that incubation of yeast mitochondria with TID-PC followed by photoactivation resulted in prominent labeling of the Gut2 protein [11]. This labeling was not the result of a preference of the TID group for Gut2, but reflects a preferential interaction of PC with this protein [11]. In the light of the results in the present study we suggest that under wild type conditions PC is involved in binding Gut2 to the membrane. Upon PC depletion binding is maintained, which suggests that either the residual PC (2% of total phospholipid) is sufficient for binding or that other phospholipids take over the role in binding. This would imply that hydrophobic interactions are important for the membrane association of Gut2, which is supported by the observation that even at 1 M NaCl the binding of Gut2 to the membrane was hardly affected.
To be able to bind hydrophobically to the membrane, Gut2 requires hydrophobic patches on its surface. The identification of these patches is complicated, because no atomic structure of Gut2 or of a homologue has been published. Analysis of Gut2 by a hydrophobic moment plot [38] and the secondary structure prediction program Jpred [39] suggested the presence of several amphipathic helices (detailed in the appendix) of which the hydrophobic sides could be involved in lipid binding. It is also known that G3P dehydrogenases from other sources are influenced by membrane lipids with a rather broad specificity [40-42], which is consistent with a prominent role of hydrophobic interactions between Gut2 and the membrane.

Acknowledgement

This research was supported by an Earth and Life Sciences (ALW) grant with financial aid from the Dutch Organization for Scientific Research (NWO).

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Appendix Calculation of the secondary structure propensities of Gut2 for membrane interaction

The release of Gut2 from mitoplasts upon carbonate wash, as observed in this study, indicates that Gut2 is a peripheral membrane protein. This contrasts the results from Triton X-114 extraction [32] discussed above. In addition, it contrasts the results of calculations with the transmembrane (TM) prediction tool TMpred [43,44], and with the dense alignment surface (DAS) algorithm [32,45], which indicate that Gut2 is an integral membrane protein with two TM helices. We have reanalyzed the occurrence of TM segments in Gut2 by the TMpred and DAS methods and observed that they do not agree on the position and on the length of the proposed TM helices (data not shown). Moreover, it is known that well-established globular proteins are often classified as integral membrane proteins by TM prediction tools [46]. Therefore, we analyzed the primary structure of Gut2 using methods that are more reliable than TMpred and DAS [47,48] and a newly developed method, MINNOU [49]. The PRO-TMHMM [48], HMMTOP [50,51]) and MINNOU [49] algorithms did not propose any TM helices in Gut2 (data not shown), in agreement with the conclusion

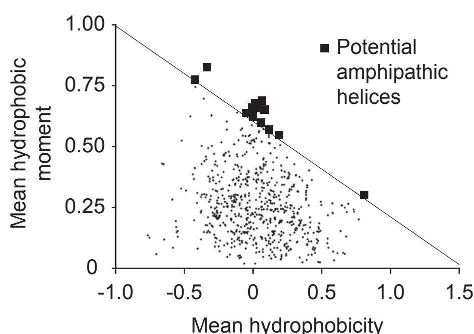

Figure 6 Hydrophobic moment plot of Gut2. For a sliding window of 11 residues the mean hydrophobic moment was calculated and plotted *vs.* its mean hydrophobicity. In case the hydrophobic moment exceeds a threshold (the oblique line), these 11 residues are predicted to be part of an amphipathic helix as indicated by the squares.

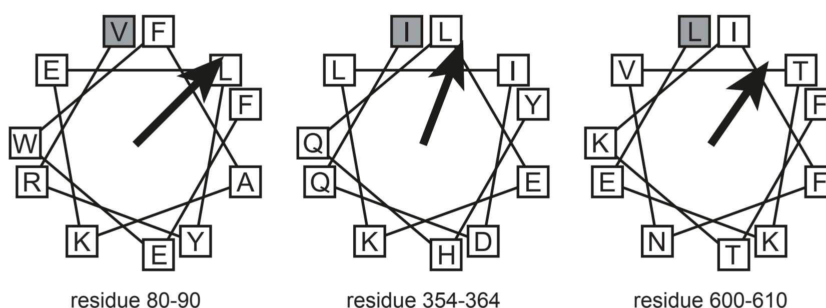

Figure 7 Helical wheel representation of potential amphipathic helices in Gut2. The length and direction of the arrow represent the hydrophobic moment of the indicated 11 residues. The N-terminal amino acids in these windows are depicted in the grey boxes.

from the carbonate extraction that Gut2 is a peripheral membrane protein. To improve the accuracy of PRO-TMHMM and HMMTOP [48], Gut2 was also analyzed together with its homologues, obtained by a BLAST search (E-value ≤ 10^{-5} [52]) in the non-redundant SwissProt database. Again no TM helices were found.
Many extrinsic membrane proteins interact with membranes via amphipathic helices. A method to predict amphipathic helices based on the amino acid sequence is by plotting the mean hydrophobic moment of amino acids *vs.* their mean hydrophobicity, using a window of 11 residues as has been described [38]. As shown in Figure 6, Gut2 contains several potential amphipathic helices according to this method.
Using the secondary structure prediction program Jpred [39], it was checked whether the candidate sequences were likely to adopt an α-helical structure. This narrowed down the number of potential amphipathic helices to the three depicted in Figure 7.
These calculations indicate that Gut2 contains amphipathic helices that could interact hydrophobically with the membrane, in agreement with the experimental data.

CHAPTER 3

Phosphatidylcholine is essential for efficient utilization of proline as nitrogen source in *S. cerevisiae*

Pieter J. Rijken, Ben de Kruijff and Anton I.P.M. de Kroon

Abstract

In a recent mRNA profiling study aimed at the identification of potentially functional relationships between proteins and phosphatidylcholine (PC), it was found that the mRNA levels of the *PUT1* and *GAP1* genes are influenced by the cellular PC content (M.C. Koorengevel *et al.*, unpublished results). This finding, combined with other observations from the literature, prompted us to analyze whether PC plays a role in the utilization of proline as a nitrogen source by *S. cerevisiae*. Depletion of the PC content in yeast PC biosynthetic mutants was found to specifically arrest growth on proline, indicating that PC is required for proline utilization *in vivo*. The underlying mechanism of this requirement was investigated, revealing that PC depletion affected both proline uptake and proline metabolism. The results suggest that the reduced rate of proline uptake is responsible for the growth defect of PC-depleted cells on proline. We speculate that PC depletion specifically affects the transport of amino acid transporters to the plasma membrane.

Introduction

PC is an abundant phospholipid building block of eukaryotic membranes. In addition, PC and its metabolism are involved in several cellular processes, such as the regulation of vesicular transport from the Golgi ([1-3] and references therein), and signal transduction (reviewed in [4,5]). These specific functions most likely require functional interactions with proteins as illustrated by the PC-dependence of β-hydroxybutyrate dehydrogenase activity [6,7].

Much of the current knowledge about the roles of PC in eukaryotic cells has been gained by using *S. cerevisiae* as a model system. This organism mainly synthesizes PC by the triple methylation of phosphatidylethanolamine (PE), catalyzed by the methyltransferases Cho2 (Pem1) and Opi3 (Pem2) [8]. The alternative route for PC synthesis, the CDP-choline or Kennedy pathway, recycles choline from PC turnover [9], and only contributes to net PC synthesis when choline is available in the growth medium [9,10]. The capacity of either route is sufficient to reach wild type PC levels, *i.e.*, 40-50% of the total phospholipid content [11].

It is known, that yeast cells with a defective PC biosynthesis have problems in maintaining their respiratory competence [12], which might be due to impaired PC-protein interactions, *e.g.*, in the cytochrome bc_1 complex [13]. However, at present the insight into functional interactions of PC with mitochondrial proteins in yeast is still very limited. To find such interactions, isolated yeast mitochondria were probed with a photoactivatable PC analogue [14]. One of the predominantly labeled proteins was the glycerol-3-phosphate dehydrogenase Gut2, and further research indicated

that PC is important for efficient functioning of this protein [15].
An alternative approach to obtain clues for functional PC-protein interactions is to monitor the cellular responses to a lowering of the cellular PC content at the level of gene transcription. A recent transcript profiling study revealed that PC depletion influenced the mRNA levels of many genes. An interesting gene was *PUT1* for which a two-fold increase of the mRNA level was observed (M.C. Koorengevel *et al.*, unpublished results). The encoded proline oxidase Put1 protein resides in the mitochondrial matrix, and is part of the pathway that allows yeast cells to use proline as a nitrogen source. Proline is converted by the FAD-dependent Put1 into Δ^1-pyrroline-5-carboxylate (P5C) [16-18]. Subsequently, the ring of P5C is broken by a spontaneous hydrolysis reaction, resulting in glutamate semialdehyde. The NAD^+-dependent P5C dehydrogenase Put2 then produces glutamate by oxidizing this semialdehyde (Figure 1). The produced glutamate serves as an intracellular nitrogen donor in biosynthetic reactions [19-21], or is further processed by the Central Nitrogen Metabolism (CNM) to glutamine, another intracellular nitrogen donor [22-24]. The proline utilization pathway is of special importance to yeast, as proline is the most abundant nitrogen source in grapes, a natural habitat of *S. cerevisiae* [25].

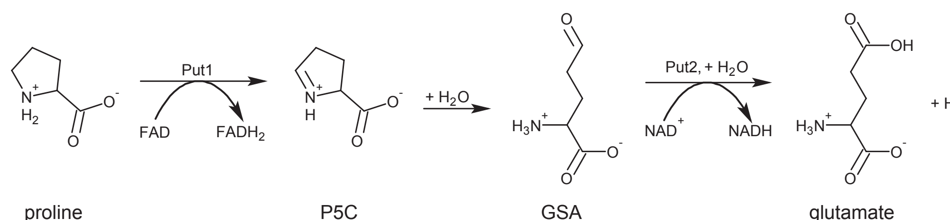

Figure 1 Proline utilization pathway in *S. cerevisiae* for the conversion of proline into glutamate. Abbrevations: $FAD(H_2)$ – (reduced) flavin adenine dinucleotide; GSA – glutamate semialdehyde; NAD^+ - nicotinamide adenine dinucleotide (NADH when reduced); P5C – Δ^1-pyrroline-5-carboxylate.

The general amino acid permease Gap1 and the high affinity proline transporter Put4 are both located in the plasma membrane, and facilitate the uptake of proline under conditions where cells use proline as their source of nitrogen [26-31]. Interestingly, the transcription of the gene encoding the Gap1 protein was also found to be increased two-fold in PC-depleted cells (M.C. Koorengevel *et al.*, unpublished results).
The transcriptional analysis thus suggests that PC may play a role in proline metabolism. Consistent with this idea, it was found that yeast cells cultured in the presence of choline, a precursor of PC in the CDP-choline route, also have altered mRNA levels of *PUT1* and *NPR2* [32], the latter gene encoding a regulator of proline import [33]. Moreover, changes in cellular PC and PE levels have been shown to be accompanied by changes in the cellular uptake capacity for several amino acids, including proline [34,35]. In addition, ongoing PC synthesis is required for the

repression of genes containing the inositol-sensitive upstream activating sequence UAS_{INO} in their promoter, which is induced when cells are transferred to a low quality nitrogen source, like proline [36]. PC might also influence proline metabolism through its importance for the respiratory competence [12] as mentioned above, as both Put1 and Put2 rely on a functional respiratory chain for their functioning [16,17].
These observations and considerations prompted us to investigate the role of PC in proline metabolism. To manipulate the cellular PC content an *opi3* mutant strain was used. This strain has a defective PE methylation pathway, and requires exogenous choline to reach wild type PC levels through the CDP-choline route [37-39]. By depriving this strain of choline, the cellular PC content can be lowered from 40% to less than 5% of total phospholipids [38].
This study provides evidence for a role of PC in the utilization of proline as a nitrogen source. The results indicate that PC is important for the efficiency of both proline uptake and proline metabolism.

Materials and methods

Materials

L-[U-^{14}C]proline (0.256 Ci/mmol), L-[5-^{3}H]proline (28 Ci/mmol), L-[G-^{3}H]glutamic acid (49 Ci/mmol) and D-[U-^{14}C]glucose (0.270 Ci/mmol) were obtained from Amersham Biosciences Benelux (Roosendaal, The Netherlands). The pU6H3HA plasmid and the antibody against Mir1 were kind gifts of dr. A. De Antoni and dr. R. Lill, respectively. The antibody against the hemagglutinin epitope was acquired from Boehringer (Mannheim, Germany). Millipore HAWP MF™ filters were purchased from Millipore (Amsterdam, The Netherlands). HPTLC silica gel 60 plates were bought from Merck (Darmstadt, Germany). All other chemicals were at least analytical grade.

Yeast strains, media and culture conditions

The yeast strains listed in Table I were maintained on YPD (1% yeast extract, 2% bactopeptone and 2% glucose). The synthetic media contained per liter: 1 g of potassium dihydrogenphosphate, 0.5 g of magnesium sulfate, 0.1 g of sodium chloride, 0.1 g of calcium chloride, 0.5 mg of boric acid, 0.04 mg of cupric sulfate, 0.1 mg of potassium iodide, 0.2 mg of ferric chloride, 0.4 mg of manganese sulfate, 0.2 mg of sodium molybdate, 0.4 mg of zinc sulfate, 20 mg of histidine, 230 mg of lysine, 40 mg of uracil, 2 µg of biotin, 400 µg of panthothenate, 2 µg of folic acid, 400 µg of niacin, 200 µg of *p*-aminobenzoic acid, and 400 µg of pyridoxine hydrochloride [40]. As nitrogen source 1 g proline (Pro medium), or 1 g glutamate (Glu medium) was added per liter, unless indicated otherwise. Galactose (5 g per liter) served as

carbon source [41]. The Glu medium was adjusted to pH 6.1, corresponding to the pH value of the Pro medium, using KOH. Media were supplemented with 75 µM inositol and where indicated with 1 mM choline. Typically, cells were grown overnight in YPD to late-log phase, and collected by centrifugation. After washing the cells twice by resuspension in water and centrifugation, the cells were resuspended in water and transferred to fresh synthetic Pro or Glu medium with or without 1 mM choline, to an initial OD600 value as indicated. All cultures were grown aerobically at 30 °C. Growth was monitored by measuring the OD at 600 nm, using a Unicam Helios Epsilon spectrophotometer.

Table I Genotypes of yeast strains used in this study

Strain	*Genotype*	*Source*
opi3	BY4742 *opi3::LEU2*	[69]
PR21	BY4742 *opi3::LEU2 PUT1-6H3HA-KanMX*	this study
PR22	BY4742 *opi3::LEU2 PUT2-6H3HA-KanMX*	this study

Epitope tagging of Put1 and Put2

Put1 was epitope-tagged at its C-terminus by inserting a histidine (His_6) and a hemagglutinin (HA_3) tag at the 3’ end of the coding strand in the genome, via homologous recombination with a PCR fragment also containing the *kan*MX cassette.

Table II Primers used in this study[1]

Primer	*Sequence*
1	5’-CAA GGC CAT AGC AAA GTC GAT TCC AAA AAG AGT AGG CCT ATC CCA CCA CCA TCA TCA TCA C-3’
2	5’-TAT AAT ATG TAT AAC TAG AAC ATC AGA ATT ATG TAT GCA TAC ACT ATA GGG AGA CCG GCA GAT C-3’
3	5’-CTT CTA CGA GTT GAC TGA TTT CAA ATA TCC ATC GAA TTA TGA ATC CCA CCA CCA TCA TCA TCA C-3’
4	5’-GAT AAT CTA ACA TTT ACA TGA GTT GAT GGA TGA AGT GAC TGA CTA TAG GGA GAC CGG CAG ATC-3’

[1] The underlined sequences correspond to fragments of the yeast chromosomal DNA, *i.e.*, for primer 1, nucleotides 1389-1428 of the *PUT1* ORF; for primer 2, nucleotides 61-102 downstream of the *PUT1* ORF (reverse-complementary); for primer 3, nucleotides 1683-1725 of the *PUT2* ORF; and for primer 4, nucleotides 31-71 downstream of the *PUT2* ORF (reverse-complementary).

This fragment was created by PCR using the plasmid pU6H3HA [42] as a template and primers 1 and 2 (Table II). The PCR product of 1817 bp was used to transform the yeast strain *opi3* and transformants were selected on G418-containing plates as

described [42]. Correct integration of the PCR fragment in the resulting strain PR21 was verified by colony PCR.
Put2 was tagged accordingly, using primers 3 and 4 (Table II), which yielded a PCR product of 1819 bp. The *opi3* strain with this PCR fragment correctly inserted was designated PR22.
Expression of the Put1-His_6HA_3 and Put2-His_6HA_3 constructs in PR21 and PR22, respectively, was verified by Western blotting, using an antibody directed against the hemagglutinin tag.

Phospholipid analysis

Cells of the *opi3* strain precultured on YPD were used to inoculate Pro and Glu media with or without choline to an initial OD600 of ~0.1. At different time points, aliquots corresponding to 50 OD units were harvested by centrifugation and freeze-dried. The method used for the subsequent lipid extraction method was based on [43]. Briefly, 10 mg of the freeze-dried cells was added to 3 mL of chloroform/methanol (2:1 v/v). The suspension was sonicated for 20 min in a bath sonicator (Branson B1200, Bransonic Ultrasonics, Danbury, CT, USA), and 1 mL of water was added. The sample was shaken vigorously for 1 min, and incubated on ice for 15 min. The lower organic layer was collected after centrifugation for 10 min at 1000 *g*. Residual lipids in the upper layer were extracted with 3 mL fresh chloroform/methanol (2:1 v/v), and the combined organic phase was evaporated to dryness under a stream of nitrogen.
To determine the phospholipid composition, the lipid extracts dissolved in chloroform/methanol (2:1 v/v) were separated by TLC based on [44]. Briefly, HPTLC plates were impregnated with 1.8% (w/v) boric acid in ethanol, dried for 5 min, and baked for 10 min at 115 °C prior to use. After applying the lipid extracts, corresponding to ~150 nmol phosphate as determined according to [45], the plates were developed in chloroform/ethanol/water/triethylamine (30/35/7/35 v/v/v/v). The phospholipids were visualized by spraying the plates with 10% (v/v) sulphuric acid, followed by gentle heating on a hot plate. The lipids were scraped off and their quantities were determined as described [45].

Cellular uptake of proline, glutamate and glucose

The *opi3* cells were cultured in Glu medium with or without choline to OD600 0.5-1.0, and harvested by centrifugation (8 sec at 15,000 *g*). In case of choline deprivation, cells had been grown for about 3 cell divisions. The procedure to monitor the uptake of proline, glutamate and glucose was based on [34]. Briefly, cells were washed once with buffer U (50 mM phosphate pH 6.3, 2 mM magnesium sulfate and 0.5% (w/v) galactose), resuspended in this buffer (at OD600 2-2.5), and incubated for 1 h at 30

°C while shaking. The cell suspension was supplemented with 0.1% (w/v; 8.7 mM) proline (doped with ^{3}H-proline to a specific radioactivity of 2.3 mCi/mmol), with 0.1% (w/v; 6.8 mM) glutamate (doped with ^{3}H-glutamate to a specific radioactivity of 2.0 mCi/mmol), or with 1 mM glucose (doped with ^{14}C-glucose to a specific radioactivity of 2.7 mCi/mmol), and incubated at 30 °C while shaking. Aliquots of 100 µL were withdrawn at the indicated timepoints, and cells were collected by filtration using Millipore HAWP MF™ filters. The extracellular radioactivity was removed by washing the filters with buffer U. Filters were dissolved in 4 mL of Insta-Gel Plus scintillation cocktail (Packard BioScience), and the radioactivity was counted using a Tri-Carb 2300TR liquid scintillation analyzer (Packard BioScience).

Determination of the cellular proline content

The *opi3* strain was cultured in Pro medium with or without choline to an OD600 of ~0.5. In case of choline deprivation, cells were grown for approximately 1.5 cell divisions. Cells (2-3 OD units) were harvested by centrifugation, washed with water and resuspended in 150 µL water. The cells were then boiled for 10 min to open them up [46]. Cellular debris was removed by centrifugation (10 min at 14,000 *g*), and the supernatant was freeze-dried to preserve the sample. The amino acids in the sample were derivatized with 9-fluorenylmethylchloroformate and proline was quantified after separation from the other amino acids by reversed phase HPLC as described [47].

In vivo proline metabolism

Cells of the *opi3* strain precultured on YPD were used to inoculate 2 mL Pro medium with or without choline to an initial OD600 of ~0.2. When the *opi3* cells had grown for approximately 1.5 cell divisions, the cultures were supplemented with 10 µCi ^{14}C-proline. After 30 min the cells were pelleted (8 sec at 15,000 *g*), washed with water, and resuspended in 10 µL water. The cell suspension was incubated for 30 sec at 99 ºC and after cooling down on ice, the cellular debris was pelleted (1 min at 15,000 *g*) and the supernatant was applied to an HPTLC plate and developed with a solution of phenol in diluted acetic acid (75 g phenol in 25 mL water with 5 mL acetic acid) [48]. The distribution of the radioactive label over the different spots on the TLC plate was quantified using a phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA) and a BioRad Molecular Imager FX. Glutamate and proline run as reference samples were visualized by spraying the plate with 0.1% (w/v) ninhydrin in isopropanol, followed by baking at 115 °C.

Other methods

Total cellular protein extracts from aliquots corresponding to 1.1 OD units were obtained, and analyzed by SDS-PAGE as described [49], which was followed by Western blotting, using antibodies directed against the hemagglutinin tag or against the mitochondrial phosphate carrier Mir1. Protein bands were visualized by ECL (Amersham) according to the manufacturer's instructions. The resulting films were quantified with a BioRad Model GS-700 Imaging Densitometer.

Results

Effect of PC depletion on the ability of yeast to grow on proline

To gain insight into the possible role of PC in the proline utilization pathway, the mutant strain *opi3* was used, which has a defective PE methylation route. This strain has wild type PC levels when choline is supplied in the medium, whereas choline deprivation after preculture in the

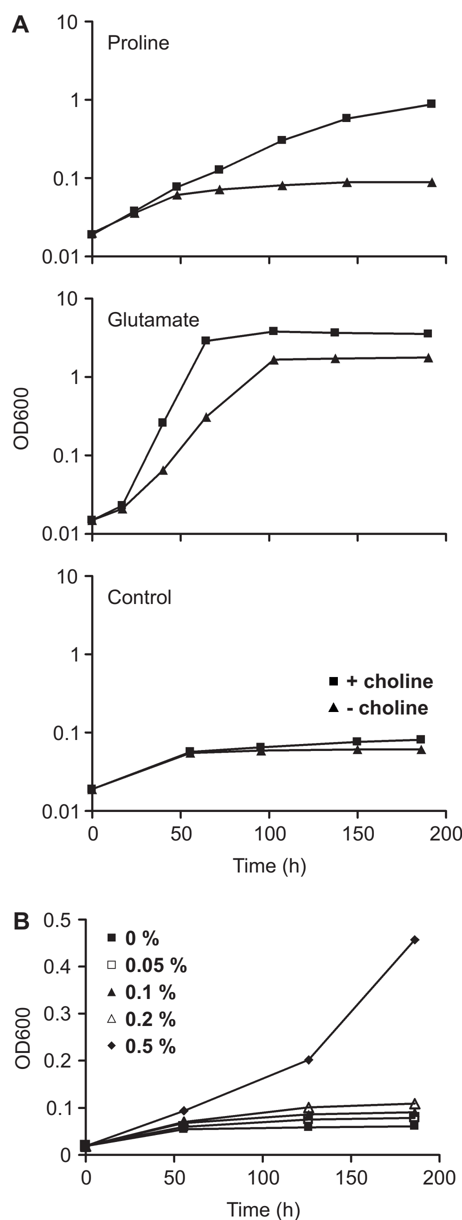

Figure 2 (A) The effect of PC depletion on the growth of the yeast *opi3* strain on different nitrogen sources. The *opi3* strain was cultured on Pro (upper panel), Glu (middle panel) or control medium (without glutamate or proline; lower panel), in the presence (squares) or absence (triangles) of 1 mM choline. Cells were precultured on YPD and transferred to the indicated media as described in Materials and Methods. Growth was monitored by measuring the OD at 600 nm. Results from a typical experiment are shown. (B) Dependence of growth of the *opi3* strain in the absence of choline on the concentration of proline. The *opi3* strain was precultured in YPD and transferred to Pro medium devoid of choline, containing increasing concentrations of proline (w/v) as indicated. Growth was monitored by measuring the OD at 600 nm. Results from a typical experiment are shown.

presence of choline leads to a decline in PC content during ongoing growth.
If PC is important for the functioning of the proline utilization pathway, PC depletion would be expected to affect growth on proline as sole nitrogen source more strongly than growth on glutamate, the product of the proline utilization pathway. To test this, the *opi3* strain was precultured in YPD and transferred to medium with either proline or glutamate as sole nitrogen source, and with or without exogenous choline (Figure 2).
In the presence of choline the *opi3* strain grew logarithmically on Pro medium, until reaching stationary phase (Figure 2A, upper panel), indicating that the cells were able to use the exogenously supplied proline as nitrogen source. Growth of the *opi3* cells on Pro medium was slow with a generation time of more than 24 h, consistent with the known slow growth of S288C-derived strains on proline [50]. During choline deprivation on Pro medium, the growth rate was comparable to the + cho situation for about 50 h and then growth abruptly ceased. On Glu medium containing choline (Figure 2A, center panel), a higher growth rate was observed than on Pro medium, consistent with glutamate being a more preferred nitrogen source than proline [50,51]. Here, the absence of choline had much less effect, with growth only being retarded compared to the + cho situation. The ability of the *opi3* strain to grow on glutamate in the absence of choline suggested that the growth arrest on Pro medium devoid of choline was caused by a specific role of PC in proline utilization, upstream of glutamate. In the absence of proline or glutamate, hardly any growth of the *opi3* strain was observed (Figure 2A, lower panel), suggesting that only minor amounts of nitrogen containing compounds were present, that most likely had accumulated intracellularly during preculture on YPD.
If the growth defect on proline in the absence of choline was due to a limitation in the availability of proline, it is expected that increasing the proline concentration in the medium can at least in part overcome the growth defect. This was tested in Figure 2B. The *opi3* strain grew for almost 2 cell divisions in the absence of proline and choline. In the presence of up to 0.2% (w/v) proline, growth was somewhat stimulated and the growth stop was delayed. A further increase of the proline concentration to 0.5% (w/v) proline apparently enabled the *opi3* strain to overcome the growth arrest and restored logarithmic growth. This strongly indicates that PC is required for growth on proline.
To correlate the effects of choline deprivation on growth with the PC levels of the *opi3* cells, cellular lipid compositions were determined as described in Materials and Methods. In the presence of choline, the *opi3* strain contained wild type PC levels, as expected [37-39], irrespective of the nitrogen source used. When choline was omitted from the Pro medium, the PC content had dropped to 11% of total phospholipids when the cells stopped growing. In the presence of glutamate as sole nitrogen source, the PC content was 3% when the cells reached stationary

phase. The ability of the *opi3* strain to grow to lower PC contents on glutamate than on proline provided further evidence that the impaired growth on proline did not result from pleiotropic effects of PC depletion, but rather was a result from a specific involvement of PC in proline utilization.

Possible mechanisms by which PC depletion could affect growth on proline are related to proline uptake and/or the processing of proline into glutamate. These possibilities were explored.

Table III Phospholipid composition (mol% of phospholipid phosphorous) of the *opi3* strain cultured on Pro or Glu medium in the presence or absence of added choline after preculture on YPD

Phospholipid class	*Pro medium*		*Glu medium*	
	+ choline	*- choline*	*+ choline*	*- choline*
PC	40	11	42	3
PI	19	27	22	28
PS	7	5	8	6
PE/PMME	26	42	23	54
PA/CL	8	14	5	9

Cells were grown either on 1 mM choline or in the absence of choline, and harvested during mid-log or late-log growth, respectively. The results of a typical experiment are shown. Abbreviations: PI – phosphatidylinositol; PS – phosphatidylserine; PMME – phosphatidylmonomethylethanolamine; PA – phosphatidic acid; CL – cardiolipin. In case two phospholipids are indicated, the sum of both is presented in the table.

Effect of PC depletion on proline uptake and cellular proline content

To investigate whether PC influences proline transport across the plasma membrane, *opi3* cells were cultured on Glu medium with or without choline. They were transferred to a nitrogen free buffer and incubated for 1 h, prior to the addition of radiolabeled proline to measure the rate of proline uptake.

As shown in Figure 3, *opi3* cells cultured on Glu medium were able to import proline (upper panel). The rate of proline uptake was found to be constant, irrespective of the presence or absence of choline, for at least 10 min after the addition of the labeled proline. However, after preculture in the absence of choline, the rate of proline uptake was reduced four-fold, suggesting that depletion of PC impairs proline uptake. To investigate whether this was a specific effect, the rates of glutamate and glucose uptake were also determined. As shown in the center panel, the rate of glutamate uptake was also reduced by PC depletion to a similar extent as the rate of proline uptake, suggesting that members of the family of amino acid permeases

[52,53] are affected by PC depletion. However, the reduction in the rate of proline and glutamate uptake upon PC depletion was not due to a general effect on plasma membrane transport, as the rate of glucose uptake in contrast was increased after PC depletion (Figure 3, lower panel).

To test whether the reduced rate of proline uptake in PC-depleted cells resulted in a decrease of the intracellular proline content, the *opi3* strain was cultured in Pro medium with or without choline. Aliquots from both cultures were obtained when the cells in the medium devoid of choline were about to enter the stationary phase, and the cellular proline content was determined as described in the experimental section. The proline content was found to be 14.4 ± 2.2 and 10.9 ± 3.5 nmol/OD unit (± SD, n = 3) for cells grown in the presence or absence of choline, respectively. These nearly identical levels of proline suggest that PC depletion also affects intracellular processing of proline.

Effect of PC depletion on proline metabolism

The metabolic fate of radiolabeled proline in normal and PC-depleted *opi3* cells was monitored in the presence or absence of choline. After incubating the cells with the radiolabeled proline for 30 min, the distribution of the radiolabel in the cell lysate was analyzed by TLC and phosphorimaging (Figure 4).

The radioactive label was recovered in different components of the cell lysate, irrespective of the presence or absence of choline in the growth medium (lanes 2 and 3). This indicated that PC depleted cells, as well as cells with a normal

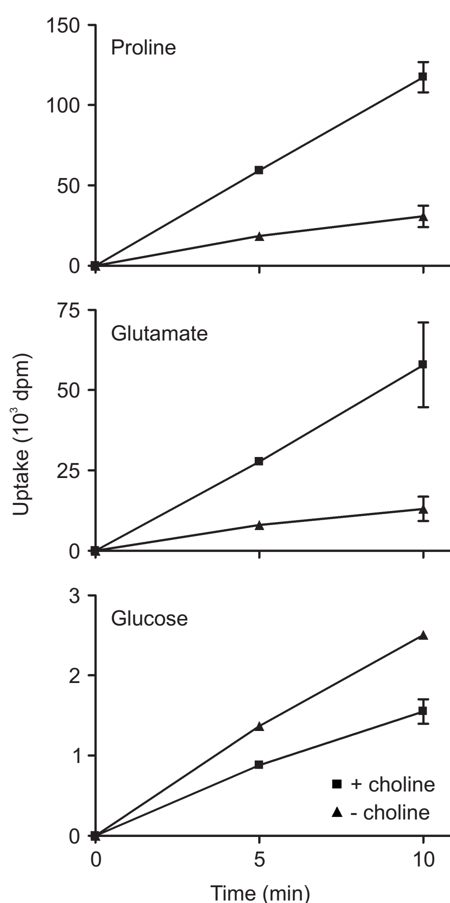

Figure 3 The effect of PC depletion on the uptake of proline, glutamate, and glucose by the *opi3* strain. The *opi3* strain was precultured on YPD, transferred to Glu medium with (squares) or without (triangles) 1 mM choline and grown for at least three generations. After subjecting the cells to nitrogen depletion for 1 h, radiolabeled proline, glutamate, or glucose was added to final concentrations of 0.1% (w/v), 0.1% (w/v) and 1 mM, respectively. Aliquots were taken at different time points and analyzed as described. The error bars at 10 min indicate the variation between two independent experiments.

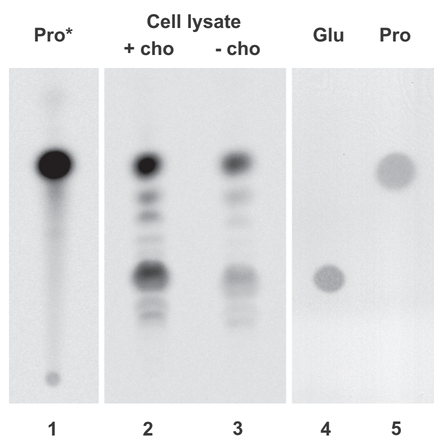

Figure 4 TLC analysis of the effect of PC depletion on the fate of ^{14}C-proline *in vivo*. The *opi3* strain was precultured on YPD and transferred to Pro medium with or without choline. After 1.5 cell divisions on Pro medium, radiolabeled proline was added to the cells. After an incubation of 30 min cell lysates were prepared from cells cultured in the presence (lane 2) or absence (lane 3) of choline. The cell lysates were subjected to TLC and phosphor imaging as described. Lane 1 shows the radiolabeled proline. In lanes 4 and 5, glutamate and proline, respectively, were run as references, and stained with ninhydrin.

PC content, were able to process the radiolabeled proline into various products. The total amount of radiolabel in lane 2 was four-fold higher than in lane 3, in agreement with the earlier observation that the rate of proline uptake is four-fold reduced in PC-depleted cells (Figure 3, upper panel). The most abundantly labeled metabolite in lanes 2 and 3 corresponds to glutamate (see reference sample in lane 4; [48]), indicating that both Put1 and Put2 were active in PC depleted *opi3* cells. The nature of the other metabolites was not further analyzed, because no major differences were seen for cells grown in the presence or absence of choline. However, quantitative analysis of the distribution of the label over proline and its products revealed that the percentage of imported proline metabolized in the + cho sample was a factor 1.5 ± 0.1 (n = 2) higher than that in the – cho sample. This indicated that the rate of proline metabolism, as mediated by Put1 and Put2, was lower after PC depletion.

Effect of PC depletion on Put1 and Put2 levels

A possible explanation for the reduced proline metabolism is that the expression levels of Put1 and Put2 were decreased by the lower cellular PC content. To enable quantification of the Put1 and Put2 levels during PC depletion, *opi3* strains were constructed with either Put1 or Put2 chromosomally tagged with a hemagglutinin epitope, yielding the strains PR21 and PR22, respectively.
The effect of choline deprivation on the growth of both modified *opi3* strains (Figure 5A) was similar to that observed for the parental *opi3* strain (Figure 2A, upper panel). Compared to the parental strain, the growth rate on proline in the presence of choline was reduced by 50% in case Put1 was tagged, and reduced by 25% when Put2 was tagged (data not shown). During growth of PR21 and PR22 on proline in the presence or absence of choline, aliquots were taken at different time points, and processed to total cell lysates to analyze Put1 and Put2 levels by Western blotting (Figure 5B).

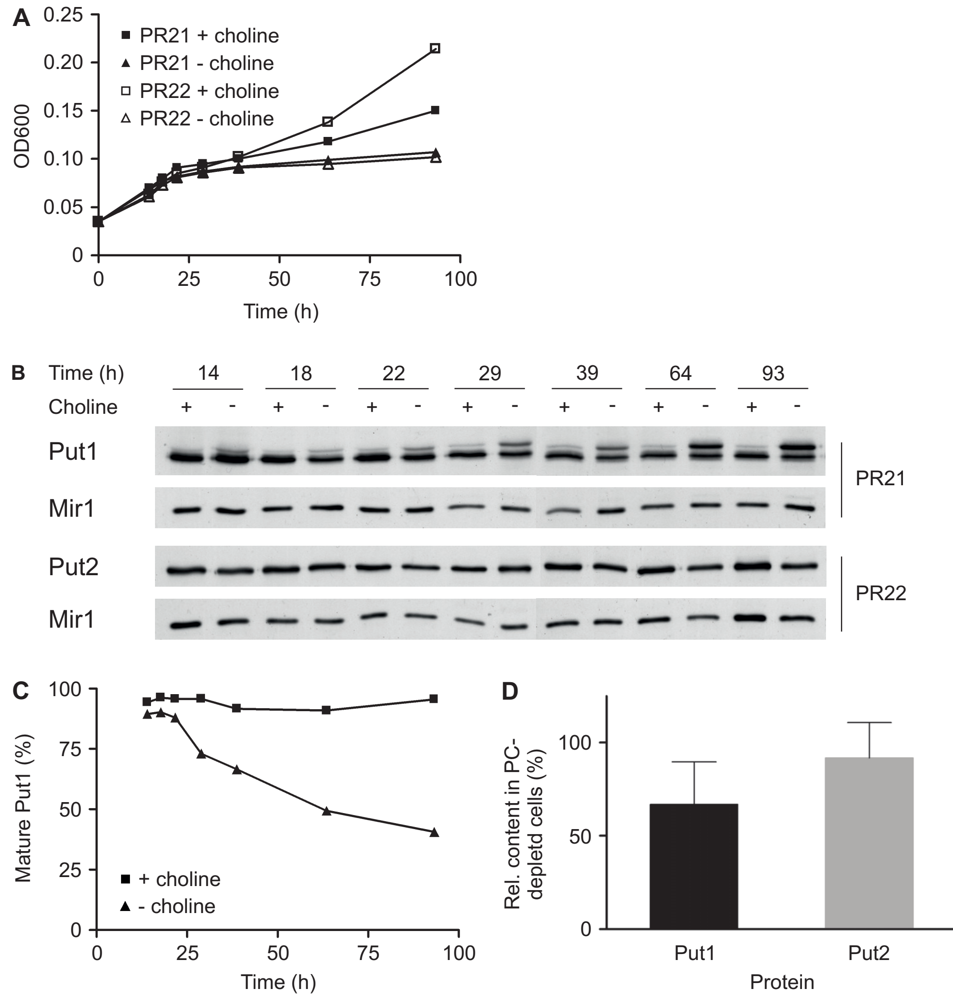

Figure 5 The effect of PC depletion on the expression levels of Put1 and Put2 in the PR21 and PR22 strains, respectively. (A) Growth curves of the PR21 (filled symbols) and PR22 strains (open symbols) on Pro medium in the presence (squares) or absence (triangles) of 1 mM choline, after preculture on YPD to late-log phase. (B) Western blot of total cell lysates of the PR21 and PR22 strains grown in Pro medium in the presence or absence of choline, prepared at different time points after transfer from YPD as in panel A. The blot was decorated with an antibody against the hemagglutinin epitope, targeting HA-tagged Put1 in the PR21 strain and HA-tagged Put2 in the PR22 strain, and with an antibody against the mitochondrial phosphate carrier Mir1 as loading control. In the case of Put1 the lower band represents mature Put1, the upper band is probably the precursor of Put1. (C) Mature Put1 as a percentage of the total (*i.e.*, mature and presumed precursor) Put1 content in the samples from panel B. (D) Quantification of the levels of Put1 and Put2 in the samples shown in panel B. The levels of both proteins were normalized to the level of Mir1 in the corresponding samples. At each time point, the Put1 and Put2 content in PC-depleted cells was calculated as a percentage of the level in cells cultured in the presence of choline. The averaged percentages are shown with the error bars representing the standard deviation (n = 7).

In the PR21 strain an additional band was observed above the Put1 band. This band most likely corresponds to the Put1 precursor, which probably contains an N-terminal mitochondrial targeting signal of 5 kDa [54]. Two observations support this notion. First, the upper band was only recovered in the total cell homogenate of PR21, prepared according to [55,56], if the metal chelator *o*-phenanthroline had been added prior to cell homogenization by douncing, whereas mature Put1 was also recovered in the absence of *o*-phenanthroline (data not shown). The metal chelator is an inhibitor of metalloproteases like MPP and MIP, which remove targeting signals from mitochondrial precursor proteins [57]. Second, when mitochondria were collected from the total cell homogenate by centrifugation as described [55,56], mature Put1 was found in the mitochondrial pellet, whereas the putative precursor remained in the supernatant (data not shown). For the cells grown in the presence of choline, the relative intensities of the mature and putative precursor bands remained constant (Figure 5B and 5C). However, during growth in the absence of choline, the relative fraction of the precursor band largely increased. This suggests that the mitochondrial import of Put1 was decreased in the PC-depleted PR21 strain.
The cellular levels of mature Put1 and Put2 were determined and normalized with respect to the level of the phosphate carrier Mir1, and the average protein levels are depicted in Figure 5D. The cellular Put1 content was found to be slightly lower in PC-depleted cells than in cells grown in the presence of choline. On the other hand, the Put2 level was not significantly affected by PC depletion in the PR22 strain. The reduced Put1 level could at least partially account for the reduced proline metabolism observed in PC depleted *opi3* cells as shown in Figure 4.

Discussion

The aim of this study was to investigate the possible influence of PC on the utilization of proline as a nitrogen source by yeast, using a mutant strain in which the cellular PC level could be manipulated. It was observed that lowering the cellular PC content caused *opi3* cells to cease growth rapidly when proline was used as sole nitrogen source. This was not due to pleiotropic effects of PC depletion, as *opi3* cells were able to grow on glutamate with lower PC contents, and as a higher proline concentration in the medium was found to restore logarithmic growth in the absence of choline. PC depletion was found to affect proline utilization at two levels, *i.e.*, uptake and metabolism.
What could underlie the reduced uptake of proline in PC-depleted cells? The similar effect of PC depletion on glutamate transport suggests that the reduced proline uptake resulted from a general mechanism acting on membrane transporters, or rather, amino acid transporters, as glucose transport was in contrast stimulated by

PC depletion. One possible reason for the reduced proline uptake is that the plasma membranes of PC-depleted cells contained less proline transporters, which might be related to a reduced biosynthesis or an impaired trafficking of these transporters to the plasma membrane. As exemplified by the inhibition of *COX1-4* mRNA translation in yeast cells devoid of PG and CL [58,59], phospholipids might play a role in the synthesis of distinct groups of proteins. Therefore, it can not be excluded that PC depletion leads to a reduced synthesis of proline transporters and of other members of the amino acid permease family, although evidence is lacking. The alternative explanation for a reduced content of proline transporters in the plasma membranes of PC-depleted cells, *i.e.*, impaired trafficking, is more likely, considering the well-known connection between PC metabolism and vesicular transport (see for instance [2] and references therein). In this context, it is of interest to note that PE depletion has been reported to reduce the uptake rates of proline and arginine, whereas the transport rate of glucose was unaffected [34]. The reduced arginine transport in PE-depleted cells was due to impaired trafficking of the arginine permease Can1, which did not reach the plasma membrane like in cells with a normal PE content, but was retained in the endoplasmic reticulum (ER) and Golgi compartments [60]. The reduced proline uptake in PE-depleted cells was attributed to a similar defect, as all amino acid permeases share the same route from the ER to the plasma membrane [61]. The effects of PC depletion on the uptake of amino acids and glucose presented here, resemble those reported in response to PE depletion, suggesting that PC depletion may also influence the trafficking of amino acid permeases.

A second possibility for the reduced proline uptake in PC-depleted cells is that the specific activity of the proline transporters was affected by PC depletion. This might have resulted from the loss of PC as a cofactor, but to our knowledge yeast amino acid transporters or homologues do not require phospholipids as cofactor. A more likely explanation for the reduced activity of the proline transporters is that PC depletion affected a regulatory mechanism involving Npr2. This proposed regulator of nitrogen permeases was shown to be required for growth on proline or glutamate as nitrogen source, and is important for proline uptake [33]. It was found that the mRNA levels of the *NPR2* gene were elevated when wild type cells were cultured in the presence of choline [32]. In PC-depleted cells this might be reversed, and the possibly resulting lower Npr2 content might have led to reduced proline and glutamate uptake rates. Alternatively, a reduced activity of the proline permeases might be due to a changed proton gradient across the PC-depleted plasma membrane, as all amino acid transporters are proton motive force driven [62]. This could also explain the reduced glutamate transport, and the unaffected glucose transport in PC-depleted cells, as the latter is not dependent on the proton gradient [63].

The other level at which PC depletion interfered with proline utilization is proline metabolism. Radiolabeled proline was found to be more slowly converted by cells

when their PC content was reduced. This might be caused by a reduced Put1 activity and/or content, since Put1 is a functionally non-redundant protein. Any change in the total cellular Put1 activity directly affects the cellular capacity to use proline as a nitrogen source. Several factors might have been responsible for a possibly lower specific activity of Put1. Lowering the PC content in the mitochondrial inner membrane might have compromised the integrity of this membrane, affecting a possible compartmentation of proline metabolism. As a consequence, accumulation of proline in the mitochondria might have been antagonized by leakage, resulting in a lower proline concentration in the mitochondrial matrix sensed by Put1 and hence in a lower Put1 activity. Another explanation for a reduced specific activity of Put1 in PC-depleted cells might be that PC acts as a cofactor of Put1 or influences regulatory mechanisms, but this is not supported by any experimental data.

A reduced Put1 content could also be responsible for the reduced proline metabolism after PC depletion. Data obtained in this study showed that the content of mature Put1 was slightly reduced in PC-depleted cells, supporting this possibility. This could be due to a decrease of the biosynthesis or the stability of Put1. With respect to stability, PC depletion could have resulted in a destabilized Put1 protein, increasing its turnover and hence decreasing the cellular Put1 content. However, Western blot analysis of total cell lysates of PR21 did not reveal any potential degradation products containing a hemagglutinin tag (data not shown), rendering this possibility unlikely. Concerning the biosynthesis of Put1, PC depletion resulted in a two-fold increase in the *PUT1* mRNA level in a *cho2opi3* strain, as mentioned earlier (M.C. Koorengevel *et al.*, unpublished results). A similar increase would be expected for the *opi3* strain under the conditions used in this study, along with an increased Put1 level. The combined levels of mature and premature Put1 were indeed found to be elevated in PC-depleted cells, indicating that PC interferes with Put1 biosynthesis at a later stage than translation of *PUT1* mRNA. An interesting possibility is that PC plays a role in the import of the precursor of Put1 by the mitochondria, where the mitochondrial targeting signal is clipped off. This is supported by the observation that the precursor most likely accumulated outside mitochondria in *opi3* cells that were subjected to PC depletion (data not shown). PC depletion does not affect the mitochondrial import of proteins in general, since no accumulation of the precursors of Put2 and of Gut2 (with cleavable targeting signals of 2 and 4 kDa, respectively [14,64]) has been found under these conditions (Figure 5B, [15]). The mechanism of and the reason for a possibly specific effect of PC depletion on the mitochondrial import of the Put1 precursor remain obscure.

It has been demonstrated that PC depletion impaired the uptake and the metabolism of proline, but what is responsible for the growth arrest of the *opi3* strain on Pro medium devoid of choline? It should be noted that it is complicated to directly translate either possibility to effects on growth rate, as exemplified for uptake by the

different growth phenotypes on proline and on glutamate in the absence of choline, despite the similarly decreased uptake rates of these amino acids under these conditions. However, two observations suggest that proline uptake is rate limiting for growth under conditions of PC depletion. First, increasing the extracellular proline concentration in the medium from 0.1% to 0.5% (w/v) enabled the *opi3* strain to grow on proline in the absence of choline. The low affinity proline uptake system (mainly Gap1) has a K_M value of 13 mM (0.16% (w/v)) [65]. Hence, the higher proline concentration increased the proline uptake capacity. Second, the addition of 0.45 mM leucine to Pro medium enabled *opi3* cells to grow in the absence of choline (P.J. Rijken *et al.*, unpublished results). Leucine is thought to increase the transcription of the *AGP1* gene more than 50-fold, even at lower concentrations [66-68]. This could have increased the proline uptake capacity sufficiently to sustain growth, through the amino acid permease Agp1 [26].
How do the results obtained compare to the observations that changes in PC metabolism and content were accompanied by changes in the mRNA levels of Put1, Gap1 and Npr2 ([32], M.C. Koorengevel *et al.*, unpublished results)? Although in these studies proline did not serve as a nitrogen source, the changes in lipid compositions might have restricted the availability of nitrogen by reducing the activity of nitrogen transporters at the plasma membrane, as had been observed earlier [35]. This has possibly triggered a cellular response to obtain nitrogen from other sources, like proline [22], by increasing the mRNA levels of the enzymes involved in transport (*i.e.*, Gap1 and Npr2) and metabolism (*i.e.*, Put1). The combined levels of mature Put1 and its putative precursor were higher in PC-depleted *opi3* cells than in cells cultured in the presence of choline, consistent with such an upregulation of *PUT1* transcription.

Acknowledgement

This research was supported by an Earth and Life Sciences (ALW) grant with financial aid from the Dutch Organization for Scientific Research (NWO).
The authors would like to thank Mr. M. van der Gaag from the Gemeenschappelijk Instrumentarium (Radboud Universiteit, Nijmegen, The Netherlands) for determining the proline content in our samples.

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CHAPTER 4

Cardiolipin molecular species with shorter acyl chains accumulate in *S. cerevisiae* mutants lacking the Acyl-Coenzyme A-binding protein Acb1

New insights into acyl chain remodeling of cardiolipin

Based on:

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Jos. F. Brouwers, Martijn C. Koorengevel, Ben de Kruijff,
Margrit Frentzen, Frédéric M. Vaz and Anton I.P.M. de Kroon (2009)
J Biol Chem 284: 27609-27619

Abstract

The function of the mitochondrial phospholipid cardiolipin (CL) is thought to depend on its acyl chain composition. The present study aims at a better understanding of the way the CL species profile is established in *Saccharomyces cerevisiae* by using depletion of the acylCoA-binding protein Acb1 as a tool to modulate the cellular acyl chain content. Despite the presence of an intact CL remodeling system, acyl chains shorter than 16 carbon atoms (C16) were found to accumulate in CL in cells lacking Acb1. Further experiments revealed that Taz1, a key CL remodeling enzyme, was not responsible for the shortening of CL in the absence of Acb1. This left *de novo* CL synthesis as the only possible source of acyl chains shorter than C16 in CL. Experiments in which the substrate specificity of the yeast CL synthase Crd1 and the acyl chain composition of individual short CL species were investigated, indicated that both CL precursors (*i.e.*, phosphatidylglycerol (PG) and CDP-diacylglycerol (CDP-DAG)) contribute to comparable extents to the shorter acyl chains in CL in *acb1* mutants. Based on the findings, we conclude that the fatty acid composition of mature CL in yeast is governed by the substrate specificity of the CL-specific lipase Cld1 and the fatty acid composition of the Taz1 substrates.

Introduction

CL is a unique anionic glycerophospholipid with dimeric structure containing four acyl chains, which is almost exclusively localized to the mitochondrial inner membrane in eukaryotic cells [1,2]. CL has been shown to co-isolate with, and to be required for optimal activity of a number of enzymes in the respiratory chain [3-5], and it has

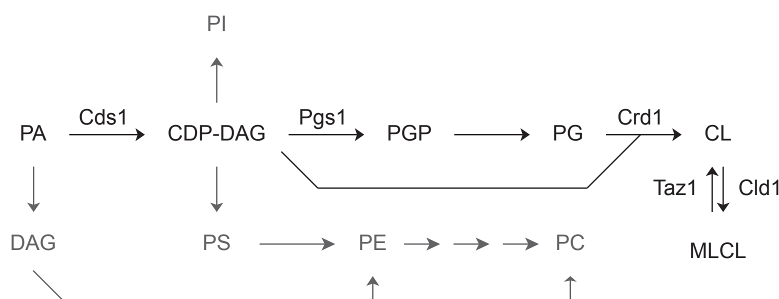

Figure 1 The cardiolipin biosynthetic pathway in the context of phospholipid biosynthesis in yeast. The enzymes of the CL biosynthetic pathway identified at the gene level are indicated: Cds1 – CDP-DAG synthase; Pgs1 – PGP synthase; Crd1 – CL synthase; Taz1 – Tafazzin; Cld1 – CL-specific deacylase. Abbreviations: DAG – diacylglycerol; MLCL – monolysocardiolipin; PA – phosphatidic acid; PC – phosphatidylcholine; PE – phosphatidylethanolamine; PGP – phosphatidylglycerolphosphate; PI – phosphatidylinositol; PS – phosphatidylserine.

been implicated in the stability and assembly of protein (super)complexes [6-8]. In the presence of divalent cations and dependent on the acyl chain composition, CL has a propensity for membrane negative curvature, a property that may be important in, *e.g.*, membrane fusion and fission [9,10]. In addition, CL is thought to serve as a proton trap in oxidative phosphorylation [11]. In recent years, CL has also been implicated in apoptosis [12,13].

CL is synthesized in the inner mitochondrial membrane by condensation of PG and CDP-DAG, catalyzed by CL synthase Crd1 (Figure 1; reviewed in [4]). Compared to the other phospholipid classes, the fatty acid composition of CL is enriched in unsaturated acyl chains, and the molecular species of CL possess a high degree of molecular symmetry [14]. The CL specific acyl chain pattern originates from substrate preferences during biosynthesis and subsequent remodeling by acyl chain exchange [15]. The finding of an aberrant CL species profile in patients suffering from Barth syndrome, which results from mutations in the tafazzin gene [16], revealed the importance of CL remodeling, and set the stage for the identification of tafazzin as the acyltransferase involved [17,18]. The *Drosophila* homologue of tafazzin was shown to be a CoA-independent phospholipid transacylase with substrate preference for CL and PC [19].

The biosynthesis and remodeling of CL have been extensively studied in the yeast *Saccharomyces cerevisiae*. After synthesis by Crd1, CL is subject to deacylation and reacylation, which involves the yeast homologue of tafazzin encoded by the *TAZ1* gene. The yeast *taz1* mutant has defects similar to those found in Barth syndrome, including a reduced CL content, an aberrant CL species profile, and an accumulation of monolyso-CL (MLCL) [20]. The bioenergetic coupling of isolated mitochondria from a *taz1* mutant is compromised [21], which may be accounted for by the impaired assembly of the III_2IV_2 supercomplex [22]. Recently, the CL-specific phospholipase Cld1 was identified that functions upstream of Taz1 [23].

Since the acyl chain composition of CL is important for its function, we investigated how the molecular species profile of CL is attained by using depletion of the 10 kDa cytosolic acylCoA-binding protein Acb1 as a tool to modify the cellular acyl chain content. Deletion of the *ACB1* gene increases the cellular levels of C14 and C16 fatty acids at the expense of C18, without having adverse effects on cell growth or on the rate of glycerophospholipid synthesis [24-26]. The changes in fatty acid composition are reflected to varying extents in the molecular species profile of phospholipids in Acb1-depleted cells as determined by electrospray ionization mass spectrometry (ESI-MS) [27,28]. We first determined by mass spectrometry that in the absence of Acb1 acyl chains shorter than C16 accumulate in CL as in the other phospholipid classes, despite the Cld1-Taz1 remodeling system. Using appropriate mutants and analysis by mass spectrometry, we investigated two possible origins of the shorter acyl chains in CL: (i) remodeling by Taz1, and (ii) *de novo* synthesis of CL from PG

and CDP-DAG.

Materials and methods

Yeast strains, plasmids, media and culture conditions

The yeast strains listed in Table I were maintained on YPD agar plates (1% yeast extract, 2% bactopeptone and 2% glucose). Strains harboring the pYPGK18 or the p416 plasmids (Table I) were obtained according to the 'Rapid Transformation Protocol' [29], and maintained on agar plates containing synthetic glucose medium (SD) lacking leucine or uracil, respectively. Synthetic media contained per liter: 6.7 g of Yeast Nitrogen Base without amino acids (Difco), 20 mg of adenine, 20 mg of arginine, 20 mg of histidine, 60 mg of leucine, 230 mg of lysine, 20 mg of methionine, 300 mg of threonine, 20 mg of tryptophan, 40 mg of uracil, and either 30 g of glucose (SD) or 22 mL 90% (v/v) lactic acid and 1 g glucose (synthetic lactate medium (SL), adjusted to pH 5.5 using KOH). Strains were cultured aerobically in SL medium at 30 °C to ensure optimal mitochondrial development. Growth was monitored by measuring the OD at 600 nm, using a Unicam Helios Epsilon spectrophotometer. Cells were harvested at late-log phase (OD600 between 0.8 and 1.0) by centrifugation (3 min, 3000 *g*), washed with water, and freeze-dried.

Table I Yeast strains and plasmids used in this study

Strain/Plasmid	*Genotype/characteristics*	*Source*
BY4742 (wild type)	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Euroscarf
BY4741 *acb1*	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 acb1::kanMX4*	Euroscarf
BY4742 *crd1*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 crd1::kanMX4*	Euroscarf
BY4741 *taz1*	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 taz1::kanMX4*	Euroscarf
BY4742 *crd1acb1*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 crd1::kanMX4 acb1::HIS5 (S. pombe)*	this study
BY4741 *taz1acb1*	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 taz1::kanMX4 acb1::HIS5 (S. pombe)*	this study
p416CYC	low copy (CEN/ARS) shuttle vector containing the *CYC1* promoter and *URA3*	N. Faergeman / [60]
p416CYC-ACB1	p416CYC derivative vector containing *ACB1* gene	N. Faergeman
pYPGK18	multicopy (2μ) shuttle vector containing the *PGK1* promoter and *LEU2*	[37]
pYPGK18-TAZ1	pYPGK18 derivative vector containing *TAZ1* gene	[37]

To analyze growth phenotypes of yeast deletion strains, cells grown in YPD to mid-log phase (OD600 between 0.6 and 0.8) were harvested by centrifugation, washed

twice with sterile water, resuspended in sterile water to an OD600 value of 1, followed by serial dilution to OD600 values of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. From each dilution, 6 μL was spotted onto agar plates, containing 1% (w/v) yeast extract, 2% (w/v) peptone, and either 2% (w/v) glucose (YPD) or 3% (v/v) glycerol (YPG). The plates were incubated at 30 °C or 37 °C for 2-6 days.

Deletion of ACB1

The *ACB1* gene was deleted via homologous recombination with a PCR construct containing the *S. pombe his5*$^{+}$ ORF flanked by coliphage *loxP* sites (kindly provided by dr. J.C. Holthuis, Utrecht University) that was created using the primers 5'- GAC TAA AAC TCT AAA ATT AGT TAA ACT AGT GTT TTC AGC AAA ATG AGG AGG GCT TTT GTA GAA AG-3' and 5'- CTA GGC CAA AAC TCC TTA CAT GGA GCT AGT ATA CCC CTT TTT TAC AAC ACT CCC TTC GTG CTT G-3', with the underlined sequences corresponding to nucleotides -42 to -1 upstream and to nucleotides 1 to 41 downstream (reverse complementary) of the *ACB1* gene, respectively. Yeast cells were transformed with the PCR product of 734 bp according to the 'High-Efficiency Transformation Protocol' [29], and transformants were selected on SD plates lacking histidine. Correct integration of the PCR fragment was verified by colony PCR.

Phospholipid extraction and TLC analysis

Total lipid extracts were prepared of freeze-dried cells as described [15]. To 10 mg of cells (dry weight), 3 mL of chloroform/methanol (2:1 v/v) was added, and the suspension was sonicated for 20 min in a Branson B1200 bath sonicator (Bransonic Ultrasonics, Danbury, CT, USA) containing ice water. Subsequently, 1 mL of water was added, and, if appropriate, 0.4 nmol tetramyristoyl-CL or 0.064 nmol dimyristoyl-PG (both from Avanti Polar Lipids, Alabaster, AL, USA) dissolved in 50 μL chloroform was added as internal standard. The mixture was shaken vigorously for 1 min, and incubated on ice for 15 min. The lower organic layer was collected after centrifugation for 10 min at 1000 *g*. Residual lipids in the upper layer were extracted with 3 mL chloroform/methanol (2:1 v/v), and the combined organic layers were evaporated under a stream of nitrogen. This procedure was applied to different amounts of cells, with volumes adjusted proportionally. Total lipid extracts were separated by thin layer chromatography (TLC) as described [30]. The phospholipid containing spots identified by running the appropriate standards, were scraped off and their quantities were determined as described [31].

Analysis of phospholipid molecular species by mass spectrometry

Total lipid extracts obtained from 10 mg of cells (dry weight) corresponding to 150-200

nmol of phospholipid phosphorous were dissolved in 150 µL of chloroform/methanol/water (50:45:5 v/v/v) containing 0.01% (w/v) NH_4OH, and 5 µL of this solution was analyzed by HPLC-MS as described [15]. Briefly, phospholipids were separated on a silica HPLC column using a linear gradient between chloroform/methanol (97:3 v/v) and methanol/water (85:15 v/v). The HPLC-eluent was introduced into a TSQ Quantum AM mass spectrometer (Thermo Electron Corporation). Mass spectra of CL, PI and PG were recorded in the negative ion mode with the following settings: source collision-induced dissociation 10 V, spray voltage 3.0 kV and capillary temperature 300 ºC. PC, PE and PS were measured in the positive ion mode, using a parent ion scan for *m/z* 184.1, and neutral loss scans for *m/z* 141.1 and 185.1, respectively. Other settings were: source collision-induced dissociation 10 V, spray voltage 3.6 kV, pressure of collision gas (argon) 0.5 mTorr, collision energy 40 V for the parent ion scan and 25 V for the neutral loss scans and capillary temperature 300 ºC. Mass spectra of each lipid were acquired during the corresponding retention time in the HPLC elution profile. The acyl chain composition of short CL species was determined by daughter scan analysis recorded in the negative ion mode with a collision energy of 50 V, Q1 peak width of 0.3 and Q3 peak width of 0.7 (FWHM).

Molecular species composition of newly synthesized PG

To label newly synthesized PG with deuterium-labeled glycerol (1,1,2,3,3-d5-glycerol; Sigma, Zwijndrecht, The Netherlands) the *crd1* and *crd1acb1* strain were cultured on SL medium. When the OD600 of the cultures had reached ~0.8, d5-glycerol was added to the medium to a concentration of 1.2% (w/v). After 10, 30, 60 and 180 min cells were harvested, washed with water by centrifugation (3 min at 3000 *g*), and the cell pellets were freeze-dried. Lipids were extracted from 20 mg of freeze-dried cells as described above. To determine the molecular species composition of d5-labelled PG, precursor scans of *m/z* 232 were recorded on a 4000 QTRAP mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Lipids were sprayed from chloroform/methanol/water (5:10:4 v/v/v) at a flow rate of 5 µL/min. Ionization was performed at 300 °C at an ion spray voltage of -4200 V. The declustering potential was set to -120 V and the collision energy to -55 V. Other parameters were optimized for a maximum signal-to-noise ratio. For analysis of unlabeled PG molecular species, precursor scans of *m/z* 227 [32] were recorded with identical settings as above. The identity of molecular species was confirmed by recording product spectra, operating the second mass filter in linear ion-trap mode with dynamic fill time. In these experiments, a collision energy of 50 V with a spread of 15 V was used, which enabled the clear identification of the $[M-H]^-$ molecular ion, the corresponding lyso-lipid fragment ions, the subsequent loss of the headgroup from this fragment ion, the fatty-acyl derived carboxylate ions and the

glycerophosphate ion (data not shown).

Fatty acid analysis

Aliquots of total lipid extracts corresponding to 1 µmol of lipid phosphorous obtained from about 50 mg of freeze-dried cells were each dissolved in 2 mL CH_3OH/H_2SO_4 (40:1 v/v), and transesterified by heating at 70 ºC for 2 h. After cooling to room temperature, 2 mL water was added and the fatty acid methylesters were extracted three times with 2 mL hexane. The composition of the fatty acid methylester mixture was determined on a Chrompack CP-9001 gas chromatograph equipped with a capillary column CP-WAX58 CB, in a 20-min run using the following temperature profile. After 2 min at 100 °C, the column was heated to 200 °C at a rate of 10 °C per min. Fatty acid methylesters were identified and signal intensities were calibrated using an equimolar mixture of the methylesters of C8:0, C10:0, C12:0, C12:1, C14:0, C14:1, C16:0, C16:1, C18:0 and C18:1 (Nu-ChekPrep, Elysian, MN, USA).

In vitro Crd1 activity assay

Crd1 activity of sucrose gradient purified mitochondria isolated from the wild type strain BY4742 [33] was determined based on published methods [15,34], with the 50 µL standard reaction mixture containing 0.1 M Bis-Tris propane/HCl buffer (pH 9), 20 mM $MgCl_2$, 24 µM CDP-dioleoyl[U-^{14}C]glycerol (42 dpm/pmol), 150 µM dioleoyl-PG and 1 to 8 µg mitochondrial protein. PG substrate specificity of Crd1 was tested in reaction mixtures containing increasing concentrations of the PG species indicated (diC16:0 PG and diC18:1 PG from Sigma-Aldrich, diC12:0 PG, diC14:0 PG, and C14:1/C17:0 PG from Avanti Polar Lipids, Alabaster, AL, USA), while the CDP-DAG substrate selectivity of the enzyme was determined by the degree of inhibition of the labeling of CL caused by increasing concentrations of different unlabeled CDP-DAG species (Sigma-Aldrich) in the standard reaction mixture. Reactions were stopped after incubation for 1 h at 37 °C, and reaction products were extracted and analyzed as described before [15].

Results

Effect of deleting the ACB1 gene on the molecular species profiles of the major membrane phospholipids

Deletion of the *ACB1* gene [24,25] or depletion of Acb1 [26-28] is known to affect the fatty acid composition of yeast cells: the average length of the fatty acids decreases, while the degree of unsaturation increases. Using mass spectrometry, we investigated how these changes translate to the molecular species compositions of the separate

membrane phospholipid classes.
The molecular species profiles of PC, PE, PI and PS in wild type and *acb1* mutant cells as determined by ESI-(MS/)MS can be found in Supplementary data, Figure S1. As yeast cells have a limited repertoire of fatty acids with saturated and mono-unsaturated C16 and C18 acyl chains as main components [25], the majority of the PC, PE, PI and PS species in wild type cells belonged to the C32 and C34 clusters (*cf.* [35]). Comparison of the *acb1* strain to the wild type revealed that the relative abundance of the C32 clusters was increased at the expense of the C34 clusters, in agreement with the average length of the acyl chains of all phospholipid classes tested being shorter in the mutant (Figure S1), as reported earlier [27,28]. Moreover, the levels of species with 30 or less carbon atoms in their acyl chains increased to extents depending on the phospholipid class, which is consistent with the rise in the abundance of fatty acids shorter than 16 carbon atoms [24,25]. For example, in PS an increased content of the C30 cluster was observed compared to wild type, whereas in the species profile of PC also significant amounts of C28 and

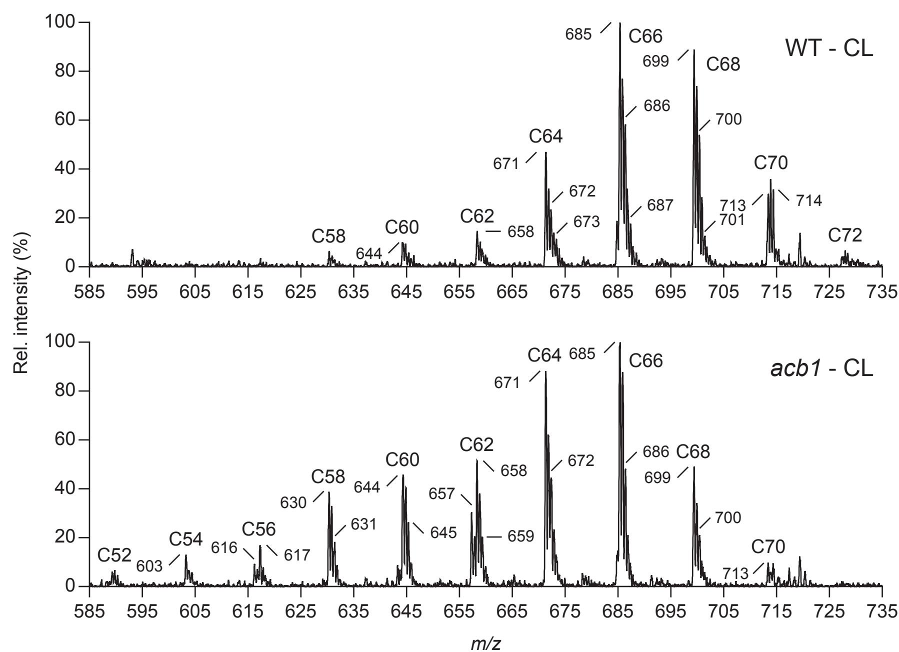

Figure 2 The effect of deleting the *ACB1* gene on the species profile of CL in yeast. Total lipid extracts were prepared of wild type and *acb1* cells cultured on SL medium to late log-phase. Phospholipid classes were separated by HPLC, and subjected to ESI-MS in the negative ion mode to determine the species composition of CL. The major doubly charged species of CL are indicated by their *m/z* values. Clusters of CL species are indicated by the total number of C-atoms in their acyl chains. In both panels, the intensity of the highest peak was set at 100%. See Table S3 for the molecular species assignment per cluster.

even C26 species were found in *acb1* cells. The deletion of the *ACB1* gene did not significantly influence the degree of saturation of PC and PE. In contrast, in *acb1* cells PI contained more unsaturated acyl chains as was observed previously [27,28], whereas for PS the content of unsaturated species was decreased.
The molecular species profiles of CL in wild type and *acb1* cells are shown in Figure 2. To interpret the mass spectra of CL it should be realized that this phospholipid is measured as a doubly charged anion implying that the measured *m/z* values correspond to half the molecular mass. In wild type cells, the CL species profile is dominated by clusters in the range of C64 to C70, as was found previously [20] and which is consistent with the abundance of C16 and C18 acyl chains in yeast. In wild type cells, the most abundant clusters are dominated by the peak with the lowest *m/z* value, *i.e.*, the one that corresponds to the tetra-unsaturated species. This peak is followed in decreasing order of intensity by the peaks representing tri- (*m/z* increased by 1), and di-unsaturated species (*m/z* increased by 2).
In the absence of Acb1, the CL species profile was profoundly changed as illustrated by the increase in the summed relative abundance of the signal intensity of the clusters smaller than C64 from approximately 10% to 40% (based on the highest peak in each cluster). The clusters smaller than C64 contain acyl chains shorter than C16, demonstrating a shortening of the acyl chains in CL. The increase in shorter CL species was at the expense of the C18-containing C68-C72 clusters, with the C70 and C72 species being virtually absent in *acb1*. Examination of the relative peak intensities within clusters indicated that the shorter CLs were on average more saturated than the longer species (Figure 2 lower panel).
Yeast lacking the *ACB1* gene has been reported to undergo a so far uncharacterized adaptation [26]. To check whether the lipid species profiles shown in Figures S1 and 2 were affected by this adaptation, *acb1* and wild type cells were transformed with plasmid p416CYC-ACB1 (Table I) carrying the wild type *ACB1* gene, or with the empty plasmid p416CYC as control. The phospholipid species profiles of the *acb1* cells were restored to wild type upon introducing the *ACB1* gene, whereas the species profiles of the *acb1* cells transformed with the empty vector and the wild type cells were not affected (data not shown). These results render it unlikely that the species profiles were affected by the adaptation, if any, of the *acb1* mutant in the BY4742 background. Moreover, the lipid profiles recorded for the *acb1* strain in this study were comparable to those found for the non-adapted yeast strain with a conditional *ACB1* knock-out [27,28].

The profound effect of *ACB1* deletion on the species profile of CL was remarkable, because the acyl chain composition of CL is thought to be important for CL function [14], and because yeast cells possess a CL remodeling system to replace inappropriate acyl chains. In the following, two possible origins of the acyl chains

shorter than C16 in CL were investigated, remodeling of CL by Taz1 and *de novo* synthesis of CL, involving the precursors PG and CDP-DAG.

Are the acyl chains shorter than C16 introduced in CL in acb1 via remodeling by Taz1?

After synthesis, CL is remodeled to obtain the correct acyl chain configuration, and the transacylase Taz1 is involved in this process [19,21,36]. To examine whether Taz1 is required for the introduction of acyl chains <C16 in CL in an *acb1* mutant, the *ACB1* gene was deleted in a *taz1* strain, yielding the *taz1acb1* strain.

Growth phenotypes on YPD at 30 and 37 °C were indistinguishable between wild type, *acb1*, *taz1*, and *acb1taz1* cells (data not shown). However, on a non-fermentable carbon source (NFCS) the *taz1* cells showed a temperature-dependent growth defect (*cf.* [22,37]) that was exacerbated in the *taz1acb1* double mutant (Figure 3).

The CL species profile of the *taz1acb1* mutant was compared to that of the *taz1* strain (Figure 4A).

Almost all CL molecules in the *taz1* mutant were found in the clusters C64 and up, as in wild type cells (*cf.* Figure 2). However, the degree of unsaturation was lower in *taz1* cells, as evidenced by a shift of the signal intensity from the *m/z* value corresponding to the tetra-unsaturated species towards that of the tri- and di-unsaturated species in each of the clusters (Figure 4B), in agreement with previous reports [20,38]. Another major difference was the high content of MLCL in *taz1*, as had been observed before [20,37]. In the absence of Taz1, deletion of the *ACB1* gene resulted in a similar CL profile as found in *acb1* (Figure 2) with increased levels of clusters containing short CL species. Moreover, the MLCL profile in *taz1acb1* resembles the profile of CL in

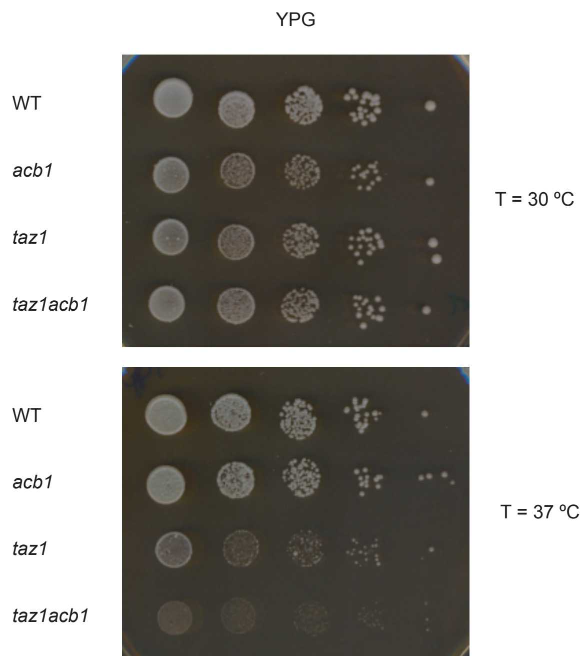

Figure 3 Growth of wild type, *acb1*, *taz1* and *taz1acb1* strains on YPG plates after preculture on YPD. The plates were incubated at 30 °C and 37 °C as indicated for 6 days. Results from a typical experiment are shown.

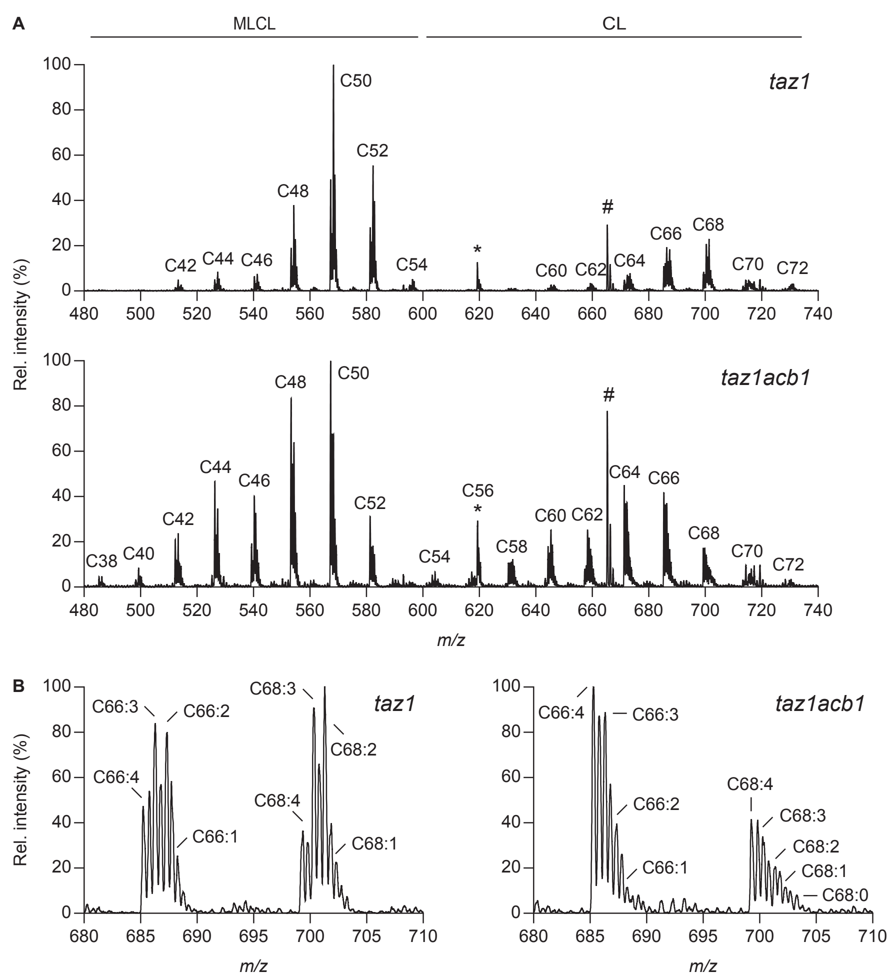

Figure 4 The effect of deleting the *ACB1* gene on the species profile of CL in a *taz1* strain. (A) Total lipid extracts of the *taz1* and *taz1acb1* strains were obtained from late-log phase cells cultured on SL medium, and the CL species profiles were determined by HPLC-MS. Peaks at *m/z* 619.5 and *m/z* 665.5 indicated by the asterisk (*) and the number sign (#) represent the internal standards TMCL and DMPG, respectively. The horizontal lines mark the *m/z* ranges of the MLCL and CL clusters that are indicated by the number of C-atoms in their acyl chains. For each panel, the intensity of the highest peak was set at 100%. (B) Enlargements of the C66 and C68 clusters of CL in *taz1* and in *taz1acb1*. The doubly charged species of CL are indicated. The intensity of the highest peak in each panel was set at 100%. Results from a typical experiment are shown. See Table S3 for the molecular species assignment per cluster.

(taz1)acb1 cells with one C16 acyl chain subtracted (Figures 4A lower panel and 2 lower panel), implying that MLCL contains relatively more chains <C16 than CL. To illustrate this, if one C16 chain is removed from CL molecules that on average contain *n* acyl chains <C16 and hence 4-*n* normal acyl chains, then the produced

MLCL will on average still have *n* acyl chains <C16, but only 3-*n* normal acyl chains. The relative content of acyl chains <C16 in the total pool of CL and MLCL is therefore higher in the absence of Taz1 than in its presence. The results shown in Figure 4 therefore indicate that Taz1 is not responsible for the presence of acyl chains <C16 in CL in *acb1*, but they don't exclude that Taz1 is able to attach shorter acyl chains to MLCL.

Interestingly, comparison of the MLCL and CL spectra in *taz1* and *taz1acb1* cells revealed that deletion of the *ACB1* gene in the *taz1* background resulted in a partial restoration of the *taz1* phenotype: within the major clusters, the balance shifted from the more saturated species towards the tetra-unsaturated species (Figure 4B) as also found for wild type CL (Figure 2 upper panel). However, with regard to the CL and MLCL contents, no significant differences were observed between the *taz1acb1* and the *taz1* strains by TLC analysis (data not shown).

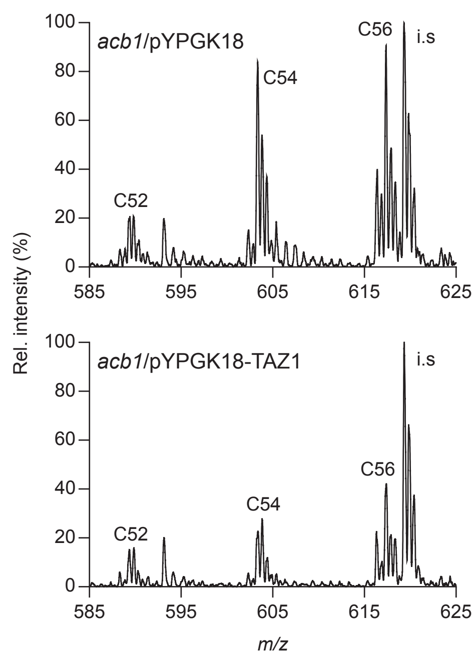

Figure 5 The influence of episomal expression of Taz1 under control of the constitutive *PGK1* promoter on the content of short CL species (up to C56) in *acb1* cells. The CL species profiles of *acb1* cells transformed with the plasmid pYPGK18-TAZ1 and with the empty vector as control, were determined by HPLC-MS. Only the parts of the mass spectra corresponding to the *m/z* range 585-625 are shown. Clusters of CL species are indicated by the total number of C-atoms in their acyl chains. The intensity of the internal standard TMCL (i.s.) was set at 100%. Results from a typical experiment are shown. See Table S3 for the molecular species assignment per cluster.

The notion that Taz1 is not required for the presence of acyl chains <C16 in CL in the *acb1* mutant was corroborated by an experiment in which *acb1* cells were transformed with the pYPGK18 plasmid carrying the *TAZ1* gene under control of the strong *PGK1* promoter. If Taz1 were responsible for the acyl chains <C16 in the short CL molecules, overexpression of this protein would be expected to increase the abundance of the short CL species. However, the opposite effect was observed. Whereas transformation with the empty plasmid did not affect the levels of the C52-C56 clusters in the CL species profile of the *acb1* strain (data not shown), overexpression of Taz1 from pYPGK18 decreased the levels of C54 and C56 CL by at least 50% relative to the internal standard (Figure 5). Episomal expression of Taz1 in *acb1* cells did not affect the species profiles of the other major membrane

phospholipids, nor did it affect growth (data not shown). We conclude that Taz1 is not required for the introduction of acyl chains <C16 into CL species in *acb1* cells, and may to some extent contribute to their replacement.

Do acyl chains shorter than C16 in CL in acb1 originate from PG and/or CDP-DAG via de novo synthesis?

The other route via which the shorter acyl chains could end up in CL is the *de novo* synthesis of CL. Crd1 synthesizes CL by transferring the activated phosphatidyl moiety from CDP-DAG to PG (Figure 1). The low cellular content of these two CL precursors due to high turnover rates interferes with the determination of the species profiles that are representative for the pools of substrates available to Crd1 [39-41]. The existence of two separate intracellular pools of CDP-DAG further complicates the analysis of this intermediate in CL synthesis [42-44]. For PG, the problem of low abundance can be circumvented by using a *crd1* strain, in which CL synthesis is blocked, resulting in increased PG levels [41,45]. To examine whether PG exhibits a similar accumulation of relatively short acyl chains in the absence of CL synthesis as CL in the *acb1* strain, a *crd1acb1* double deletion strain was constructed. Growth phenotypes of the *crd1acb1* strain, the congenic single deletion mutants and the wild type strain are shown in Figure 6.

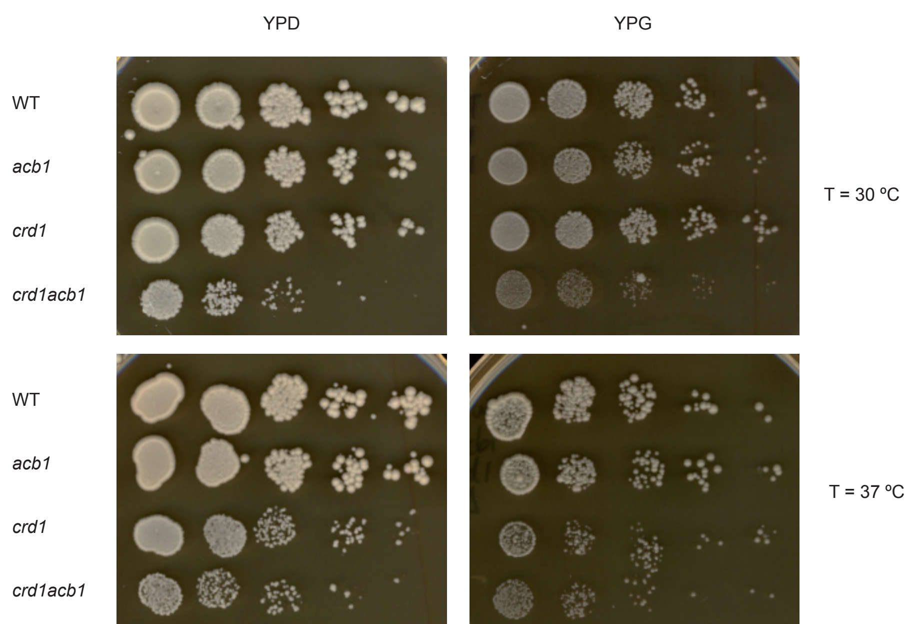

Figure 6 Growth of wild type, *acb1*, *crd1* and *crd1acb1* on YPD and YPG plates after preculture on YPD. The plates were incubated at 30 or 37 °C for 2-5 days. Results from a typical experiment are shown.

On YPD and YPG at 30 °C, growth of the *crd1acb1* mutant was somewhat impaired compared to that of the *acb1* and *crd1* single mutants and the parental wild type. At 37 °C, the poorer growth phenotype of the *crd1* strain compared to *acb1* and wild type (*cf.* [46,47]) was slightly exacerbated in the *crd1acb1* mutant.

To check whether the *crd1* strain provides a representative background to study the effect of *ACB1* deletion on the sorting of acyl chains, the fatty acid compositions of total lipid extracts were compared between wild type and *crd1* cells, and between *acb1*and *crd1acb1* cells. No significant differences were observed (see Supplementary data, Table S1), validating the *crd1acb1* mutant strain as model for investigating the sorting of acyl chains.

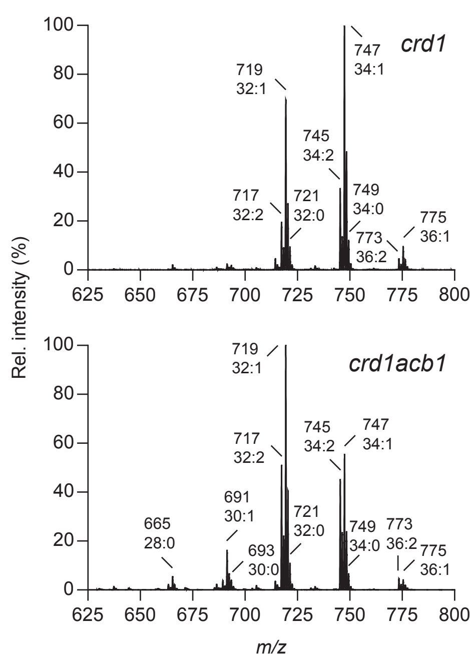

Figure 7 The effect of deleting the *ACB1* gene on the species profile of PG in the *crd1* strain. Total lipid extracts were obtained from late-log phase *crd1* and *crd1acb1* cells, and the species composition of PG was analyzed by HPLC-MS, as detailed in the Experimental Procedures section. The major $[M-H]^-$ species of PG are indicated. The intensity of the highest peak was set at 100%. Representative results are shown. See Table S2 for the molecular species assignment per cluster.

The species profile of PG in the *crd1acb1* double mutant was determined by mass spectrometry and compared to that in the *crd1* strain (Figure 7). In the latter strain, the PG profile was dominated by C32 and C34 clusters with a minor contribution of C36. In the *crd1acb1* mutant, the PG molecules were on average shorter with C32 as most abundant cluster. In addition, about 10% of the PG signal originated from the C28 and C30 clusters, which were virtually absent in *crd1*. These results are in line with the earlier observations for the other phospholipid classes in the *acb1* mutant (Figure 2 and Supplementary data, Figure S1). With respect to degree of saturation, most PG species in the *crd1* cells were mono-unsaturated; deletion of the *ACB1* gene resulted in a slight increase in the relative levels of di-unsaturated species.

Considering that the shortest PG in *acb1* cells contained at least 28 carbon atoms in its combined acyl chains, PG is unlikely to be the *sole* source of acyl chains shorter than C16 in CL in view of the presence of the C52-C58 clusters in the *acb1* CL profile (Figure 2). CDP-DAG must also contribute acyl chains <C16 to CL, unless the PG profile of the *crd1acb1* mutant is not representative for the pool of

PG available as substrate for Crd1 in the *acb1* mutant due to species selective turnover [48] or remodeling of PG. To test the latter possibility, the species profile of newly synthesized PG was monitored over time by stable isotope labeling in conjunction with tandem mass spectrometry [49]. Logarithmically growing *crd1* and *crd1acb1* cells were pulsed for different periods of time with d5-glycerol to label PG. The incorporation of d5-glycerol in the backbone or head group of PG increases the *m/z* values of the PG species by 5, thus enabling the selective detection of newly synthesized PG by scanning for precursors of *m/z* 232, corresponding to the d5-glycerophosphate glycerol minus H_2O fragment ion [32]. The parent ion scans of newly synthesized PG obtained after 3 h of labeling of *crd1* and *crd1acb1* cells (Figure 8A) are consistent with the ESI-MS spectra of unlabeled PG (Figure 7) when taking into account that the chances of ion fragmentation decrease with increasing *m/z* value, resulting in a more sensitive detection of parent ions as the *m/z* value decreases. Unfortunately, the species profiles of unlabeled PG could not be obtained by parent ion scanning (*m/z* 227) because of interference by C14 acyl chains that yield a fragment ion with the corresponding *m/z* value (data not shown), rendering a direct comparison impossible.

Figure 8B shows the time course of the relative intensities of d5-labeled PG species after different times of labeling with d5-glycerol. In the *crd1* strain, the species profile of d5-labeled PG shows only minor changes over time, arguing against extensive species selective turnover or acyl chain remodeling of PG. The same holds true for the *crd1acb1* strain in which, with the possible exceptions of C26:0, C28:1 and C32:1, no large changes in d5-PG species distribution occur. Although newly synthesized PG apparently contained slightly shorter species than steady state PG (C26 vs. C28, respectively), the results corroborate the above conclusion that CDP-DAG also serves as source of acyl chains shorter than C16 in CL in *acb1*.

Since CDP-DAG is a precursor of PG, it might seem a trivial notion that acyl chains <C16 end up in CL via both PG and CDP-DAG, but it should be kept in mind that Crd1 might prefer long species of one substrate and short species of the other (*cf.* [15]), and/or that Pgs1 and Crd1 (Figure 1) might have access to different CDP-DAG pools. To gain more insight into the relative contributions of CDP-DAG and PG to the acyl chains <C16 in CL in *acb1* cells, the substrate specificity of Crd1 was examined *in vitro* in isolated mitochondria from the wild type strain. First, to assess whether substrate specificity of Crd1 could have led to a preferred use of short PG over short CDP-DAG or vice versa, CL synthesis by isolated wild type mitochondria in the presence of various species of both CL precursors was evaluated.

The PG species specificity of Crd1 was analyzed by determining the CL synthesis rates from labeled CDP-dioleoylglycerol and increasing concentrations of various PG species. As depicted in Figure 9A, Crd1 displayed the highest activity with the shortest and the most unsaturated PG species tested, and the lowest activity with the

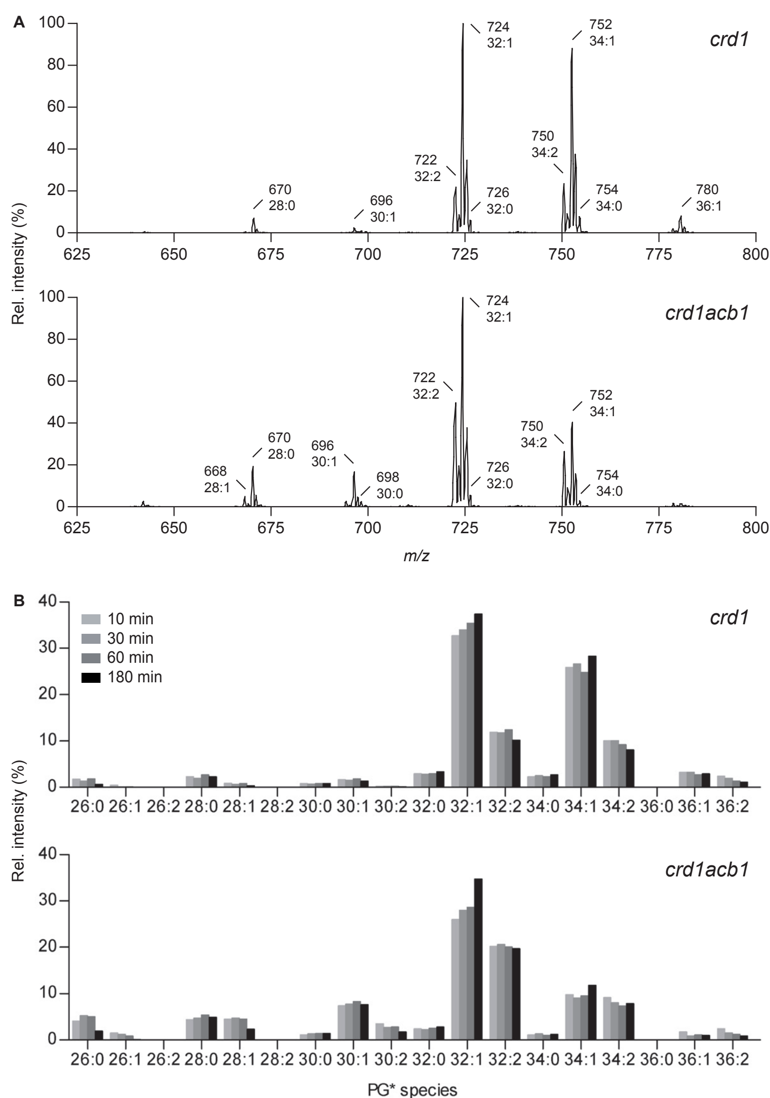

Figure 8 The effect of deleting *ACB1* on the species profile of newly synthesized PG in *crd1*. To selectively detect newly synthesized PG, *crd1* and *crd1acb1* cells cultured to mid-log phase in SL medium were labeled with d5-glycerol for the times indicated. The species compositions of the labeled PG (denoted by PG*) were analyzed in total lipid extracts by MS/MS as detailed in the Experimental Procedures section. (A) Parent ion scans at *m/z* 232 of d5-labeled PG in total lipid extracts of *crd1* and *crd1acb1* cells after 3 h of incubation with d5-glycerol. The major $[M-H]^-$ species of PG* are indicated. The intensity of the highest peak is set at 100%. (B) The time-dependent evolution of the species profiles of labeled PG in *crd1* and *crd1acb1* strains during labeling with d5-glycerol. The panels show the relative signal intensities of the molecular species indicated, with the total PG signal intensity set at 100%. Representative results are shown. See Table S2 for the molecular species assignment per cluster.

dipalmitoyl species. The observed PG species specificity of Crd1 was less distinct than those of CL synthases in more complex eukaryotes [15,50,51]. CDP-DAG competition experiments revealed that Crd1 also preferentially used CDP-DAG species with shorter or unsaturated acyl chains (Figure 9B). Again, the selectivity of the yeast enzyme was only weak in comparison to the selectivity of CL synthases from mammalian cells [15] and higher plants [51]. Hence, the results suggest that in yeast mitochondria *de novo* synthesized CL species can to a large extent be determined by the substrate species composition available to the Crd1. In addition, based on the observed similar species specificity of Crd1 for both its substrates, it is unlikely that Crd1 preferentially sources acyl chains shorter than C16 from either PG or CDP-DAG.

To shed more light on the relative contributions of acyl chains <C16 by PG and CDP-DAG, the dominant CL species in the C52-C56 clusters were subjected to MS/MS-analysis. Based on the acyl chains that were identified, the most likely acyl chain compositions were determined for each species (Table II), taking into account the mass and the degree of saturation. Surprisingly, all CL molecules were found to contain two regular (C16 or C18) and two short (C10 and/or C12) chains. No C14-containing molecules were found. Since it is highly improbable that the two shorter chains both originated from a single precursor, the shortest PG and CDP-DAG species used for CL synthesis are apparently C26 and C28, consistent with the results obtained for PG (Figure 8). The data further imply that short PG and

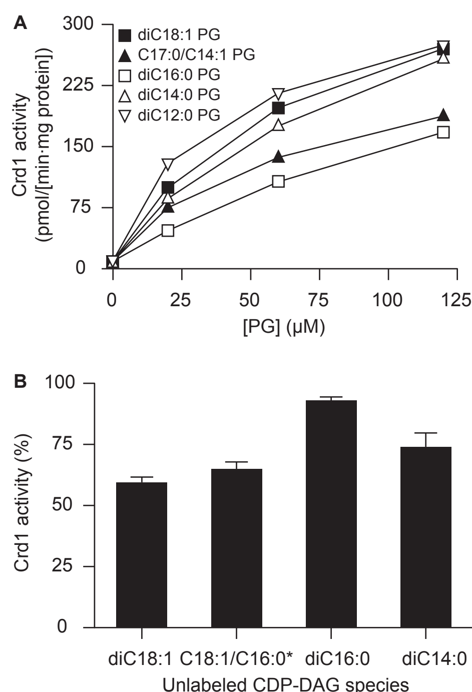

Figure 9 Substrate species specificity of Crd1. (A) Formation rates of CL by Crd1 as a function of the concentrations of diC18:1 (■), C17:0/C14:1 (▲), diC16:0 (□), diC14:0 (△) and diC12:0 (▽) PG species under otherwise standard assay conditions. (B) Incorporation of 24 μM CDP-dioleoyl[U-^{14}C]glycerol into CL remaining in the presence of 24 μM of the indicated unlabeled CDP-DAG species under otherwise standard assay conditions. The amount of labeled CL produced in the absence of unlabeled CDP-DAG is set at 100%. Similar results were obtained when the assays were conducted with two- to three-fold higher concentrations of unlabeled CDP-DAG species or five-fold lower concentrations of labeled CDP-dioleoylglycerol (data not shown). *18:1/16:0 CDP-DAG is a mixture of CDP-DAG species prepared from egg lecithin which predominantly consists of the 18:1/16:0 species.

CDP-DAG species predominantly have chains with unequal lengths, for instance C10 and C16 instead of C12 and C14. This remarkable finding was confirmed by daughter scan analysis of C28:0 PG from *crd1acb1*, which revealed C12:0 and C16:0 as most abundant acyl chains (data not shown).

Table II Molecular structures of short CL species in *acb1* cells as resolved by MS/MS analysis of the peak of highest intensity in the C52-C56 clusters

CL species	*Parent m/z*	*Most abundant acyl chains*[a]	*Possible acyl chain compositions*[b]
C52:2	589.37	C10:0 and C16:1	$(C10:0)_2(C16:1)_2$
C54:2	603.38	C10:0, C12:0 and C16:1	$(C10:0)(C12:0)(C16:1)_2$
C56:2	617.40	C10:0, C12:0, C16:1 and C18:1	(C10:0)(C12:0)(C16:1)(C18:1) and $(C12:0)_2(C16:1)_2$

[a] Based on peak heights. [b] Acyl chain compositions were determined, based on the acyl chains that were identified and the mass and number of double bonds of each species.

We conclude that PG and CDP-DAG are to similar extents responsible for the presence of acyl chains shorter than C16 in CL in the *acb1* strain.

Discussion

The present study revealed that deletion of *ACB1* gene leads to an altered molecular species profile of CL, as was observed previously for other phospholipid classes [27,28]. Since the acyl chain composition is thought to be important for the functioning of CL [14,52,53], we expected beforehand that the CL profile would be relatively resistant to changes, also in view of the available CL remodeling system. Therefore, the mechanism by which acyl chains shorter than C16 accumulate in CL in the absence of Acb1 was elucidated using MS analysis of lipid extracts from various yeast mutant strains.

The results showed that CL remodeling by Taz1 was not required for the accumulation of acyl chains <C16 in CL. Analysis of PG in *crd1acb1* cells (steady state and newly synthesized) demonstrated PG species containing acyl chains <C16, but not to the extent that PG could solely account for the shortest CL molecules in *acb1* cells. Therefore, a substantial portion of the acyl chains <C16 in CL had to stem from the other CL precursor, *i.e.*, CDP-DAG. The conclusion that both CL precursors contribute to comparable extents to acyl chains <C16 in CL in *acb1* cells was supported by the species selectivity of Crd1 *in vitro*, and the distinct acyl chain compositions found for short CL species in *acb1* cells. We will discuss the implications of our findings for CL remodeling in yeast.

Why did the CL remodeling enzymes not restore the wild type species profile of CL in the absence of Acb1? It could be argued that CL remodeling depends on the

supply of acylCoA by Acb1. However, since Acb1 does not localize to mitochondria [54], and since the only CL remodeling acyltransferase identified so far, Taz1, is thought to be a transacylase that does not require acylCoA [19], we consider the direct involvement of Acb1 in CL remodeling unlikely. An indirect effect of Acb1 on CL remodeling, *e.g.*, resulting from a possible requirement for Acb1 of the re-acylation of acyl chain donors of CL remodeling, cannot be excluded. CL remodeling could have been hampered by a shortage of appropriate acyl chains. Phospholipids most likely serve as acyl chain donors in Taz1-mediated remodeling [19]. In *acb1* the vast majority of phospholipids belongs to the C32 and C34 mono- and di-unsaturated species (Figure S1), rendering a shortage of C16 and C18 chains highly improbable. Even if other compounds than phospholipids serve as donor in a transacylation, no impact on CL remodeling is expected based on the fatty acid composition in *acb1* total lipid extracts (Table S1).

In view of the absence of even the slightest accumulation of MLCL in *acb1* cells, and because overexpression of Taz1 had only a minor effect on the overall CL profile in *acb1* cells, it is unlikely that the remodeling capacity of Taz1 is insufficient.

Therefore, we postulated that the acyl chains <C16 in CL may not be recognized as substrate by the CL phospholipases. In support of this idea, a qualitative comparison of the CL and MLCL profiles in *taz1acb1* (Figure 4A) in terms of relative peak heights within the clusters suggested that MLCL was produced by the cleavage of predominantly C16 chains rather than by removal of acyl chains <C16, with *e.g.*, C46 MLCL originating from C62 CL, and C50 MLCL from C66 CL. During the preparation of this paper, the identification of the yeast CL phospholipase Cld1 was reported [23]. Cld1 was shown to have a strong preference for removing C16:0 acyl chains from CL, fully consistent with our hypothesis. Further, circumstantial evidence is provided by the daughter scan of the most intense peak in the C52 CL cluster from *acb1* cells, corresponding to C52:2 CL, which revealed that this minor species was built from C10:0 and C16:1 chains (Table II). Remarkably, within the C26 cluster of PG, C26:0 PG was the most abundant species (data not shown), suggesting that a C16:0 chain originating from PG had been replaced during CL remodeling by a C16:1 chain.

The specificity of Cld1 allows an elegant reduction of the number of different acyl chains in remodeled CL, thereby contributing to the symmetry in CL [14,52]. Selective removal of C16:0 is therefore sufficient to establish a CL pool with mainly C16:1 and C18:1 in wild type cells. The specificity of the CL phospholipase for C16:0 explains why the C18-rich clusters C70 and C72 are not dominated by tetra-unsaturated species in wild type and *acb1* cells (Figure 2), and also why acyl chains <C16 in *acb1* are not adequately removed, resulting in the accumulation of short (and relatively saturated) CL species in *acb1* compared to wild type.

Based on our findings and those of Beranek *et al.* [23], we propose the following mechanism for CL remodeling in yeast: Cld1 is the prime phospholipase acting

on CL, and responsible for the recognition and cleavage of C16:0 chains from CL. Subsequently, Taz1 is primarily responsible for the reacylation of the MLCL produced. The transacylase Taz1 uses the acyl chains available from donor phospholipid(s). The combined actions of Cld1 and Taz1 lead to an enrichment in CL of C16:1 and C18:1 (the major acyl chains in yeast next to C16:0 [38]) at the expense of C16:0. Recently, a lack of acyl specificity has been reported in transacylase reactions catalyzed by rat liver, human and *Drosophila* tafazzin [55], supporting the proposed mechanism.
In conclusion, the fatty acid composition of mature CL in yeast is governed by the specificity of Cld1 and the fatty acid composition of the Taz1 substrates.

The partial restoration of the CL species profile observed in the *acb1taz1* double mutant compared to the *taz1* mutant can be accounted for by the increased content of unsaturated fatty acids in *acb1* cells (Choi, 1996; Table S1). The increased unsaturation is reflected in the *de novo* synthesized CL rather than in the other phospholipid classes (Figure S1), consistent with the preference of Crd1 for unsaturated substrates. Even though the partial restoration of the CL species profile in *acb1taz1* seemed to be accompanied by a trend towards restoration of mitochondrial protein (super)complexes [56], it did not suppress the growth defect of *taz1* cells cultured on NFCS at 37 °C (Figure 3), suggesting that processes other than oxidative phosphorylation limit growth.
Although the pronounced growth defect of *crd1acb1* on NFCS was not explored further, we speculate that the underlying cause for the retarded growth is related to the altered acyl chain composition of PE in this double mutant compared to *crd1*. The PE content in *crd1acb1* was found to be similar to that in *crd1* (data not shown). PE has been shown to be essential for the viability of yeast cells lacking CL, probably because PE and CL can both promote hexagonal lipid phases [10,57], and can substitute for each other in mitochondrial processes where this non-bilayer propensity is required. A functional relationship between PE and CL is further suggested by the concerted regulation of the synthesis of these lipids [58]. In *crd1acb1* cells, PE is on average shorter than in *crd1* (data not shown), which implies a decreased propensity of PE in the double mutant to form non-bilayer structures [59]. When CL is present, the impact of the shortening of PE on mitochondrial function might be limited. Since the double mutant *crd1acb1* lacks CL, it might be less able to cope with the altered PE species profile, resulting in the severe retardation of growth. An alternative explanation for the growth defect of *crd1acb1* would be that the altered acyl chain composition of PG due to Acb1 depletion renders PG less suited to replace CL. However, since this replacement is thought to rely primarily on the net negative charge shared by PG and CL [45], alterations in the acyl chain composition of PG are likely to have less of an impact than the changes in PE.

Acknowledgement

This research was supported by an Earth and Life Sciences (ALW) grant with financial aid from the Dutch Organization for Scientific Research (NWO). The work was further supported by grants of the Prinses Beatrix Fonds (WAR05-0126) and the Barth Syndrome Foundation to F.M.V. The authors would like to thank dr. Henry Boumann for performing pilot studies, and the mass spectrometry section of the Laboratory of Genetic Metabolic Diseases for technical assistance. The p416CYC plasmids were a kind gift of dr. Nils Faergeman.

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Supplementary data

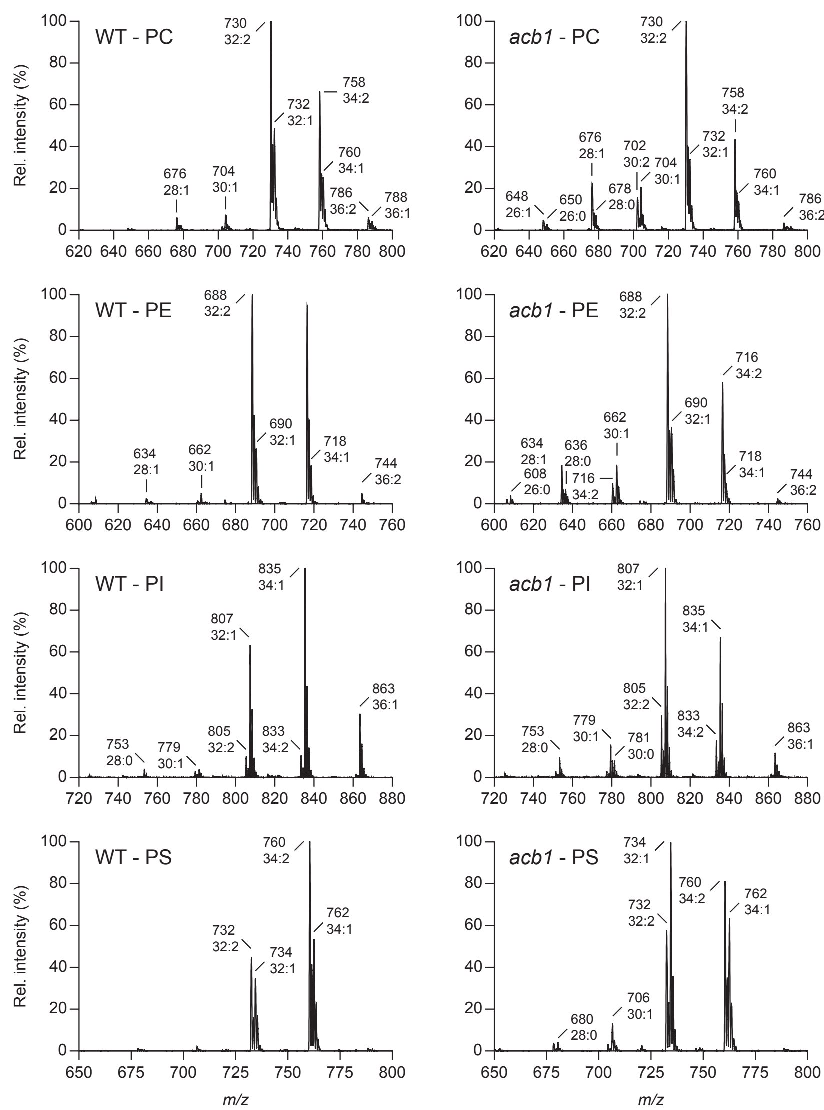

Figure S1 The effect of deleting the *ACB1* gene on the species profiles of phospholipids in yeast. Wild type (WT) and *acb1* cells were cultured in SL medium, harvested at late log-phase, and total lipid extracts were prepared. Phospholipid classes were separated by HPLC, and subjected to MS(/MS) to determine the species compositions of PC, PE, PI, and PS, as indicated in the respective panels. The major $[M+H]^+$ species of PC, PE and PS, as well as the major $[M-H]^-$ species of PI are indicated by their *m/z* values. The species labels indicate total acyl carbons:total acyl double bonds. In each panel, the intensity of the highest peak was set at 100%. See Table S2 for the molecular species assignment per cluster.

Table S1 Relative abundance of fatty acids (mol%, ± SD, n = 4) in total lipid extracts of WT, *acb1*, *crd1* and *crd1acb1* strains grown in SL medium. Lipids were transesterified and the fatty acid methyl esters were quantified by gas chromatography.

Strain	*Fatty acids*						
	C12:0	*C14:0*	*C14:1*	*C16:0*	*C16:1*	*C18:0*	*C18:1*
WT	0.6 ± 0.2	1.2 ± 0.1	0.4 ± 0.0	17.1 ± 1.3	52.0 ± 1.4	5.0 ± 0.9	23.7 ± 1.6
acb1	2.0 ± 0.2	2.3 ± 0.9	2.5 ± 0.6	14.0 ± 1.2	58.5 ± 0.2	3.3 ± 0.3	17.6 ± 0.8
crd1	0.5 ± 0.2	1.1 ± 0.1	0.3 ± 0.0	17.0 ± 0.3	50.2 ± 2.6	5.3 ± 1.6	25.7 ± 1.2
crd1acb1	1.8 ± 0.4	2.5 ± 0.1	2.7 ± 0.1	12.7 ± 0.5	60.2 ± 1.4	2.6 ± 0.2	17.4 ± 0.7

Table S2 Molecular species assignment for PC, PE, PI, PS and PG per cluster with the theoretical *m/z* values indicated

Cluster	*# double bonds*	*Possible acyl chain compositions*[a]	*m/z for indicated phospholipid class*				
			PC	*PE*	*PI*	*PS*	*PG*[b]
C26	0	(16:0)(C10:0), (C14:0)(C12:0)	650	608	725	652	637
	1	(16:1)(C10:0), (C14:1)(C12:0)	648	606	723	650	635
C28	0	(C18:0)(C10:0), (C16:0)(C12:0), $(C14:0)_2$	678	636	753	680	665
	1	(C18:1)(C10:0), (C16:1)(C12:0), (C14:1)(C14:0)	676	634	751	678	663
	2	$(C14:1)_2$	674	632	749	676	661
C30	0	(C18:0)(C12:0), (C16:0)(C14:0)	706	664	781	708	693
	1	(C18:1)(C12:0), (C16:1)(C14:0), (C16:0)(C14:1)	704	662	779	706	691
	2	(C16:1)(C14:1)	702	660	777	704	689
C32	0	(C18:0)(C14:0), $(C16:0)_2$	734	692	809	736	721
	1	(C18:1)(C14:0), (C16:1)(C16:0), (C18:0)(C14:1)	732	690	807	734	719
	2	(C18:1)(C14:1), $(C16:1)_2$	730	688	805	732	717
C34	0	(C18:0)(C16:0)	762	720	837	764	749
	1	(C18:1)(C16:0), (C18:0)(C16:1)	760	718	835	762	747
	2	(C18:1)(C16:1)	758	716	833	760	745
C36	0	$(C18:0)_2$	790	748	865	792	777
	1	(C18:1)(C18:0)	788	746	863	790	775
	2	$(C18:1)_2$	786	744	861	788	773

[a] Only combinations of C10-C18 acyl chains are listed. Note: not all combinations are present in equal amounts, since some acyl chains are more abundant than others (see also Table S1). [b] For peak assignments in Figure 8A, the listed *m/z* values should be increased by 5 to account for the presence of d5-glycerol in labeled PG.

Table S3 MLCL and CL molecular species assignment per cluster with the theoretical *m/z* values indicated

Lipid	*Cluster*	*Possible acyl chain compositions*[a]	*m/z for indicated # of double bonds*				
			0	*1*	*2*	*3*	*4*
MLCL	C38	(C18)(C10)$_2$, (C16)(C12)(C10), (C14)$_2$(C10), (C14)(C12)$_2$	486	485	484	-	-
	C40	(C18)(C12)(C10), (C16)(C14)(C10), (C16)(C12)$_2$, (C14)$_2$(C12)	500	499	498	-	-
	C42	(C18)(C14)(C10), (C18)(C12)$_2$, (C16)$_2$(C10), (C16)(C14)(C12), (C14)$_3$	514	513	512	511	-
	C44	(C18)(C16)(C10), (C18)(C14)(C12), (C16)$_2$(C12), (C16)(C14)$_2$	528	527	526	525	-
	C46	(C18)$_2$(C10), (C18)(C16)(C12), (C18)(C14)$_2$, (C16)$_2$(C14)	542	541	540	539	-
	C48	(C18)$_2$(C12), (C18)(C16)(C14), (C16)$_3$	556	555	554	553	-
	C50	(C18)$_2$(C14), (C18)(C16)$_2$	570	569	568	567	-
	C52	(C18)$_2$(C16)	584	583	582	581	-
	C54	(C18)$_3$	598	597	596	595	-
CL	C52	(C18)(C14)(C10)$_2$, (C18)(C12)$_2$(C10), (C16)$_2$(C10)$_2$, (C16)(C14)(C12)(C10), (C16)(C12)$_3$, (C14)$_3$(C10), (C14)$_2$(C12)$_2$	591	590	589	588	-
	C54	(C18)(C16)(C10)$_2$, (C18)(C14)(C12)(C10), (C18)(C12)$_3$, (C16)$_2$(C12)(C10), (C16)(C14)$_2$(C10), (C16)(C14)(C12)$_2$, (14)$_3$(C12)	605	604	603	602	-
	C56	(C18)$_2$(C10)$_2$, (C18)(C16)(C12)(C10), (C18)(C14)$_2$(C10), (C18)(C14)(C12)$_2$, (C16)$_2$(C14)(C10), (C16)$_2$(C12)$_2$, (C16)(C14)$_2$(C12), (C14)$_4$	619	618	617	616	615
	C58	(C18)$_2$(C12)(C10), (C18)(C16)(C14)(C10), (C18)(C16)(C12)$_2$, (C18)(C14)$_2$(C12), (C16)$_3$(C10), (C16)$_2$(C14)(C12), (C16)(C14)$_3$	633	632	631	630	629
	C60	(C18)$_2$(C14)(C10), (C18)$_2$(C12)$_2$, (C18)(C16)$_2$(C10), (C18)(C16)(C14)(C12), (C18)(C14)$_3$, (C16)$_3$(C12), (C16)$_2$(C14)$_2$	647	646	645	644	643
	C62	(C18)$_2$(C16)(C10), (C18)$_2$(C14)(C12), (C18)(C16)$_2$(C12), (C18)(C16)(C14)$_2$, (C16)$_3$(C14)	661	660	659	658	657
	C64	(C18)$_3$(C10), (C18)$_2$(C16)(C12), (C18)$_2$(C14)$_2$, (C18)(C16)$_2$(C14), (C16)$_4$	675	674	673	672	671
	C66	(C18)$_3$(C12), (C18)$_2$(C16)(C14), (C18)(C16)$_3$	689	688	687	686	685
	C68	(C18)$_3$(C14), (C18)$_2$(C16)$_2$	703	702	701	700	699
	C70	(C18)$_3$(C16)	717	716	715	714	713
	C72	(C18)$_4$	731	730	729	728	727

[a] Only compositions based on C10-C18 acyl chains are listed. Note: not all combinations are present in equal amounts, since some acyl chains are more abundant than others (see also Table S1).

CHAPTER 5

Summarizing discussion

The aim of the research described in this thesis was to gain insight into the significance of phosphatidylcholine (PC) for the functioning of mitochondrial proteins and to advance the understanding of acyl chain remodeling of the specific mitochondrial lipid cardiolipin (CL). In the current chapter, the findings will be briefly summarized and discussed in the light of (recent) literature. In addition, suggestions for future experiments will be made.

Based on the prominent labeling of Gut2 by the photoactivatable PC analogue TID-PC and other considerations, it was hypothesized that Gut2 activity depends on PC. Such a dependence might serve to align carbon/energy and lipid metabolic pathways. From the results of the *in vivo* and *in vitro* experiments with PC-biosynthetic mutants described in Chapter 2, it was concluded that PC is most likely involved in binding the protein to the membrane, rather than in stimulating Gut2 activity. However, it could not be excluded that residual PC remaining under the conditions of PC depletion used, was sufficient for Gut2 activity. The specific activity apparently remained unchanged after a decrease in PC content from about 42 to 2% of total phospholipids, rendering a role of PC in modulating Gut2 activity to regulate glycerol-3-phosphate (G3P) availability for lipid synthesis unlikely.
Interestingly, deletion of the Gut2 homologue in the yeast *Yarrowia lipolytica* was recently found to increase the rate of lipid accumulation [1], providing evidence that the flux through lipid synthetic pathways indeed depends on Gut2 activity. However, *Y. lipolytica* might not be exemplary for other eukaryotes, considering its rather unique lipid degradation (where it took its name from), utilization and storage capabilities. Furthermore, as discussed in Chapter 2, a decreased Gut2 activity in *S. cerevisiae* was accompanied by an increased glycerol excretion – wasting G3P which could have been used for lipid synthesis.
To determine whether the activity and/or membrane association of Gut2 strictly relies on (residual) PC, several studies were envisioned with purified recombinant Gut2 and lipid vesicles with different lipid compositions. Despite extensive efforts over almost a year, no Gut2 could be obtained that was both active and pure (data not shown). Briefly, plasmid-born N-terminally His-tagged Gut2 expressed in the cytosol of *S. cerevisiae* was active, but purification via metal-affinity and/or ion-exchange chromatography was unsuccessful. The use of *E. coli* as expression system resulted in the production of inclusion bodies containing relatively pure His-tagged Gut2. Initial purification attempts indicated that the His-tag did not have an added value (most impurities were found to contain the His-tag as well, suggesting they were the result of Gut2 degradation), and hence, a new plasmid encoding Gut2 without this tag was constructed. Inclusion bodies produced by *E. coli* after transformation with

this plasmid yielded Gut2 with about 90% purity following urea-solubilization and ion-exchange chromatography. Unfortunately, high-throughput screening of refolding factors did not result in the identification of suitable refolding conditions to get Gut2 in an active conformation.

In the absence of pure and active Gut2, *cho2opi3* cells cultured in the presence of propanolamine might provide an alternative approach to determine whether residual PC is required for Gut2 activity and membrane association. As reported by Choi *et al.* and discussed in Chapter 1, these cells in which PC has been replaced by phosphatidylpropanolamine (PPrN) are viable and can grow on the non-fermentable carbon source (NFCS) lactate [2]. It will be worthwhile to investigate the ability of *cho2opi3* cells to grow on glycerol as sole carbon source in propanolamine-supplemented medium devoid of choline. In case the cells are able to utilize glycerol, it can be concluded that PC is not crucial for Gut2 to be functional. Investigation of the membrane association of Gut2 in these cells will also reveal whether Gut2 requires PC for membrane anchoring. If Gut2 is found to be detached from the membrane, it might also be concluded that binding to the membrane is not important for Gut2 functioning.

The membrane association of Gut2 in the complete absence of PC could also be studied in lactate-grown cells, if glycerol does not support growth. Alternatively, it can be investigated whether mutations in the three potential amphipathic helices have an impact on membrane binding, TID-PC labeling and/or enzyme activity. In this context, it is interesting to note that the recently elucidated structure of the Gut2 homologue GlpD in *E. coli* was found to contain at least one helix embedded horizontally in the membrane [3]. Unfortunately, alignment of the GlpD and Gut2 sequences using a BLAST algorithm (http://blast.ncbi.nlm.nih/gov/Blast.cgi) did not reveal an obvious match between the membrane helix in GlpD and either one of the three predicted amphiphatic helices in Gut2, despite overall good sequence similarity (E-value $4 \cdot 10^{-40}$; data not shown).

In a recent study, Gut2 was found to be crosslinked by two new photoactivatable PC analogues [4]. It could be argued that the used analogues are not representative for PC due to the attachment of the photoactivatable moieties to the choline headgroup, and hence that the labeling of various proteins merely reflects their association with the membrane rather than a specific interaction with PC. Nevertheless, the fact that Gut2 as a relatively minor protein is abundantly labeled by three different PC analogues unlike many other (trans)membrane proteins strongly suggests that Gut2 indeed specifically interacts with PC, urging for further research into the significance of this interaction.

In Chapter 3, the focus was on the possible role of PC in the utilization of proline as nitrogen source, involving the mitochondrial protein Put1. The investigation was

based on the finding in an mRNA profiling study that the transcript levels of the *PUT1* and *GAP1* genes were increased in PC-depleted cells and on other observations reported in the literature. *In vivo* and *in vitro* experiments demonstrated that PC indeed plays a role in proline utilization. Although a reduced proline uptake appeared to be the bottleneck for growth of *opi3* cells in the absence of choline, PC depletion was also found to impact proline metabolism. These results have already been discussed in Chapter 3, and no relevant articles have been published since. Nevertheless, the intriguing observation that the presumed precursor of specifically Put1 gradually accumulated in cells subjected to PC depletion needs an additional thought. As discussed, the impaired Put1 import was most likely not due to a generic defect in the import and processing of proteins with a cleavable presequence. Almost 20 years ago, Kerber and Soll suggested that a direct interaction with PC was needed for a precursor protein to be imported by chloroplasts [5]. Although to my knowledge such a role for PC has never been confirmed in other studies, it is known that lipids are able to bind with various affinities to mitochondrial presequences [6,7]. Although speculative, it would therefore be interesting to investigate whether PC functions as a chaperone specifically for the Put1 precursor, or whether PC is involved in recruiting this precursor to the import machinery. However, the latter option is less likely, considering that PC is found throughout the cell: the risk of mistargeting the precursor to organelles other than the mitochondria would be high. A possible starting point would be to compare the affinity and specificity by which the presequences of Put1 and Put2 bind to phospholipids or to study a mutant in which the presequences of both proteins have been swapped.

Like for Gut2, yeast cells in which PC has been replaced by PPrN might be a valuable tool to shed light on the function and dispensability of PC in proline utilization, both in proline uptake and metabolism (including the biosynthesis and trafficking of the enzymes involved).

Photolabeling with TID-PC and mRNA profiling following PC depletion did not generate many promising leads besides Gut2 and Put1. Assuming that PC indeed is crucial for the functioning of mitochondrial proteins, the following causes might have hampered the detection and identification of those proteins through photolabeling with TID-PC:

1) Exchange of endogenous PC bound to proteins by TID-PC might be slow or non-existent, especially if proteins hold to PC with high affinity, if the lipid binding pocket is not (easily) accessible, or if the TID moiety (sterically) interferes with binding;
2) Less abundant proteins are easily missed, due to scavenging of TID-PC by abundant proteins and, obviously, due to lack of sensitivity in the readout;
3) The photoactivatable moiety might not have access to PC-interacting proteins that are soluble or only shallowly penetrate the membrane.

As to mRNA profiling, pleiotropic effects might have masked relevant interactions. Furthermore, it should be kept in mind that many enzymes are not regulated at the level of gene transcription or mRNA stability.

How to generate more leads for studying the role of PC in mitochondrial functioning? The following two approaches might help:

1) The use of yeast cells with PPrN replacing PC might allow the photolabeling of proteins that would otherwise have bound PC with high affinity (by the way, it would be interesting to find out whether the addition of *N,N,N*-trimethyl-methanolamine would be able to rescue *cho2opi3* cells in the absence of choline);
2) Yeast cells might be fed radiolabeled choline and 10-azi-stearic acid (10-ASA; a photoactivatable fatty acid) to allow the biosynthesis of photoactivatable and radioactively labeled PC. Upon UV-irradiation, proteins interacting with this PC analogue might be crosslinked *in vivo* and visualized by virtue of the radioactive label. This approach has been shown to work for mammalian cells [8] and is particularly elegant, considering that (i) labeling occurs *in vivo*, (ii) the photoactivatable PC might be incorporated in newly synthesized proteins and protein complexes (avoiding that high-affinity binding pockets for PC are already occupied by regular PC), and (iii) the photoactivatable moiety is so small that it is less likely to interfere with interactions involving the acyl chains.

To investigate the possible functional interactions between PC and a protein of interest, PC depletion might not be the most suited approach, considering the pleiotropic effects it invokes. Instead, one could consider the use of cells in which PC has been replaced to varying extents by PPrN – for instance by supplementing the cells with choline and propanolamine in different ratios – to study protein activities, membrane associations and protein complex stabilities. It should be kept in mind, that although PC might be essential for the functioning of a particular protein, the protein itself might not be essential for the viability or growth of the cell, and as a consequence *in vivo* experiments might not always be fruitful in shedding light on the function of a particular PC-protein interaction.

In the second part of the thesis, *acb1* mutants were exploited to gain insight into CL remodeling, triggered by the finding that CL from Acb1-depleted cells contains elevated levels of acyl chains shorter than C16. The experimental data demonstrated that the CL remodeling system in *S. cerevisiae* does not strive for the removal of those shorter chains, but rather, that it specifically focuses on the replacement of C16:0

chains in CL molecules. This conclusion was consistent with the reported substrate preference of the newly discovered CL-specific lipase Cld1 [9]. The CL remodeling mechanism proposed in Chapter 4 (selective outflow of acyl chains via Cld1 followed by non-selective inflow via Taz1) is able to account for the CL profile observed in *S. cerevisiae* including those in *taz1* and *acb1* cells, and for the accumulation of MLCL in the former and not in the latter mutant strain.

As discussed in Chapter 1, two other hypotheses have been postulated in literature with respect to the mechanism of CL remodeling: the Lands' cycle and Taz1-mediated remodeling without involvement of a CL lipase. To start with the latter, recent calculations based on this model demonstrated that a high degree of symmetry in CL molecules could be established despite the lack of a distinct substrate specificity of tafazzin [10], lending it credibility. However, it would be interesting to check whether similar calculations based on selective outflow and non-selective inflow of acyl chains by the combined action of Cld1 and Taz1 or based on the Lands' cycle with most likely selective inflow would also result in accurate predictions of CL profiles. Furthermore, lipase-independent exclusive Taz1-mediated CL remodeling does not provide a plausible explanation for the accumulation of MLCL in the absence of Taz1. It has been suggested that abnormal CL in the absence of Taz1 (or its homologues in other eukaryotes) is more susceptible to degradation [11], but this raises questions as to why MLCL apparently is not degraded further, why abnormal CL in *acb1* cells is not degraded too and why differences in CL profiles between tissues within the same organism [12] are tolerated. Another explanation for the MLCL accumulation in the absence of Taz1 that has been put forward is that acylCoA-dependent reacylation of lysophospholipids can no longer drive the reacylation of MLCL [11]. However, this does not explain why there is so much MLCL in the first place. Furthermore, the explanation seems to be conflicting with the proposed model itself in which lysophospholipids only serve as catalytic intermediates and in which remodeling depends on the entry or exit of CL and phospholipids (and not of lysophospholipids).

Although the Lands' cycle does not play a major role, if any, in the remodeling of CL in *S. cerevisiae* considering the involvement of the acylCoA-independent Taz1, it is increasingly receiving attention for other eukaryotes. A recent study has provided compelling evidence for the existence of an acylCoA-dependent MLCL acyltransferase in human cells [13], supporting earlier results for rats [14,15] and pigs [16]. Interestingly, unlike Taz1, the acylCoA-dependent enzyme displays a clear substrate specificity [13] allowing selective inflow of acyl chains into the CL pool. In several organisms, a calcium-independent phospholipase A_2 has been found which deacylates CL [17-19]. It is currently unknown, whether this lipase acts in concert with the acylCoA-dependent MLCL acyltransferase and whether it is the functional homologue of Cld1, or both. Further research is needed to shed more light on the physiological roles of these remodeling enzymes.

In spite of years of research, not much is known about the biological significance of CL remodeling. Why do cells invest in a remodeling system for CL? What is the added value of a specific acyl chain composition for CL functioning, in CL-protein interactions and/or as a membrane constituent? Although the acyl chains of CL might be actively involved in a number of CL-protein interactions, it is conceivable that only a minority of the interactions (if any) depends on the presence of a particular CL species. Hence, also without CL remodeling, sufficient amounts of the required species should be available in the CL pool. Furthermore, if CL-protein interactions would drive the need for CL remodeling, one would expect similar CL species across tissues of the same organism, since they contain the same proteins (albeit at different expression levels). However, significant differences in the CL species profile have been recorded between tissues of the same organism [12]. Lastly, analysis of protein complexes in wild type, *acb1*, *taz1* and *taz1acb1* mitochondria indicated that the CL and/or monolyso-CL (MLCL) content have a larger impact on complex stability than the CL acyl chain composition [20].
Considering the arguments above and the fact that the acyl chain composition is an important determinant of the properties of membrane lipids, it is more likely that remodeling aims to render CL fit for its role as a membrane constituent than as an interaction partner for proteins. In this context, it is interesting to recall that the non-bilayer lipid phosphatidylethanolamine (PE) is required for viability in the absence of CL (Chapter 1 and [21]). The non-bilayer propensity of CL itself largely depends on its acyl chains and especially the degree of unsaturation of those chains. Considering that remodeling in yeast comprises the selective replacement of C16:0 by other chains that are usually unsaturated (Chapter 4 and [9]), it is conceivable that CL remodeling aims to increase the non-bilayer propensity of CL through the introduction of unsaturated acyl chains. It has been pointed out that the degree of unsaturation in CL greatly varies between and even within organisms [12]. However, it should be kept in mind that this variation might be caused by differences in mitochondrial demand, available acyl chains for CL remodeling, environmental conditions like the presence of divalent cations (which can also promote the hexagonal phase of CL; see Chapter 1) and the overall membrane composition.
The high degree of unsaturation generally observed renders CL susceptible to peroxidation, so what would be the benefit for mitochondria of non-bilayer CL compared to for instance PE? I speculate that remodeled CL is found in close association with respiratory proteins, forming a relatively fluid environment for those proteins without grossly affecting the properties of the bulk membrane. The increased fluidity might promote the mobility of electron carriers and the dynamics of respiratory proteins. Alternatively, or additionally, remodeled CL with its high intrinsic curvature might more readily segregate into curved CL-rich membranes,

like the cristae in mitochondria [22], thereby facilitating the formation of a proton sink and consequently enhancing the performance of the ATP synthase [23]. Thus, the remodeling of CL might enhance mitochondrial functioning and/or efficiency. In this context, it is worth mentioning that the C16:0 content in CL was distinctly lower in wild type yeast cells grown on NFCS compared to glucose [24].

Testing the hypothesis that remodeling aims to increase the non-bilayer propensity of CL might be difficult, considering that yeast cells are even able to cope with the complete absence of CL – changes in the acyl chain composition of this lipid will be less of an issue for cells. Nevertheless, a start could be to study the growth of wild type, *cld1*, *cld1psd1*, *cld1acb1* and *cld1psd1acb1* cells on lean media, as well as the respiratory competence (*e.g.*, P/O ratios) of their mitochondria as a function of the lipid composition (including species profiles of each lipid class, especially PE). Additionally, the effect of divalent cations on mitochondrial activity might be investigated, considering that they influence the inclination of CL to form non-bilayer membrane structures. More elaborate approaches could include site-directed mutagenesis of Cld1 to change the substrate specificity, or the expression of the recently identified human MLCL acyltransferase [13] in *taz1*cells, both to ultimately change the CL species profile. Since mitochondria are such complex and delicate structures, caution should be exercised so as not to create artifacts.

As mentioned above, CL from glucose-cultured yeast cells contains significantly more C16:0 than CL from cells grown on a NFCS. This difference supports the notion that the acyl chain composition of CL is important for mitochondrial functioning, but it also suggests that CL remodeling is subject to some form of regulation. Furthermore, it would be interesting to investigate whether this difference also exists when cells are cultured successively on NFCS and glucose. If so, although speculative, there might be a system for ‘reverse’ remodeling: the replacement of unsaturated acyl chains by C16:0 when cells switch to glucose metabolism.

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Nederlandse samenvatting

Biologische cellen en intracellulaire compartimenten met specifieke functies (organellen geheten) zijn gescheiden van hun omgeving door vliezen met een dikte van slechts ongeveer 35 Å (1/10.000 van een menselijke haar). Deze vliezen, hierna aangeduid als membranen, worden gevormd door lipiden die in twee lagen tegenover elkaar liggen. De hydrofobe ('water-vrezende') vetzuren of acylketens van tegenover elkaar liggende lipiden in een membraan zijn naar elkaar toe gericht, terwijl de wateroplosbare kopgroepen van de lipiden juist naar buiten gericht zijn. De kopgroepen schermen zo de hydrofobe acylketens af van de waterige omgeving van het membraan (Figure 1 in Chapter 1). Door hun hydrofobe kern zijn membranen nagenoeg ondoordringbaar voor veel stoffen, waardoor verschillen tussen de omgevingen aan beide kanten van een membraan gecreëerd en in stand gehouden kunnen worden. Om toch bijvoorbeeld voedings- en afvalstoffen over membranen te transporteren, zijn er in membranen speciale eiwitten aanwezig die fungeren als kanalen of pompen.
Naast deze transporters bevatten membranen diverse andere membraaneiwitten. Er worden twee typen onderscheiden; de zogeheten transmembraaneiwitten steken dwars door een membraan heen, terwijl de perifere membraaneiwitten slechts oppervlakkig met een membraan verbonden zijn. Aangezien in elk compartiment andere processen plaatsvinden, bevat elk membraan een eigen, karakteristieke set membraaneiwitten. Zelfs binnen membranen kunnen er domeinen onderscheiden worden met specifieke taken en bijbehorende membraaneiwitten.
Diversiteit in membranen zit echter niet alleen in verschillen qua gehuisveste eiwitten, maar ook qua lipidensamenstelling. Een groot deel van de lipiden behoort tot de groep van fosfolipiden, die onderverdeeld wordt in klassen op basis van de kopgroep (Figure 5 in Chapter 1). Afhankelijk van het membraan, kunnen er van elke klasse meer of minder lipiden aanwezig zijn (zie bijvoorbeeld Table I in Chapter 1). Deze verscheidenheid geeft aan dat lipiden niet slechts bouwstenen van membranen zijn, maar dat ze ook andere, meer gespecialiseerde functies hebben. Een van deze functies is dat ze een geschikte omgeving creëren voor het functioneren en/of de stabiliteit van membraaneiwitten en complexen daarvan. Kennis van de synthese, eigenschappen en functies van lipiden is daarom cruciaal voor het goed begrijpen van cellulaire processen.

Om deze kennis te vergroten is en wordt vaak gebruik gemaakt van het ééncellige organisme *S. cerevisiae*, oftewel bakkersgist. Naast bekend verondersteld toepassingen in het maken van brood, bier en wijn, is bakkersgist ook een geliefd studieobject. Gistcellen groeien snel onder uiteenlopende omstandigheden, ze kunnen makkelijk en ingrijpend genetisch gemodificeerd worden, en er zijn geen

ethische kwesties waarmee rekening gehouden dient te worden, zoals bij proefdieren. Het belangrijkste is echter dat de cellulaire processen in bakkersgist erg lijken op die in de mens, ondanks de grote uiterlijke verschillen. Conclusies gebaseerd op experimenten met bakkersgist kunnen dan ook vaak geëxtrapoleerd worden naar de mens.

Het werkterrein in dit proefschrift is het mitochondrion, een celorganel dat het beste omschreven kan worden als de 'energiecentrale' van de cel. Mitochondriën hebben een bijzondere structuur, omdat ze in tegenstelling tot de meeste andere organellen omsloten zijn door twee onderscheiden membranen (Figure 2 in Chapter 1). Het buitenste membraan is relatief permeabel door de vele kanaal-vormende eiwitten. De mitochondriële matrix, waarin bijvoorbeeld het mitochondriële genoom zich bevindt, is omsloten door de binnenste membraan. Dit ingestulpte membraan bevat onder andere de grote eiwitcomplexen die deel uitmaken van de zogeheten ademhalingsketen (zie hieronder). De ruimte tussen de beide membranen wordt de tussenmembraanruimte (IMS) genoemd. Hoewel mitochondriën vaak afgebeeld worden als een soort 'jelly beans', vormen ze in de cel een vertakt en buisvormig dynamisch netwerk (Figure 2 in Chapter 1).

Naast diverse functies die hier verder onbesproken blijven, is het genereren van ATP via de ademhalingsketen de belangrijkste taak van de mitochondriën. ATP is een energierijk molecuul, dat overal in de cel gebruikt wordt om de benodigde energie voor chemische omzettingen en andere processen te leveren. In afwezigheid van zuurstof kunnen gistcellen alleen energie halen uit fermenteerbare koolstofbronnen, zoals glucose. Elk glucose molecuul levert na omzetting via de zogeheten glycolyse route twee pyrodruivenzuur, twee ATP en twee NADH moleculen. Pyrodruivenzuur kan vervolgens verder omgezet worden in ethanol, onder verbruik van de gevormde NADH. In de aanwezigheid van zuurstof kan pyrodruivenzuur (na decarboxylering) volledig geoxideerd worden tot CO_2 via de citroenzuur- of Krebscyclus in de mitochondriële matrix. De energie die hierbij vrijkomt, wordt gebruikt om FAD en NAD^+ te reduceren tot respectievelijk $FADH_2$ en NADH, en om guanosinetrifosfaat (GTP) te vormen. Opgemerkt dient te worden dat de NADH die tijdens glycolyse in het cytosol gevormd wordt, niet in de mitochondriële matrix kan komen. Diverse systemen in de binnenste membraan, waaronder de glycerol-3-fosfaat (G3P) shuttle (Figure 3 in Chapter 1), zijn in staat om de in NADH opgeslagen energie toch ten goede te laten komen aan ATP synthese.
Via de ademhalingsketen worden de energierijke $FADH_2$ en NADH weer geoxideerd tot respectievelijk FAD en NAD^+. De energie die hierbij vrijkomt, wordt gebruikt om protonen vanuit de mitochondriële matrix naar de IMS te pompen. Door het stuwmeer aan protonen vanuit de IMS via het ATP synthase eiwitcomplex terug naar de matrix

te leiden, kan ATP gemaakt worden. Dankzij de ademhalingsketen levert elk glucose molecuul ongeveer 30 in plaats van 2 ATP moleculen. Voor niet-fermenteerbare koolstofbronnen, zoals glycerol, melkzuur en ethanol, is de ademhalingsketen absoluut noodzakelijk voor de generatie van ATP.

Het doel van het onderzoek beschreven in dit proefschrift was om inzicht te krijgen in:

1) Betekenis van fosfatidylcholine (PC), het meest voorkomende fosfolipide in mitochondriën, voor het functioneren van mitochondriële eiwitten;
2) Acylketen remodeling (vervanging van een acylketen in een lipide door een andere) van het specifiek mitochondriële lipide cardiolipine (CL).

PC kan door gistcellen op twee manieren gemaakt worden, te weten via i) methylering van het lipide fosfatidylethanolamine (PE) door de eiwitten Cho2 en Opi3, en ii) koppeling van choline aan het lipide diacylglycerol (DAG) door het eiwit Cpt1 (Figure 6 in Chapter 1). Als de eerste route (genetisch) uitgeschakeld is, kunnen gistcellen nog steeds normale hoeveelheden PC maken via de tweede route, mits er voldoende choline aanwezig is in hun omgeving.
Door cellen zonder Cho2 en Opi3 eerst te laten groeien in de aanwezigheid van choline en hen vervolgens over te brengen naar een choline-vrije omgeving, kan de hoeveelheid PC per cel verlaagd worden: bij elke celdeling moet de aanwezige PC verdeeld worden over de moeder- en dochtercel. Na 4 of 5 celdelingen in afwezigheid van choline is het gehalte aan PC in elke cel zover gedaald dat de cellen stoppen met groeien. Gistcellen die alleen Cho2 of Opi3 missen, kunnen nog wel voldoende PE methyleren tot PC om te overleven in afwezigheid van choline, maar het PC gehalte is beduidend lager dan in normale cellen.
Ondanks het feit dat gistcellen kunnen overleven met minder PC, kan depletie van dit lipide wel gevolgen hebben voor het functioneren van de mitochondriën. Uiteraard kan dit voor een deel veroorzaakt worden door verstoringen in de structuur van de mitochondriële membranen, waarvan PC een belangrijke component is, maar daarnaast is het waarschijnlijk dat er specifieke eiwitten zijn die adequate hoeveelheden PC nodig hebben om optimaal te functioneren.
Om dergelijke eiwitten te identificeren, werden mitochondriën uit gistcellen geïsoleerd en in contact gebracht met PC waarin een normale acylketen vervangen was door een foto-activeerbare variant. Na bestraling met UV-licht wordt deze acylketen reactief, zodat het lipide via een chemische reactie gekoppeld kan worden aan naburige eiwitten. De G3P dehydrogenase Gut2 werd erg prominent gelabeld door dit foto-activeerbare lipide, wat een speciale interactie suggereerde tussen PC en dit eiwit. Gut2 is essentieel voor het gebruik van glycerol als koolstof- en energiebron (Gut komt van Glycerol utilization); het oxideert G3P tot dihydroxyacetonfosfaat (DHAP).

Deze omzetting is ook onderdeel van de hierboven genoemde G3P shuttle (Figure 3 in Chapter 1), een systeem dat NADH uit het cytosol regenereert tot NAD^+. Energie uit NADH die hierbij vrijkomt, kan via de G3P shuttle doorgegeven worden aan de ademhalingsketen en zo gebruikt worden voor de synthese van ATP.
Een mogelijke verklaring voor de interactie tussen Gut2 en PC is dat de activiteit van het eiwit afhangt van de PC concentratie. Een dergelijke afhankelijkheid zou een rol kunnen spelen in het afstemmen van de Gut2 activiteit op de cellulaire behoefte aan fosfolipiden. In geval van een tekort aan fosfolipiden zou Gut2 minder gestimuleerd worden door PC, waardoor er meer G3P overblijft voor de aanmaak van fosfolipiden (zie ook Figure 6 in Chapter 1). Bij een overschot aan fosfolipiden (inclusief PC) zou Gut2 extra gestimuleerd worden, zodat de aanmaak van nieuwe lipiden afgeremd wordt door een verlaagd G3P gehalte. Deze hypothese werd gesteund door indirect bewijs, wat hier verder onbesproken blijft.
In Chapter 2 is het onderzoek naar de mogelijke invloed van PC op het functioneren van Gut2 beschreven. PC depletie resulteerde in een grotere afname van de groei van gistcellen op glycerol dan op koolstofbronnen waar Gut2 niet essentieel is. Daarnaast werd er meer glycerol uitgescheiden naarmate het PC gehalte in cellen lager was. Beide resultaten suggereren dat in intacte cellen het functioneren van Gut2 inderdaad afhankelijk is van het PC gehalte.
Om inzicht te krijgen in het onderliggende mechanisme, werden mitochondriën uit cellen in verschillende stadia van PC depletie geïsoleerd en nader bestudeerd. Het bleek dat de totale activiteit van Gut2 in mitochondriële preparaten (gecorrigeerd voor de hoeveelheid Gut2) niet daalde, zelfs niet wanneer het PC gehalte gereduceerd was van ruim 40% tot zo'n 2% van het totaal aan fosfolipiden. Ook werd er geen effect van PC depletie op de binding van Gut2 aan het membraan waargenomen, wat een rol had kunnen spelen in de regulering van de Gut2 activiteit.
Hoewel niet kon worden uitgesloten dat de kleine hoeveelheid PC die ook na maximale depletie achter was gebleven, voldoende was voor maximale stimulering en/of binding van Gut2 aan het membraan, is een afstemming van de Gut2 activiteit door PC op de synthese van fosfolipiden niet waarschijnlijk.
De resultaten van de *in vivo* experimenten die in tegenstelling tot die van de *in vitro* experimenten wel een functionele interactie tussen PC en Gut2 suggereren, reflecteren mogelijk een indirect effect van PC depletie op het functioneren van Gut2, bijvoorbeeld door verstoorde cellulaire structuren. De interactie tussen PC en Gut2, zoals gevonden met behulp van de foto-activeerbare PC variant, zou een bijdrage kunnen leveren aan de binding van Gut2 aan het membraan, hoewel andere fosfolipiden hier via hydrofobe interacties met Gut2 ook een rol zouden kunnen spelen.
Vervolgonderzoek zou zich kunnen richten op de mogelijke betekenis van achtergebleven PC op de activiteit van Gut2 en op mogelijke interacties van PC met

Gut2 in de context van membraan binding.

Een andere strategie om functionele interacties tussen eiwitten en PC te identificeren, is te bestuderen hoe cellen reageren op PC depletie op het niveau van gentranscriptie. Om te compenseren voor het verlies aan activiteit van PC-afhankelijke eiwitten zouden cellen de productie van deze eiwitten kunnen stimuleren door verhoogde transcriptie van de corresponderende genen. Met andere woorden, in cellen met een verlaagd PC gehalte zou de mRNA concentratie van PC-afhankelijke eiwitten omhoog kunnen gaan. Een zogeheten mRNA profiling studie resulteerde onder andere in de ontdekking dat de mRNA niveaus van de *GAP1* en *PUT1* genen verhoogd waren in cellen met een gereduceerd PC gehalte. De gecodeerde eiwitten, te weten Gap1 and Put1, zijn respectievelijk betrokken bij de opname van proline en de omzetting ervan tot pyrroline-5-carboxylaat (P5C; zie Figure 4 in Chapter 1). Deze omzetting door Put1 in de mitochondriën is onmisbaar om proline als stikstofbron te kunnen gebruiken – de naam Put komt van Proline utilization.
Het verhoogde mRNA niveau van genoemde genen vormde samen met gegevens uit de literatuur aanleiding om te onderzoeken of PC een rol speelt in het gebruik van proline als stikstofbron. Uit dit onderzoek, beschreven in Chapter 3, bleek dat PC depletie de groei van gistcellen veel sterker remde op proline dan op glutamaat als stikstofbron, wat sterk suggereerde dat PC inderdaad een specifieke rol speelt in de processen die nodig zijn voor de omzetting van proline in glutamaat. Verdere experimenten gaven aan dat met name de opname van proline een knelpunt was voor de groei van cellen met een verlaagd PC gehalte – mogelijk door een verminderde hoeveelheid proline transporters in het plasma membraan. Echter, ook de omzetting van proline naar glutamaat door Put1 en Put2 bleek minder efficiënt te gaan in PC-gedepleteerde cellen. Analyse van de hoeveelheid Put1 en Put2 suggereerde dat dit veroorzaakt zou kunnen zijn door een verlaagd Put1 gehalte in de mitochondriën, alhoewel andere oorzaken niet uitgesloten kunnen worden. Een interessante waarneming was dat de mogelijke ‘precursor’ van Put1 (de precursor bevat ten opzichte van normaal of ‘mature’ Put1 een extra stukje peptide dat de eindbestemming van het eiwit aangeeft – in dit geval de mitochondriële matrix waar dit stukje peptide afgeknipt wordt) zich meer en meer ophoopte buiten de mitochondriën bij voortschrijdende PC depletie. Deze ophoping werd niet gevonden voor Gut2 en Put2, waarvan de precursor ook een dergelijke peptide bevat. Verder onderzoek zal licht moeten werpen op een mogelijk specifieke taak van PC bij de import van Put1 door de mitochondriën. Daarnaast kan de betekenis voor de activiteit van Put1 nog verder uitgezocht worden, net als de rol van PC voor de activiteit en/of lokalisering van aminozuurtransporters.

In Chapter 5 zijn suggesties gedaan voor vervolgonderzoek naar de rol van PC

voor de mitochondriële eiwitten Gut2 en Put1, en voor alternatieve aanpakken om eiwitten te identificeren met een mogelijke afhankelijkheid van PC. Bij de bestudering van mogelijke PC-eiwit interacties dient terdege rekening gehouden te worden met mogelijke pleiotrope effecten van PC depletie.

Het tweede lipide dat centraal staat in dit proefschrift is CL. CL wordt alleen in mitochondriën gevonden, waar het een belangrijke bouwsteen is van met name het binnenste mitochondriële membraan. Daarnaast stabiliseert en/of stimuleert CL diverse essentiële mitochondriële eiwitcomplexen. CL wordt door Crd1 in de mitochondriën gemaakt door samenvoeging van de lipiden fosfatidylglycerol (PG) en cytidinedifosfaat-diacylglycerol (CDP-DAG; zie Figure 6 in Chapter 1). Hierdoor heeft het vergeleken met andere fosfolipiden een unieke structuur met vier in plaats van de gebruikelijke twee acylketens. Na de synthese van CL kan een zogeheten remodelingsmechanisme acylketens vervangen door andere en zo grotendeels de acylketensamenstelling bepalen van CL, die geacht wordt belangrijk te zijn voor de functies van dit lipide.
In een onderzoek naar eiwitten die mogelijk betrokken zijn bij remodeling van PC was opgevallen dat in de afwezigheid van Acb1 (acylCoA-binding protein) niet alleen de acylketensamenstelling van PC afweek van het normale stramien, maar ook die van CL. Ondanks de aanwezigheid van een intact remodelingsmechanisme voor CL werd er een sterk verhoogd gehalte aan korte acylketens (minder dan 16 koolstofatomen lang; <C16) gevonden. Blijkbaar was het remodelingssysteem niet in staat of werd het niet geactiveerd om de korte ketens te vervangen; dit maakte gistcellen met een uitgeschakeld *ACB1* gen interessant voor onderzoek naar de remodeling van CL.
In Chapter 4 is het onderzoek naar de onderliggende reden van de ophoping van korte acylketens in CL in *acb1* cellen beschreven. Met behulp van massaspectrometrie (een techniek om componenten in een mengsel te onderscheiden op basis van hun moleculaire massa) werd CL van verschillende gistmutanten geanalyseerd om de afkomst van de korte acylketens te identificeren: *de novo* synthese of remodeling. Uit de resultaten bleek dat de korte acylketens niet van remodeling afkomstig waren, en dat ze dus via de precursors PG en CDP-DAG in CL terecht waren gekomen. Bestudering van beide precursors is om diverse redenen lastig, onder andere vanwege hun lage concentratie. Er werd daarom gebruik gemaakt van een *crd1* mutant, die niet in staat is CL te maken en waarin zich daarom PG ophoopt. In de afwezigheid van Crd1 en Acb1 werden er korte acylketens gevonden in PG, maar niet in die mate dat PG alleen verantwoordelijk kon zijn voor de korte ketens in CL in *acb1* cellen.
Om meer inzicht te krijgen in de relatieve bijdragen van PG en CDP-DAG, werd de substraat specificiteit van Crd1 als functie van de acylketensamenstelling van beide

precursors bepaald, evenals de acylketensamenstelling van CL moleculen met de laagste massa. Met name de observatie dat kort CL hoofdzakelijk combinaties van reguliere (>C14) en hele korte (<C14) acylketens bevatte en dus niet vier acylketens van vergelijkbare lengte, suggereerde dat beide precursors in gelijke mate bijdroegen aan de korte acylketens in CL. Verder onderzoek bevestigde dat kort PG inderdaad combinaties van korte en reguliere acylketens bevatte.
In antwoord op de vraag waarom het CL remodelingsmechanisme de korte ketens in CL in *acb1* cellen niet vervangt, werd geconcludeerd dat de capaciteit van dit mechanisme voldoende zou moeten zijn, maar dat de korte ketens niet herkend worden als zijnde ongeschikt. Gebaseerd op de data in Chapter 4 en de resultaten van een andere onderzoeksgroep werd gesteld dat CL remodeling in gist zich richt op de vervanging van verzadigde C16 acylketens: de lipase Cld1 verwijderd specifiek deze acylketen uit CL, waarna Taz1 de ontstane monolyso-CL (MLCL) weer voorziet van een nieuwe acylketen. Deze cyclus van deacylering-reacylering kan net zo lang doorgaan tot alle verzadigde C16 acylketens vervangen zijn, met als uiteindelijk resultaat een verrijking aan onverzadigde C16 en C18 acylketens ten koste van verzadigd C16.
Samenvattend, de acylketensamenstelling van CL in gist wordt bepaald door de substraat-specificiteit van Cld1 en de acylketensamenstelling van de Taz1-substraten. Recente data van andere groepen geeft echter aan dat er ook alternatieve mechanismen zouden kunnen zijn, in ieder geval in andere organismen.

In Chapter 5 wordt nader ingegaan op de vraag wat de reden kan zijn dat cellen investeren in CL remodeling. Remodeling wordt niet geacht een toegevoegde waarde te hebben voor interacties tussen CL en eiwitten. Het meest waarschijnlijk is dat CL door remodeling (meer) geschikt gemaakt wordt voor het functioneren als membraancomponent, wat met name een positief effect zou kunnen hebben op het functioneren van de ademhalingsketen.

Dankwoord

In de afgelopen jaren zijn er velen geweest die direct of indirect hebben bijgedragen aan de totstandkoming van dit proefschrift. Nu, aan het eind van een enerverende periode, wil ik een aantal mensen publiekelijk bedanken.

Toon, als co-promotor heb je het grootste stempel gedrukt op dit boekje. Ik heb het nooit bijgehouden, maar bij elkaar opgeteld zullen we weken en misschien wel maanden in een goede sfeer samen hebben gezeten om resultaten, vervolgexperimenten en manuscripten te bespreken. Wat me hierbij altijd opviel, was je uitgebreide en parate kennis van oude en recente literatuur. In besprekingen wist je mij ook vaak na te laten denken over details waar ik zelf in eerste instantie geneigd was overheen te stappen. Bewonderenswaardig was ook hoe je omging met de verwikkelingen rond je aanstelling – ik ben blij dat alles uiteindelijk toch goed is terecht gekomen.

Vervolgens Ben. Jouw deskundige begeleiding als promotor was onmisbaar. De snelheid waarmee je manuscripten nakeek en er de pijnpunten uithaalde, was ongekend, evenals je vermogen om door alles heen de hoofdlijnen in de gaten te houden. Het belangrijkste wat ik van jou geleerd heb (of waar je me op z'n minst bewust van hebt gemaakt) is dat ogenschijnlijk tegenstrijdige resultaten niet direct afgeschreven moeten worden: er valt vaak toch nog wel een positieve conclusie te trekken. Ik ben dankbaar dat ik je als promotor heb mogen hebben en ik wens je alle goeds toe, ook voor na je aanstaande emeritaat.

Toon en Ben, naast de concrete bijdrage aan dit boekje, hebben jullie mij de afgelopen jaren geholpen bij het ontwikkelen van competenties waar ik nu dagelijks profijt van heb; bedankt daarvoor. Ook heb ik de ruimte gewaardeerd die jullie gegeven hebben om het proefschrift af te ronden naast mijn huidige werk.

Andere substantiële bijdragen aan dit boekje kwamen van de diverse co-auteurs. Fred Vaz en Riekelt Houtkooper van het AMC, zonder jullie MS resultaten en grondige kennis van CL remodeling was er geen hoofdstuk 4 geweest. Bedankt voor de samenwerking! Fred, ook bedankt voor je bereidheid om plaats te nemen in de beoordelingscommissie. Jos Brouwers, jou ben ik dank verschuldigd voor de analyses van deuterium-gelabelde lipiden, waarvan de resultaten ook in hoofdstuk 4 terecht zijn gekomen. Another major contribution to Chapter 4 was made by two people from Aachen University: Margrit Frentzen en Hana Akbari, thanks a lot for your efforts to determine the substrate species specificity of Crd1!

Uit de groep Biochemie van Membranen ben ik Martijn erkentelijk voor het grondwerk

voor hoofdstuk 3. Bedankt hiervoor, maar ook voor je vetzuur analyses die dan wel in een supplement van hoofdstuk 4 terecht zijn gekomen, maar die daardoor niet minder waardevol waren. Van jou heb ik ook geleerd dat een goede mito-isolatie gepaard gaat met mekkerende schapen.

Via Martijn kom ik bij de andere leden van de TLC, waarmee de resultaten bediscussieerd werden: Matthijs, Henry, Jacob en Cedric. Henry, jou wil ik bedanken voor het leggen van de basis voor hoofdstuk 4, maar ook voor je gezelschap op het lab tot 's avonds laat. Verder m'n jaar- en baai-genoot Jacob: altijd in voor een gesprek, wat eigenlijk maar zelden over het werk ging (waarover wel, weet ik niet meer…). De oprichting van de ABP is je bij deze vergeven. Cedric, gelukkig was ik zo'n beetje klaar met het experimentele werk toen jij met tientallen petrischalen de stoven begon te bezetten. Ik wens je veel succes met het schrijven van je proefschrift.

Simone, jou wil ik hier ook vermelden. Ondanks je inzet hebben we nooit zuiver en actief Gut2 in handen kunnen krijgen. Desalniettemin vond ik het leuk en leerzaam om je te mogen begeleiden. Het is overigens wel heel toevallig dat je promotor familie van mij is!

Naast degenen die hierboven genoemd zijn, waren er vele anderen die weliswaar hun bijdrage niet concreet terug zien in het proefschrift, maar die wel de nodige ondersteuning gaven.

Irene, bedankt voor het regelen van diverse praktische zaken, maar ook voor het zijn van een stabiele factor in een groep die behoorlijk veranderde in de afgelopen jaren. Een andere belangrijke rol was weggelegd voor Dick. Door je vertrek is er een brok technische kunde en een onderhandelingstalent verloren gegaan voor de 6^{e} verdieping. *Even* langskomen in je atelier was er nooit bij, maar onze gesprekken heb ik altijd erg op prijs gesteld. Het scharnier voor het zonnescherm doet het nog steeds (en heb ik inmiddels nagemaakt voor de andere kant)!

Verder ben ik dank verschuldigd aan de vele kamer- en labgenoten, voor de gezelligheid en voor het lenen van glaswerk, reagentia en andere dingen: Dragomir, Edgar, Els, Erica, Henry, Hester, Ingrid, Mobeen, Nick, Robert, Robin, Suat, Vincent en Yvonne. Ingrid, van stagebegeleidster werd je collega/kamergenoot, vervolgens plaatsgenoot en wie schetste mijn verbazing toen bleek dat ik bij je buren in Leiden was gaan werken! Ook met jou heb ik vele goede gesprekken kunnen voeren over van alles en nog wat. Robin, jij bent de enige met wie ik van begin tot eind dezelfde kamer gedeeld heb en dat was zeker geen straf. Ik heb goede herinneringen aan de cursus in Nijmegen (samen met Vincent in de Micra) en de tochten naar de

Spar en Tricolore (gebruikmakend van de buienradar om het geschiktste moment te kiezen). De laatste kamer werd ook gedeeld met Yvonne. Je inlevingsvermogen en belangstelling voor alles en iedereen bewonder ik enorm. Veel succes met je eigen promotie een week later dan de mijne. Vincent kan om meerdere redenen niet onvermeld blijven, maar aangezien die al bij iedereen bekend zijn, behoeft dat geen verdere uitleg. De enige die hem mogelijk evenaarde qua kennis van (obscure) muziek was Robert.

De goede sfeer was ook de verdienste van de andere collega's: analisten, AiO's, postdocs en staf – bedankt hiervoor! De reorganisatie heb ik niet meer aan den lijve ondervonden, maar ik wens iedereen succes in de nieuwe structuur.

Ook mijn collega's in Leiden en met name Harold wil ik bedanken voor de getoonde belangstelling en flexibiliteit. Dit geldt ook voor alle familie en vrienden (misschien dat dit boekje eindelijk duidelijk kan maken waar ik mee bezig geweest ben?), die tevens zorgden voor afleidingen tijdens het hele traject.

Pa en ma, ook jullie bedankt voor de steun en mogelijkheden die jullie gegeven hebben - ook voor het begin van de AiO-periode.

Aan het eind van alles gekomen, wil ik jou bedanken, Heleen. Samen met Aron en Masha heb je de nodige offers gebracht. Dit boekje is ook van jou.

Pieter

Curriculum vitae

List of publications

Curriculum vitae

Pieter Johannes Rijken was born in Hedel, the Netherlands, on November 4th, 1980. After graduating with a gymnasium diploma from the Van Lodensteincollege in Amersfoort in 1998, he studied chemistry at Utrecht University. A short research project on the interaction between lipids and sugars in the department Biochemistry of Membranes of the Faculty of Chemistry under supervision of dr. I.J. Vereyken was followed by a major in the department Membrane Enzymology. Here, he investigated the substrate specificity of patatin and the possible application of this protein in the synthesis of monoacylglycerols under supervision of prof.dr. M.R. Egmond. In 2002, he received his MSc degree in chemistry (*cum laude*). Subsequently, he enrolled as PhD student in the department Biochemistry of Membranes in Utrecht. Under supervision of prof.dr. B. de Kruijff and dr. A.I.P.M. de Kroon, he studied phosphatidylcholine-protein interactions and cardiolipin remodeling in yeast mitochondria, as described in this thesis.
While completing the manuscript of this thesis, the author has been employed by Crucell in Leiden. Initially, he worked as scientist biochemical assays & formulation, but the focus rapidly shifted towards formulation & comparability. As of March 2010, he heads the formulation group as senior scientist with a special interest in antibodies and adenoviruses. Earlier this year, he took up the position of chairman of Crucell’s works council.

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