

# Mitochondrial Cardiolipin Involved in Outer-Membrane Protein Biogenesis: Implications for Barth Syndrome

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## Summary

The biogenesis of mitochondria requires the import of a large number of proteins from the cytosol [1, 2]. Although numerous studies have defined the proteinaceous machineries that mediate mitochondrial protein sorting, little is known about the role of lipids in mitochondrial protein import. Cardiolipin, the signature phospholipid of the mitochondrial inner membrane [3–5], affects the stability of many inner-membrane protein complexes [6–12]. Perturbation of cardiolipin metabolism leads to the X-linked cardio-skeletal myopathy Barth syndrome [13–18]. We report that cardiolipin affects the preprotein translocases of the mitochondrial outer membrane. Cardiolipin mutants genetically interact with mutants of outer-membrane translocases. Mitochondria from cardiolipin yeast mutants, as well as Barth syndrome patients, are impaired in the biogenesis of outer-membrane proteins. Our findings reveal a new role for cardiolipin in protein sorting at the mitochondrial outer membrane and bear implications for the pathogenesis of Barth syndrome.

## Results and Discussion

Mitochondria play crucial roles in cellular bioenergetics, programmed cell death, and the metabolism of amino acids, lipids, heme, and iron [1, 2, 19]. To fulfill these tasks, mitochondria contain ~1000 different proteins, 99% of which have to be imported from the cytosol [1, 2]. Of the mitochondrial lipids, the dimeric phospholipid cardiolipin is characteristic for this organelle [3–5]. Cardiolipin is synthesized in the inner

membrane and affects the stability of various inner-membrane protein complexes, including respiratory chain complexes and metabolite carriers [6–12]. Mitochondria from mutants defective in cardiolipin biosynthesis have a reduced inner-membrane potential  $\Delta\psi$ , leading to a reduction of the  $\Delta\psi$ -dependent protein translocation into the inner membrane [6, 11, 15, 20]. Mutations of the phospholipid transacylase tafazzin, which remodels the fatty acyl side chains of cardiolipin, cause the severe human disease Barth syndrome [13, 14, 17]. The mitochondrial defects of tafazzin mutants have been attributed to defects of inner-membrane protein complexes [15–18]. Interestingly, tafazzin was not only found in the inner membrane, but also in the outer membrane [15, 21]. However, different views have been reported on the presence of cardiolipin in the outer membrane [3, 22–24], and little has been known on a possible function of outer-membrane cardiolipin, the only exception being a controversial discussion on the role of cardiolipin in outer-membrane permeabilization in apoptosis [23–26]. Because apoptotic defects have not been found in Barth syndrome [26], experimental evidence for a functional relation of Barth syndrome and the outer mitochondrial membrane has been lacking.

To determine the presence of cardiolipin in the outer membrane of *Saccharomyces cerevisiae* mitochondria, we prepared highly pure outer-membrane vesicles from isolated yeast mitochondria. The liquid chromatography/mass spectrometry profile demonstrated the presence of cardiolipin in the outer membrane (Figure 1A, top; total mitochondrial membranes are shown for comparison, bottom). A quantitative determination revealed that the concentration of cardiolipin in the outer membrane was 25% of the cardiolipin concentration of total mitochondrial membranes (Figure 1B). To determine the purity of the outer-membrane preparation, we performed a western blot titration from 100% to 1%, demonstrating that a contamination with inner-membrane marker proteins (Cox1, Sdh4) was below the detection limit and thus far below 1% (Figure 1B). In contrast, a fraction of tafazzin (Taz1) was clearly present in the purified outer membranes. With an antiserum against the authentic (untagged) protein, we found Taz1 at a 16% level in the outer membrane (Figure 1B).

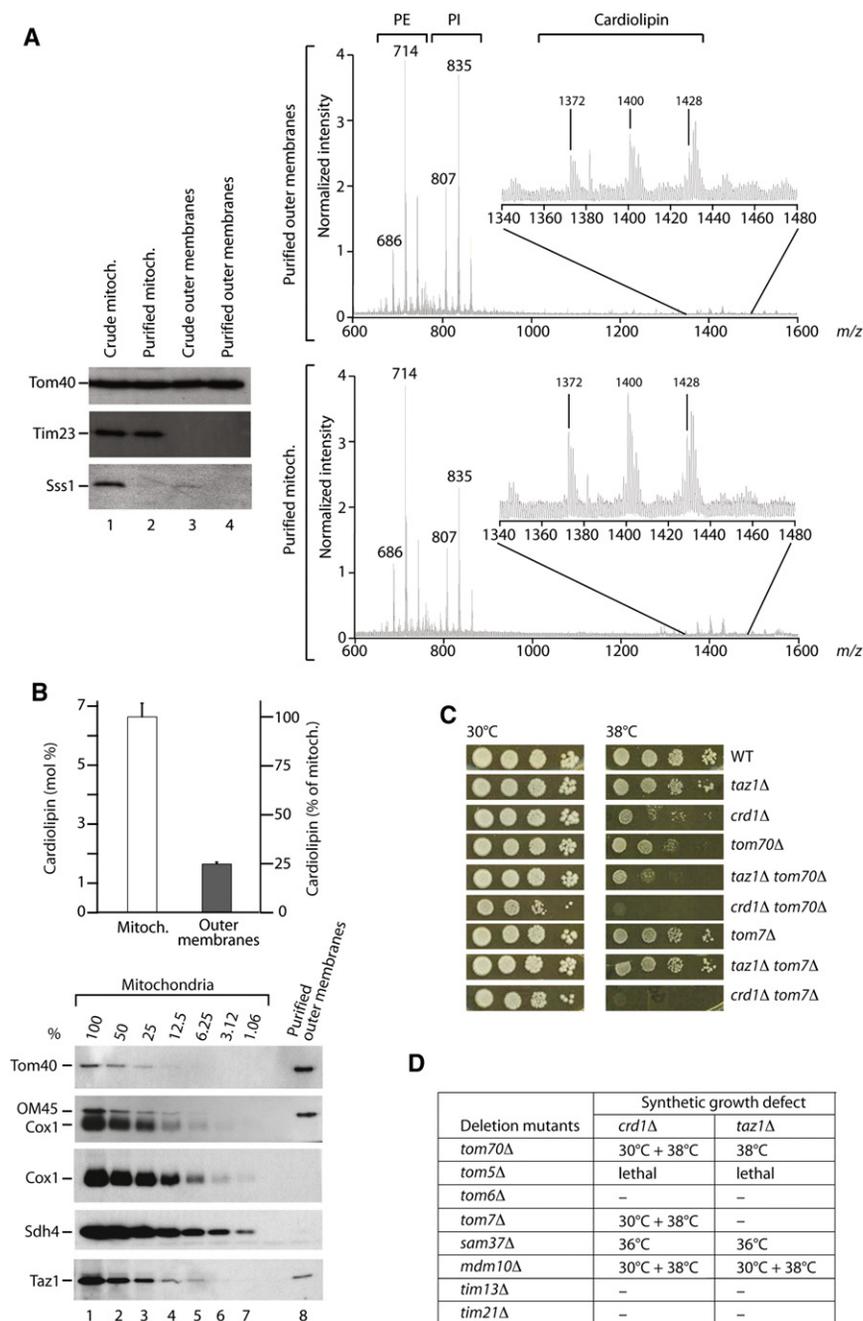
To study a possible role of cardiolipin in the outer membrane, we used yeast mutants that lacked either cardiolipin synthase (*crd1Δ*) or tafazzin (*taz1Δ*). *crd1Δ* cells lack cardiolipin, whereas in *taz1Δ* cells, the cardiolipin levels are lower and immature cardiolipin species (lacking unsaturated fatty acids) and monolysocardiolipin are observed [4, 5, 17]. We generated double-deletion mutants with genes encoding components of the two protein transport machineries of the outer membrane: the translocase of the outer membrane (TOM complex) and the sorting and assembly machinery (SAM complex). The majority of *tom/sam* deletion mutants showed synthetic growth defects with the cardiolipin mutants, including a synthetic lethality of *tom5Δ crd1Δ* and *tom5Δ taz1Δ* (Figures 1C and 1D). These results indicate a genetic interaction of cardiolipin mutants with components of outer-membrane protein import.

To define the molecular steps affected in protein import, we analyzed the translocases and import capability of isolated

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**Figure 1. Outer-Membrane Lipids and Genetic Interaction of Cardiolipin Mutants with *tom/sam* Mutants**

(A) Purified yeast mitochondrial outer-membrane vesicles were analyzed by immunoblotting (left) and liquid chromatography/mass spectrometry (right). The following abbreviations are used: PE, phosphatidylethanolamine; PI, phosphatidylinositol; Sss1, endoplasmic reticulum protein.

(B) Cardiolipin was quantified [11] with tetramyristoyl cardiolipin as internal standard. Data from two independent outer-membrane preparations are represented as mean  $\pm$  range. The purity of outer-membrane vesicles was determined by a western blot titration of outer-membrane proteins (Tom40, OM45) and inner-membrane proteins (Cox1, Sdh4).

(C and D) Genetic interactions and synthetic growth defects. Cells were grown at 30°C in liquid YPD to the early stationary phase, serially diluted, spotted on YPD plates, and incubated at the indicated temperatures. Synthetic growth defects of double-deletion mutants are indicated; “-” represents no synthetic growth defect.

mitochondria. Because cardiolipin mutants are temperature sensitive for growth [6, 11], we searched for conditions that minimized indirect, pleiotropic effects. We found that growth of the cells at very low temperature (21°C) yielded *crd1Δ* and *taz1Δ* mitochondria that contained the components of the protein-import machineries of outer and inner membranes in amounts close to that of wild-type mitochondria (Figure 2A). Thus, alterations of steady-state protein levels, which would indirectly affect mitochondrial biogenesis and function, were minimized (facilitating the identification of primary mutant defects by a kinetic analysis of protein import, as outlined below). The isolated mutant mitochondria contained an intact outer membrane because the intermembrane space-exposed Tim23 subunit of the translocase of the inner membrane was

protected against externally added Proteinase K (unless the outer membrane was disrupted by swelling) (see Figure S1A available online). To analyze the TOM complex, we lysed mitochondria with digitonin and separated the protein complexes by blue native electrophoresis. The mobility of the TOM complex was altered in *crd1Δ* and *tam41Δ* mitochondria that lack cardiolipin (Figure 2B) (Tam41 is required for an early step of cardiolipin biosynthesis [11]). For comparison, the mobility of the inner-membrane TIM22 complex, but not of the Tim9-Tim10 complex of the intermembrane space, was also altered in *crd1Δ* mitochondria as reported (Figure S1B) [11]. In *taz1Δ* mitochondria, the mobility of the TOM complex and the TIM22 complex was close to that of wild-type mitochondria (Figures 2B; Figure S1B). These results indicated that the lack of cardiolipin affected the organization of the TOM complex. We thus performed a 2D electrophoresis (blue native electrophoresis followed by SDS polyacrylamide gel electrophoresis [SDS-PAGE]) to determine whether the lack of cardiolipin influenced the association of Tom subunits. The core components Tom40, Tom22, and the small Tom subunits were present in the TOM complex independently of the presence or absence of cardiolipin (Figure 2C). However, the association of the receptor Tom20 with the TOM complex was clearly impaired (Figure 2C). Tom20 is more loosely associated with the TOM complex, and thus only a fraction of this receptor migrated with the TOM complex under wild-type conditions. In *crd1Δ* mitochondria, this fraction was considerably reduced (Figure 2C), indicating that cardiolipin influences the interaction of Tom20 with the TOM complex.

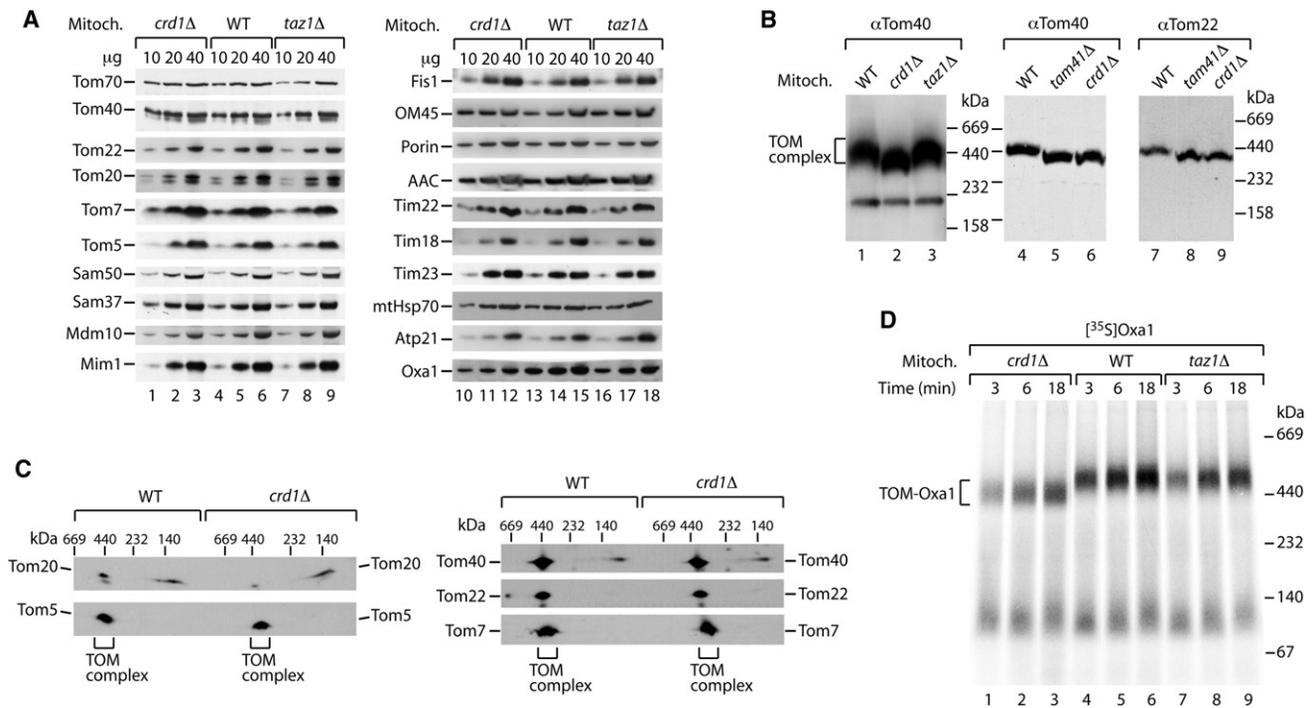


Figure 2. Cardiolipin Mutant Mitochondria Are Impaired in Precursor Accumulation at the Outer Membrane

(A) Steady-state protein levels. Mitochondria (Mitoch.,  $\mu\text{g}$  protein) from wild-type (WT), *crd1Δ*, and *taz1Δ* yeast grown at 21°C in liquid YPG were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. (B) Isolated mitochondria were analyzed by blue native electrophoresis and immunoblotting. The band at ~230 kDa in the Tom40 western blot is only visible after long exposure. (C) 2D analysis. Isolated mitochondria were separated by blue native electrophoresis followed by SDS-PAGE and immunoblotting. (D) Oxa1 was imported into isolated mitochondria (BY4741 background) at 24°C in the absence of an inner-membrane potential ( $\Delta\psi$ ). Samples were analyzed by blue native electrophoresis and autoradiography.

To monitor protein import, we synthesized and radiolabeled the precursor proteins in a cell-free system and incubated them with isolated mitochondria. The  $\Delta\psi$ -dependent import of hydrophilic precursor proteins across the inner membrane (F1 $\beta$ , Su9-DHFR) was not affected or was only mildly affected in mitochondria lacking Crd1 or Taz1 (Figure S2A), in agreement with the observation that cardiolipin-deficient cells grown at low temperature are able to generate a  $\Delta\psi$  [11]. (It has been shown that at higher temperature, cardiolipin-deficient mitochondria are partially impaired in protein import across the inner membrane, concomitant with a decrease of  $\Delta\psi$  [6, 11].) However, import of the multispanning inner-membrane protein Oxa1 was of lower efficiency in *crd1Δ* mitochondria compared to wild-type mitochondria and was also partially impaired in *taz1Δ* mitochondria (Figure S2B). The steady-state levels of Oxa1 were also moderately reduced in the mutant mitochondria (Figure 2A). It has been shown that the Oxa1 precursor accumulates as a translocation intermediate at the TOM complex in the absence of a  $\Delta\psi$  (the intermediate can be directly visualized by blue native electrophoresis [27, 28]), whereas hydrophilic precursor proteins destined for the matrix pass through the TOM complex and do not form stable TOM intermediates on blue native gels. We thus asked whether the alteration of the TOM complex in cardiolipin mutants affected the accumulation of the Oxa1 intermediate. The amount of TOM-Oxa1 intermediate was considerably decreased in *crd1Δ* mitochondria (and the blue native mobility was altered as expected) (Figure 2D). Interestingly, the amount of TOM-Oxa1 was also reduced in *taz1Δ* mitochondria

(Figure 2D). Here, the mobility of the TOM complex was close to that of wild-type mitochondria (Figures 2B and 2D) because cardiolipin was still present, yet the altered cardiolipin species partially impaired the accumulation of the Oxa1 intermediate. Thus, lack or alteration of cardiolipin impairs the import of Oxa1 at the level of the TOM complex, i.e., at an early step before the  $\Delta\psi$ -dependent insertion into the inner membrane.

We thus asked whether further steps of protein biogenesis of the outer membrane were affected by cardiolipin defects. The outer membrane contains two main classes of proteins:  $\alpha$ -helical proteins and the characteristic  $\beta$  barrel proteins [29, 30]. The biogenesis pathway of  $\beta$  barrel proteins involves both the TOM complex and the SAM complex. Blue native electrophoresis resolves several forms of the SAM complex [30, 31]: the SAM<sub>core</sub> complex consisting of Sam35, Sam37, and Sam50; the SAM<sub>holo</sub> complex that additionally contains Mdm10; and a smaller form containing Sam35 and Sam50 (Figure 3A). In *crd1Δ* mitochondria, more of the smaller complex was observed (Figure 3A), indicating that the lack of cardiolipin affected the organization of the SAM complex. With the radiolabeled precursor of the  $\beta$  barrel protein Tom40, the biogenesis steps can be monitored by blue native electrophoresis and dissected into three steps [30, 31]: first, an intermediate of ~250 kDa, representing the association of the precursor with the SAM complex; second, a smaller intermediate (Int-II); and third, the assembly into the mature TOM complex of ~400 kDa. *crd1Δ* mitochondria were strongly inhibited in forming the assembly steps of Tom40, yet *taz1Δ* mitochondria were also impaired (Figure 3B, top). Similarly,

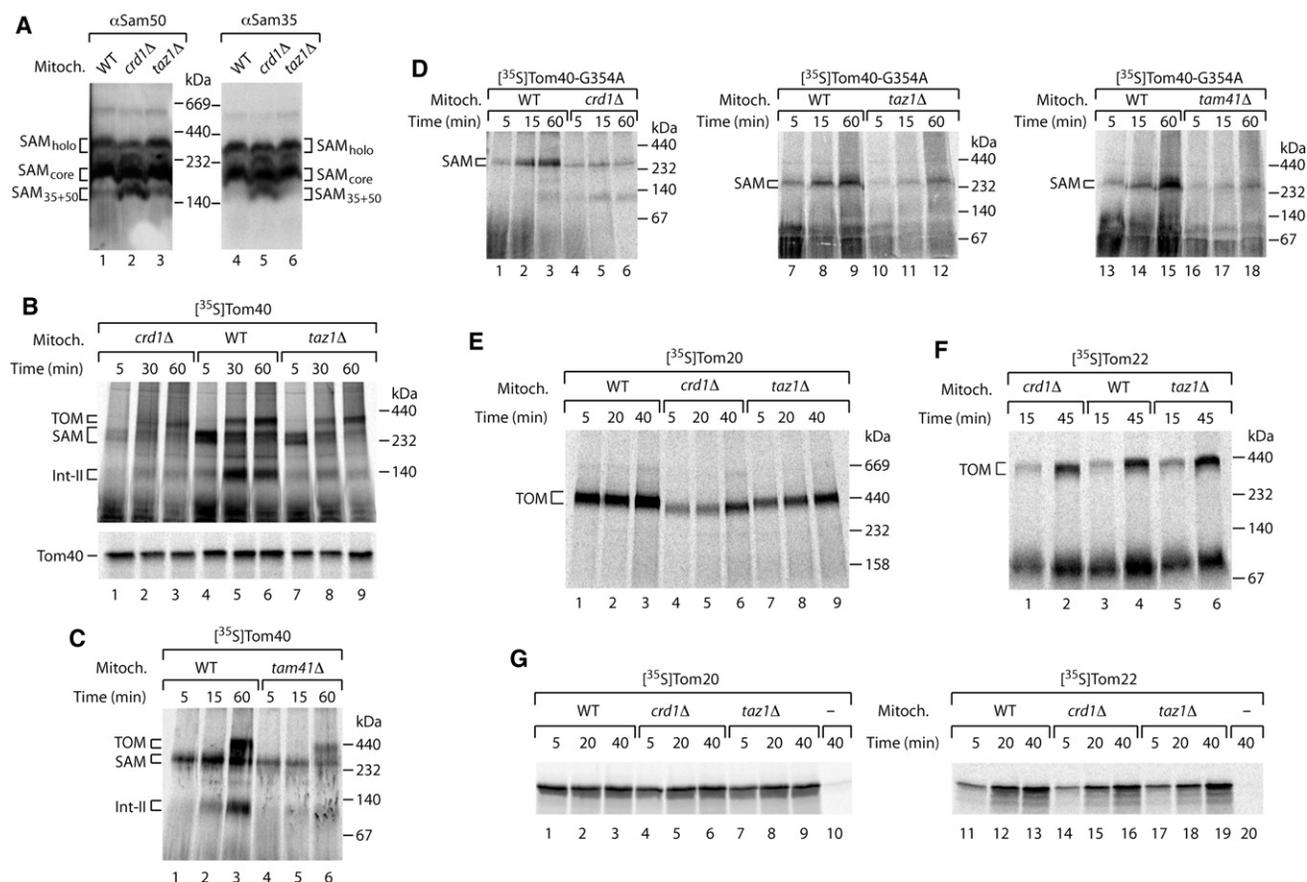


Figure 3. Cardiolipin Mutant Mitochondria Are Impaired in the Assembly of Outer-Membrane Proteins

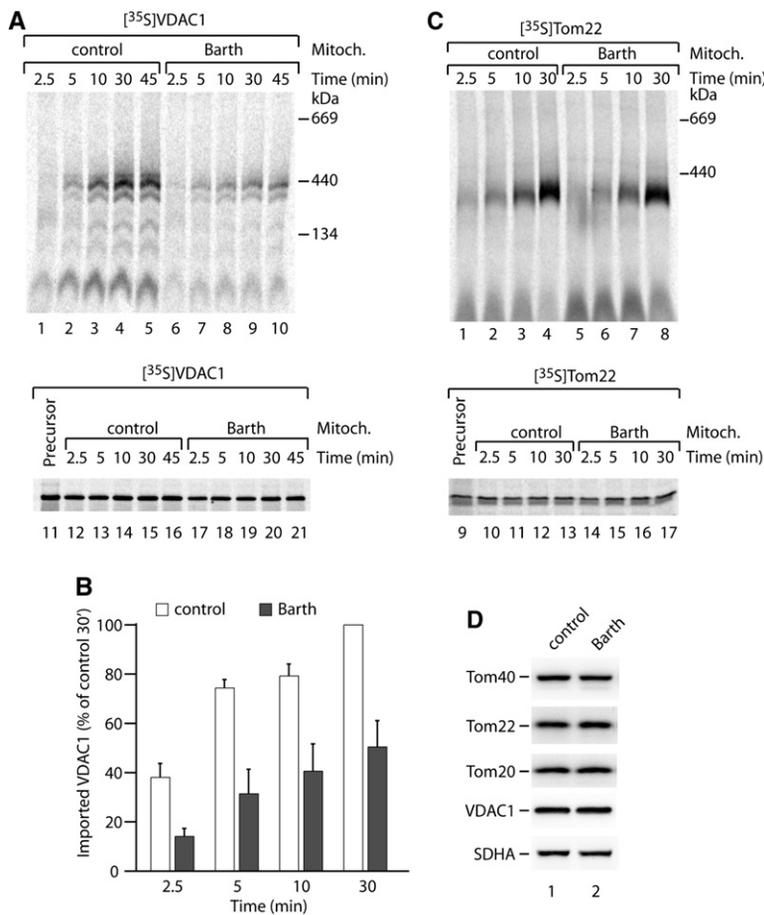
(A) Isolated yeast mitochondria were separated by blue native electrophoresis and analyzed by immunoblotting. (B) [<sup>35</sup>S]Tom40 precursor was incubated with WT, *crd1Δ*, and *taz1Δ* mitochondria (min). Mitochondria were reisolated and analyzed by blue native electrophoresis (top) or SDS-PAGE (bottom) and autoradiography. (C) Tom40 precursor was imported and analyzed by blue native electrophoresis. (D) Tom40-G354A precursor was imported in isolated mitochondria. (E) [<sup>35</sup>S]Tom20 was incubated with WT, *crd1Δ*, and *taz1Δ* mitochondria at 24°C (min). Samples were analyzed by blue native electrophoresis and autoradiography. (F) Tom22 precursor was imported and analyzed as in (E). (G) After import of [<sup>35</sup>S]Tom20 (lanes 1–10) or Tom22 (lanes 11–20), mitochondria were incubated with 0.1 M Na<sub>2</sub>CO<sub>3</sub> for 30 min. Pellets were analyzed by SDS-PAGE and autoradiography. Samples 10 and 20 did not contain mitochondria.

*tam41Δ* mitochondria were impaired in the assembly pathway of Tom40 (Figure 3C). The initial targeting of the precursor to mitochondria was not blocked because the total amount of Tom40 precursor bound to mitochondria was only moderately affected in *crd1Δ* and *taz1Δ* mitochondria (Figure 3B, bottom). To analyze the step of interaction with the SAM complex in more detail, we used a mutant form of the Tom40 precursor where one conserved glycine residue was replaced by alanine, leading to an accumulation of the precursor at the SAM complex in wild-type mitochondria [30]. All three cardiolipin mutant mitochondria showed a strong defect in precursor accumulation at the SAM complex (Figure 3D), demonstrating that the lack or alteration of cardiolipin impaired the assembly pathway of the β barrel precursor already at the level of the SAM complex.

To determine a possible influence of cardiolipin on the biogenesis of α-helical outer-membrane proteins, we used two precursors that are imported by different pathways: Tom20, which is imported via the outer-membrane protein Mim1 and subsequently assembles onto the TOM complex [32–34], and Tom22, which uses the SAM complex for

membrane insertion and, with the help of Mdm10, assembles into the TOM complex [35, 36]. The assembly of Tom20 with the TOM complex was impaired in both *crd1Δ* and *taz1Δ* mitochondria (Figure 3E), whereas the assembly of the precursor of Tom22 was not affected or was only mildly affected (Figure 3F) (β barrel precursors and Tom22 show different characteristics of SAM dependence, including access to SAM from the inter-membrane space or cytosolic side, respectively [35, 36]). The initial insertion of Tom20 and Tom22 into the outer membrane, as analyzed by treatment of the membranes at alkaline pH, was not inhibited by the lack of cardiolipin (Figure 3G). Thus, the lack of cardiolipin does not impair the Mim1-mediated membrane insertion of the Tom20 precursor but inhibits the subsequent assembly step. Because the precursor of Tom22 is correctly imported and assembled in cardiolipin mutant mitochondria, we conclude that the outer membrane and the SAM complex are not generally (unspecifically) damaged in the mutant mitochondria.

We asked whether these observations with yeast mitochondria bear relevance for the biogenesis of outer-membrane proteins in Barth syndrome mitochondria. We isolated



**Figure 4. Assembly of Outer-Membrane Proteins in Barth Syndrome Mitochondria**

(A–C) Mitochondria isolated from control or Barth syndrome patient lymphoblasts were incubated at 37°C (min) in the presence of [<sup>35</sup>S]VDAC1 or [<sup>35</sup>S]Tom22. Following import, samples were analyzed by blue native electrophoresis (top), SDS-PAGE (bottom), and autoradiography. A sample of reticulocyte lysate (representing 50% of added precursor/import) is also shown on the SDS-PAGE gel. For (B), assembled VDAC1 was quantified from four independent experiments (three for 2.5 min). Data are represented as mean ± standard error of the mean (SEM).

(D) Control and Barth syndrome mitochondria were analyzed by SDS-PAGE and immunoblotting. SDHA indicates a 70 kDa subunit of succinate dehydrogenase.

mitochondria from a human lymphoblast cell line derived from a Barth syndrome patient with a *TAZ* splicing defect that is expected to result in no production of tafazzin protein [16]. We used precursors for which the assembly kinetics in lymphoblast mitochondria could be monitored by blue native electrophoresis: the major  $\beta$  barrel protein VDAC1 and  $\alpha$ -helical Tom22. The <sup>35</sup>S-labeled precursors were imported into isolated Barth syndrome and control mitochondria. The assembly kinetics of the  $\beta$  barrel precursor were significantly delayed in the mutant mitochondria (Figures 4A and 4B) (the total binding of the precursor to the mutant mitochondria was only mildly affected; Figure 4A, bottom). The assembly of Tom22 into the mutant mitochondria was similar to control mitochondria (Figure 4C). Steady-state protein levels, including Tom proteins, were comparable between mutant and control mitochondria (Figure 4D). Together with the efficient import of Tom22, this indicates that the impaired import kinetics of VDAC1 import were not caused by general (unspecific) damage of the outer membrane. We conclude that Barth syndrome mitochondria show a similar defect in biogenesis of outer-membrane proteins as cardiolipin mutant mitochondria from yeast.

Our results suggest a new role of cardiolipin for the biogenesis of mitochondrial outer-membrane proteins. By a quantitative analysis of highly pure outer membranes, we demonstrate that a fraction of cardiolipin as well as tafazzin is present in the outer membrane. In yeast mitochondria isolated from cardiolipin mutants and human mitochondria isolated from a Barth syndrome patient, the assembly of  $\beta$  barrel proteins is delayed. How does the lack or alteration of cardiolipin affect the

mitochondrial outer membrane? The outer membrane was not generally damaged in the cardiolipin mutants, because the initial targeting of precursor proteins to mitochondria still took place, and different types of  $\alpha$ -helical precursors exhibited selective defects (impaired assembly of Tom20, but not of Tom22). These findings suggest a more specific role of the non-bilayer-forming phospholipid cardiolipin for protein sorting and influencing the activity of preprotein translocases. Studies with inner-membrane protein complexes revealed a specific interaction of cardiolipin with respiratory chain complexes, metabolite carriers, and further proteins; in several cases, alteration of the complexes in cardiolipin mutants could be visualized by blue native gels [6, 10–12, 15, 37]. Indeed, the blue native

pattern of the TOM and SAM complexes was altered in cardiolipin-deficient mitochondria, indicating that cardiolipin is involved in the organization of the translocase complexes. In tafazzin mutant mitochondria, cardiolipin is not absent, but altered species are observed and thus the defects are not as strong as in cardiolipin-deficient mitochondria [14–18]. The functional assays of precursor binding to TOM and SAM indeed revealed that the altered cardiolipin led to a partial impairment of precursor binding. We conclude that the outer membranes of cardiolipin mutant mitochondria are not unspecifically damaged, but cardiolipin is involved in the assembly pathways of  $\beta$  barrel precursors and some  $\alpha$ -helical precursors. Outer-membrane protein assembly pathways are delayed but not blocked in cardiolipin mutants, because a block of TOM or SAM functions would be lethal even to yeast cells. We suggest that mitochondrial defects of Barth syndrome patients are not only caused by inner membrane defects but also involve the role of cardiolipin in the biogenesis of outer-membrane proteins. In summary, we conclude that loss or alteration of cardiolipin impairs the assembly pathways of several mitochondrial outer-membrane proteins.

#### Experimental Procedures

##### Yeast Strains, Growth, and Analysis

The *S. cerevisiae* strains used in this work were derived from YPH499, BY4741, and BY4742 (EUROSCARF). *crd1* $\Delta$ , *taz1* $\Delta$ , and *tam41* $\Delta$  strains have been described [11, 15] (EUROSCARF). The strains used for import studies are listed in Table S1; cells were grown on nonfermentable medium (liquid YPG or YPLac [11]) at 21°C. Mutants of *TOM*, *SAM*, or *TIM* genes were screened for genetic interactions with *crd1* $\Delta$  and *taz1* $\Delta$ . The *crd1* $\Delta$ ::*URA3*

and *taz1Δ::URA3* mutants in the *Mat $\alpha$*  background were crossed to *Mata* deletion mutants of *TOM* or *SAM* genes disrupted with *KanMX4*. Diploids were selected on double-dropout synthetic medium lacking methionine and lysine; the resulting heterozygous diploids were sporulated and subjected to meiotic tetrad analysis. Double mutants were identified by growth on *Ura<sup>-</sup>* and G418 (200  $\mu$ g/ml) medium. The lethality observed in double mutants was not due to defective spore germination, because a few tiny colonies from the spores inferred to be the double mutants could be seen under the microscope.

#### Import of Precursor Proteins into Mitochondria

Yeast mitochondria were isolated by differential centrifugation. Precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [<sup>35</sup>S]methionine. Import into isolated mitochondria was performed in import buffer (3% [w/v] bovine serum albumin [BSA], 250 mM sucrose, 80 mM KCl, 5 mM methionine, 5 mM MgCl<sub>2</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM MOPS-KOH, [pH 7.2], 2 mM NADH, 4 mM ATP, 20 mM creatine phosphate, 0.1 mg/ml creatine kinase) at 24°C. The import reaction was stopped on ice or by addition of AVO (8  $\mu$ M antimycin A, 1  $\mu$ M valinomycin, 20  $\mu$ M oligomycin). To dissipate  $\Delta\psi$ , we added AVO before the import experiment. Where indicated, samples were treated with 50  $\mu$ g/ml Proteinase K for 15 min on ice. For native analysis, mitochondria were solubilized in digitonin-containing buffer (0.4%–1% digitonin, 20 mM Tris/HCl [pH 7.4], 0.1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaCl, 10% glycerol) and resolved by blue native electrophoresis.

Lymphoblast cell lines were derived from a control subject and Barth syndrome patient with a *TAZ* splicing defect (c.527-1G > A) [16]. Cells were grown at 37°C and 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen) and 50  $\mu$ g/ml uridine. In vitro import and assembly of radiolabeled human Tom22 and mouse VDAC1 into freshly isolated lymphoblast mitochondria was performed as previously described [38]. Following import, samples were solubilized in digitonin, and aggregates were removed by a clarifying spin. Samples were split and analyzed by blue native electrophoresis or SDS-PAGE.

#### Miscellaneous

The preparation of highly pure mitochondria and isolation of outer-membrane vesicles via sucrose gradient centrifugation were performed as described [39]. Lipid analysis by liquid chromatography/mass spectrometry was performed as reported [11, 40].

#### Supplemental Data

Supplemental Data include two figures and one table and can be found online at [http://www.cell.com/current-biology/supplemental/S0960-9822\(09\)01982-4](http://www.cell.com/current-biology/supplemental/S0960-9822(09)01982-4).

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