The Cardiolipin Transacylase, Tafazzin, Associates with Two Distinct Respiratory Components Providing Insight into Barth Syndrome

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Mutations in the mitochondrial cardiolipin (CL) transacylase, tafazzin (Taz1p), result in the X-linked cardio-skeletal myopathy, Barth syndrome (BTHS). The mitochondria of BTHS patients exhibit variable respiratory defects and abnormal cristae ultrastructure. The biochemical basis for these observations is unknown. In the absence of its target phospholipid, CL, a very large Taz1p complex is missing, whereas several discrete smaller complexes are still observed. None of the identified Taz1p complexes represents Taz1p homodimers. Instead, yeast Taz1p physically assembles in several protein complexes of distinct size and composition. The ATP synthase and AAC2, both required for oxidative phosphorylation, are identified in separate stable Taz1p complexes. In the absence of CL, each interaction is still detected albeit in reduced abundance compared with when CL is present. Taz1p is not necessary for the normal expression of AAC2 or ATP synthase subunits or assembly of their respective complexes. In contrast, the largest Taz1p complex requires assembled ATP synthase and CL. Mitochondria in Δtaz1 yeast, similar to ATP synthase oligomer mutants, exhibit altered cristae morphology even though ATP synthase oligomer formation is unaffected. Thus, the Taz1p interactome defined here provides novel insight into the variable respiratory defects and morphological abnormalities observed in mitochondria of BTHS patients.

INTRODUCTION

The mitochondrial inner membrane (IM) forms a barrier that not only compartmentalizes numerous critical cellular activities, including iron–sulfur cluster formation and the tricarboxylic acid cycle, but additionally maintains the electrochemical gradient established by the electron transport chain and harnessed by the ATP synthase to generate ATP. The composition of the mitochondrial IM is unique, containing a distinctively high ~3–4:1 protein:phospholipid ratio. In contrast, the mitochondrial outer membrane (OM) ratio is ~1–1.6:1 (Sperka-Gottlieb et al., 1988; Ardail et al., 1990; Simbeni et al., 1991). The IM also contains cardiolipin (CL), the signature phospholipid of mitochondria. CL is a structurally unusual phospholipid, with one negative charge associated with its two headgroups at physiological pH and four associated fatty acyl chains (Schlame et al., 2000; Haines and Dencher, 2002). CL is intimately associated with all of the major players in oxidative phosphorylation, including complexes I, III, IV, and V, and the major carrier proteins for adenine nucleotides and phosphates (Schlame et al., 2000). Moreover, CL is required to fully reconstitute the activity of respiratory complex IV and the ADP/ATP carrier (AAC) in vitro (Hoffmann et al., 1994; Sedlak and Robinson, 1999). In organello, CL acts as a glue that stabilizes the assembly of individual respiratory complexes into so-called respiratory supercomplexes (Zhang et al., 2002, 2005; Pfeiffer et al., 2003) that function to increase the efficiency of oxidative phosphorylation (Boumans et al., 1998; Zhang et al., 2005). Which aspect of CL (e.g., its two phosphate headgroups or four associated acyl chains) is responsible for these unique functional properties of CL is at present unknown.

Cardiolipin synthase, Crd1p, synthesizes CL in the matrix-facing leaflets of the mitochondrial IM (Schlame and Haldar, 1993). Newly synthesized CL undergoes a remodeling process, the end result of which is the incorporation of more unsaturated fatty acyl chains and the establishment of a high degree of acyl chain symmetry (Schlame et al., 2005). One pathway of CL remodeling is mediated by the CL transacylase, tafazzin (Taz1p; Xu et al., 2006b), the mutant gene product associated with the X-linked disease Barth syndrome (BTHS). BTHS is characterized by cardiac and skeletal myopathies and cyclic neutropenia (Barth et al., 1983, 1999, 2004); the disease presents in infants and if undiagnosed, is often fatal due to cardiac failure or sepsis. There are three hallmarks of the loss of Taz1p activity in the mitochondria of BTHS patients (Vreken et al., 2000; Valian-
expected associations that collectively provide new insight into some of the pathologies observed in BTHS patients.

**MATERIALS AND METHODS**

**Yeast Strains**

All strains were derived from the wild-type (wt) parental S. cerevisiae yeast strain GA74-1A (MAT a, his3-11,15, leu2, ura3, trpl, ade8, rho’), mit−). The Δtas1 (MAT a, leu2, ura3, trpl, ade8, Δtas1::HISMX6) and Δtas1 (MAT a, his3-11,15, leu2, ura3, ade8, Δtas1::TRP1) strains have been described (Claypool et al., 2006; Claypool et al., 2008). To generate the Δtas1Δtas1 (MAT a, leu2, ura3, ade8, Δtas1::HISMX6, Δtas1::TRP1) and Δtap2 (MAT a, leu2, ura3, trpl, ade8, Δtap2::HIS6) strains, the entire open reading frame of each gene was replaced using the PCR-mediated one-step gene replacement strategy (Wach et al., 1994).

**Molecular Biology**

To place the CNAP tag (amino acid sequence: MEDQVDPIDGK-GGAGG-HHHHHHHHH: the Protein C [PC] epitope tag is underlined, and the Hiss tag is in bold) onto the N-terminus of Tas1p but still under control of the Tas1p promoter, overlap extension was performed (Ho et al., 1989). The sequence of every construct was verified by DNA sequencing. The sequences of all primers are available upon request.

**Antibodies**

Most of the antibodies used in this work were generated in the Schatz lab or our lab and have been described previously. Other antibodies used were as follows: mouse anti-Sec62p (kind gift of Dr. David Meyers, University of California, Los Angeles), mouse anti-β-actin (Abcam, Cambridge, MA), mouse anti-Myc tag (9E10; Evan et al., 1985); obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Iowa City, IA), mouse anti-Y23 (clone 6H8; Panneels et al., 2003), and mouse anti-PC (Roche, Indianapolis, IN) monoclonal antibodies, and horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL).

**Electron Microscopy**

Conventional electron microscopy was performed as previously described (Rieder et al., 1996). Briefly, the cells were fixed in 3% glutaraldehyde contained in 0.1 M Na cacodylate, pH 7.4, 4.5 mM CaCl2, 5 mM MgCl2, and 2.5% sucrose for 1 h at 25°C with gentle agitation; spheroplasts, embedded in 2% ultra-low temperature agarose (prepared in water); cooled; and subsequently cut into small pieces (1 mm3). The blocks were washed thoroughly four times with ddH2O, 10 min total; transferred to 1% thiocarbohydrazide at room temperature for 3 min; washed in ddH2O (four times, 1 min each); and transferred to 1% OsO4/1% potassium ferrocyanide contained in 0.1 M cacodylate/5 mM CaCl2, pH 7.4, for 30 min at room temp. The blocks were washed thoroughly four times with ddH2O, 10 min total; transferred to 1% tiochlorobiodazole at room temperature for 3 min; washed in ddH2O (four times, 1 min each); and transferred to 1% OsO4/1% potassium ferrocyanide in cacodylate buffer, pH 7.4, for an additional 3 min at room temperature. The cells are then washed four times with ddH2O (15 min each); en bloc-stained in Kellenberger’s uranyl acetate (UA) for 2 h overnight; dehydrated through a graded series of ethanol; and subsequently embedded in Spurr resin. Sections were cut on a Reichert Ultracut T ultramicrotome; poststained with UA and lead citrate; and observed on a Philips TEM 420 microscope (Mahwah, NJ) at 80 kV. Images were recorded with a Soft Imaging Systems Megaview III digital camera (Olympus Soft Imaging Solutions, Lakewood, CO), and figures were assembled in Adobe Photoshop 10.0 (San Jose, CA).

**Miscellaneous**

Subcellular fractionation, isolation of mitochondria, alkali extraction, submicronodular localization, and immunblotting were as described (Claypool et al., 2006); 2D Blue native/SDS-PAGE, consecutive nondenaturing affinity purification (CNAP), immunoprecipitation (IP), and liquid chromatography–tandem mass spectrometry (LC-MS/MS) were as described (Claypool et al., 2008). The performed experiments used mitochondria harvested from yeast grown at 30°C to OD600~3 as follows: in Figures 1, 3A, 7D, and 8 and Supplemental Figure S1, rich lactate medium (1% yeast extract, 2% tryptone, 0.05% dextrose, and 2% lactic acid, 3.4 mM CaCl2·2H2O, 8.5 mM NaCl, 2.95 mM MgCl2·6H2O, 7.35 mM KH2PO4, and 18.7 mM NH4Cl); in Figures 2, 3, B and C, 4, and 5; B and C, and in Supplemental Figures S2, S3, and S4, synthetic lactate medium (1% yeast extract, 2% tryptone, 0.05% dextrose, 0.2% dropout mix synthetic minus Leu, 0.05% dextrose, 3.4 mM CaCl2·2H2O, 8.5 mM NaCl, 2.95 mM MgCl2·6H2O, 7.35 mM KH2PO4, and 18.7 mM NH4Cl); and in Figures 5A, 3, and 5A; YP-dextrose. Protein synthesis was inhibited by the addition of 200 μg/ml cycloheximide for the final 4 h of growth before isolating mitochondria. Phospholipid labeling and extraction and data collection was as described (Claypool et al., 2006) except that the extracted phospholipids were
Supplemental Table S1 that presents the LC-MS/MS data generated for
ization studies of CNAPTaz, and immunoblots after CNAP, respectively, and
chondrial membranes that line the IMS in
ase K sensitivity profile as Tim54p (IM protein with large
implies that CL is not a determinant of proper mitochondrial
the context of CL-containing or CL-deficient membranes
CL (Figure 1B). Equal mitochondrial expression of Taz1p in
regardless of whether mitochondrial membranes contained
onstrated that Taz1p levels were statistically the same
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pholipid, CL (Supplemental Figure S1A). A more quantita-
not affected by the presence or absence of its target phos-
suggested that the absolute expression level of Taz1p was
(Figure 1A). A Taz1p immunoblot of whole cell extracts
intermediate in the CL remodeling pathway, accumulated
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the context of CL-containing or CL-deficient membranes
implies that CL is not a determinant of proper mitochondrial
targeting by Taz1p. Additionally, based on a similar protein-
K sensitivity profile as Tim54p (IM protein with large
domain residing in IMS), Taz1p still associated with mito-
chondrial membranes that line the IMS in Δcrd1 mitochondria
(Supplemental Figure S1B).

Yeast Taz1p associates with mitochondrial membranes via
an unusual membrane anchor that protrudes into, but not all
the way through, the lipid bilayer (Claypool et al., 2006). This
type of integral interfacial membrane association is bio-
chemically indistinguishable from classical integral mem-
brane proteins when performing alkali extraction. Alkali
extraction is used to distinguish between integral (retained
in pellet) and peripheral (released into supernatant) mem-
brane proteins. In fact, when applied over a pH range,
integral membrane proteins are released into the superna-
tant providing a signature alkali extraction profile for each
protein. Interestingly, the alkali extraction profile of Taz1p
was different in Δcrd1 than wt mitochondria (Supplemental
Figure S1C). Specifically, Taz1p was significantly less ex-
tractable at pH 11.5 in the absence versus the presence of CL
(Figure 1C). This was not a general feature as the extraction
profile of Tim23p (four transmembrane domains) was the
same in wt and Δcrd1 mitochondria. The assembly of Taz1p
into complexes was analyzed by 2D blue native/SDS-PAGE
(BN/SDS-PAGE; Figure 1D). In the presence or absence
of CL, the majority of Taz1p resolved at or below 67 kDa, likely
reflecting a Taz1p monomer (44 kDa); however, several dis-
crete larger complexes were readily detected. Intriguingly,
very large Taz1p-containing complex (highlighted by red
arrow) was only observed in wt mitochondrial extracts and
not in either Δcrd1 or ΔΔcrd1 extracts. Whether the absence
of this largest complex explains the distinct alkali extraction
profiles of Taz1p in wt and Δcrd1 mitochondria is unclear.
Thus, in the absence of CL, the membrane association and
complex assembly of Taz1p is altered.

**Taz1p Does Not Form Homodimers**

On the basis of migration as determined by 1D BN-PAGE,
Brandner et al. (2005) suggested that Taz1p might assemble
as a homodimer. To determine if Taz1p does in fact form
homodimers, a differential epitope-tagging strategy was
used. Specifically, ΔΔcrd1 yeast expressing either MycTaz
(Myc tag on N–terminus) or TazHis8Taz (His8 within coding
sequence just after amino acid 154) constructs alone on
high-copy (1 copy) plasmids or coexpressing MycTaz and
TazHis8Taz on low-copy (1 copy) plasmids were generated.
Previously, we have demonstrated that Taz1p constructs con-
taining epitope tags on either the N-terminus or within the
Taz1p-coding sequence just after aa 154 are expressed at equi-
valent levels and functional (Claypool et al., 2006); indeed based
on the lack of accumulation of MLCL, MycTaz and TazHis8Taz
were functional (Supplemental Figure S2). Importantly, Myc-
Taz and TazHis8Taz assembled normally when expressed alone
or coexpressed (Figure 2A), indicating that the Taz1p
interactome was preserved for each Taz1p variant. To deter-
mine if Taz1p does form homodimers, TazHis8Taz was affinity-
purified by Ni2+NTA chromatography, and bound mate-
rial was analyzed by immunoblot for total Taz1p or MycTaz
(Figure 2B). When expressed individually, TazHis8Taz but not
MycTaz bound to the Ni2+ resin, as expected. When coex-
pressed, none of the bound Taz1p contained a Myc tag. Thus,
we conclude that yeast Taz1p does not form homodimers.

To confirm this conclusion, we determined whether a
previously characterized BTHS mutant tafazzin, G230R, acted
in a dominant-negative manner when expressed in the
context of endogenous Taz1p (Figure 2C). As BTHS is X-
linked, the absence of a female carrier with disease symp-
toms is explained by X-chromosome inactivation. Thus, the
ability of a mutant Taz1p to interfere with wt Taz1p function
has not been documented. Of the four BTHS mutations that
we have characterized, the G230R mutant was selected be-
cause, in contrast to the three other mutants that are mislo-
calized to the mitochondrial matrix, the G230R BTHS mu-
tant is still resident to IMS-facing membranes (Claypool et al.,
2006). Consistent with the conclusion that Taz1p does not
function as an obligate homodimer, the G230R BTHS mutant
does not act in a dominant-negative manner when coex-
pressed with endogenous Taz1p.

**Taz1p Associates with AAC2 and the ATP Synthase in
Distinct Complexes**

Because Taz1p assembled in several distinct complexes and
the largest complex either depends on CL for its stability or
represents a physical association between Taz1p and Crlp1,
we defined the Taz1p interactome. A yeast strain lacking
both the TAZ1 and CRD1 genes (ΔΔcrd1Δcrd1) was generated.
Analysis and quantitation of the mitochondrial phospho-
lipid profile in the ΔΔcrd1Δcrd1 strain demonstrated that the
ΔΔcrd1Δcrd1 strain phenocopied the Δcrd1 strain (Figure 3A).
This had been expected but never previously demonstrated
and is consistent with Taz1p acting downstream of Crd1p.
Second, we appended onto the N-terminus of Taz1p, but still
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The exposure of each set of immunoblots is designated at the bottom. n
digitonin extracts from mitochondria derived from the indicated strains were resolved by 2D BN/SDS-PAGE, and Taz1p complexes were
in carbonate at each pH determined as follows: S/(S + P) × 100, where S is the volume of protein detected in the supernatant at a given pH and P is the volume associated with the pellet at the same pH. The asterisks indicates a statistically significant difference in the extractability
mitochondria as calculated by the Student’s t test, p < 0.001 (mean ± SD, n = 4–5). (D) 100 μg of 1.5% (wt/vol) digitonin extracts from mitochondria derived from the indicated strains were resolved by 2D BN/SDS-PAGE, and Taz1p complexes were revealed by immunoblot. The exposure of each set of immunoblots is designated at the bottom. n = 3.

To identify associating proteins, we performed a preparative scale CNAP (schematized on the left in Figure 4). As a negative control, CNAP was performed on mitochondrial extracts containing untagged Taz1p (I, ∆taz1[Taz1p]). In comparison to this negative control, when CNAP was performed on extracts containing CNAPTaz and CL (II, ∆taz1[CNAPTaz]), numerous unique co-affinity–purified bands were detected upon SYPRO Ruby staining. The abundance of many of these copurified bands was decreased in CNAPed samples derived from extracts containing CNAPTaz but not CL (III, [CNAPTaz]). Surprisingly, the major ADP/ATP carrier, AAC2 (highlighted in orange) and five different subunits of the ATP synthase (blue) were identified by LC-MS/MS analysis of tryptic digests from the indicated bands. Additional proteins that

<table>
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<th>Strain</th>
<th>Taz1p Expression pg/mg Mitochondrial Protein</th>
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<tr>
<td>WT</td>
<td>93.72 ± 15.9</td>
</tr>
<tr>
<td>∆crd1</td>
<td>104.23 ± 17.8</td>
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Figure 1. Altered membrane association and complex formation of Taz1p in absence of CL. (A) Phospholipids were extracted from 1 mg of mitochondria isolated from the indicated strains and separated by TLC, and phospholipids were visualized using molybdenum blue. Scanned images were analyzed using Quantity 1 software and lane analyses functions. The determined phospholipid profile for each mitochondrial sample was calculated after backgro...
copurified with CNAPtaz included three heat-shock proteins (two Hsp70 proteins, encoded by SSC1 and SSC3, and Hsp60), Cor1p, and Sdh2p. These identifications were deemed likely contaminants as the proteins either reside within a different mitochondrial compartment as Taz1p (all three heat-shock proteins are resident to the matrix) or participate in multisubunit complexes and none of the other three heat-shock proteins are resident to the matrix) or participate in multisubunit complexes and none of the other three heat-shock proteins are resident to the matrix.

Multiple approaches were used to independently confirm the interactions between Taz1p and AAC2 and Taz1p and the ATP synthase. First, after CNAP, the affinity-purified material was analyzed by immunoblot (Supplemental Figure S4). When membranes contained CL, CNAPtaz, but not untagged Taz1p, co-affinity-purified subunits of the ATP synthase (F1α and F1β) and a small amount of AAC, which as expected, failed to recognize untagged Taz1p in either the starting material or after the ATP synthase was IPed (Figure 5B). In contrast, CNAPtaz was greater when mitochondria contained CL and AAC was no longer detected. Second, we used a mAb specific to the N-terminus of yeast AAC2 (Panneels et al., 2003) to determine if endogenous Taz1p and AAC2 interact (Figure 5A). Indeed, Taz1p, but not subunits of the ATP synthase or porin, was co-immunoprecipitated (co-IP) with AAC2 from wt mitochondrial extracts. Importantly, Taz1p was not co-IPed with AAC2 in the absence of either Taz1p or AAC2 expression. Interestingly, although Taz1p was co-IPed with AAC2 from extracts lacking CL, the abundance of copurified Taz1p was reduced. The failure to co-IP the ATP synthase with AAC2 from wt mitochondrial extracts suggested that the association of Taz1p with AAC2 does not depend on, nor is it mediated through the ATP synthase. Indeed, although in reduced quantities, Taz1p was still co-IPed with AAC2 from extracts lacking assembled ATP synthase (Δatp2, Atp2p = F1β).

Third, the interaction of Taz1p with the ATP synthase was analyzed by co-IP immunoblot utilizing a polyclonal anti-serum raised against the ATP synthase holoenzyme. Unfortunately, our Taz1p antiserum is cross-reactive to the F1α and/or F1β subunits (migrate slightly above Taz1p by SDS-PAGE) of the ATP synthase, preventing us from using this reagent to confirm this interaction with endogenous proteins. Therefore, the association of Taz1p and the ATP synthase was investigated utilizing the CNAPtaz panel. To detect the CNAPtaz constructs, an anti-PC epitope tag mAb was utilized, which as expected, failed to recognize untagged Taz1p in either the starting material or after the ATP synthase was IPed (Figure 5B). In contrast, CNAPtaz was co-IPed with the ATP synthase and the abundance of co-IPed CNAPtaz was greater when mitochondria contained CL. Critically, CNAPtaz, although readily detected, failed to be co-IPed from Δatp2[CNAPtaz] extracts, demonstrating the specificity of this interaction. That AAC2 was not co-IPed with the ATP synthase from Δtaz1[CNAPtaz] extracts indicates that the association of Taz1p with the ATP synthase does not include AAC2.

To determine whether Taz1p associates with the ATP synthase and AAC2 in distinct complexes, the concentrated eluates after CNAP were analyzed by 2D BN/SDS-PAGE.
Figure 3. CNAPTaz is physiologically relevant. (A and B) Steady-state $^{32}$P labeling and analyses were performed as described in Figure 2. In A, the relative abundance of each phospholipid is expressed as a % of the total phospholipid in each strain (mean ± SEM, n = 3–5). Statistical significance (p < 0.05) was determined by one-way ANOVA, with Holm-Sidak pairwise comparisons. In B, the relative abundance of MLCL and CL is expressed as a % of the total phospholipid in each strain (mean ± SEM, n = 3–10) (C) Digitonin extracts from Δtaz1[CNAPTaz] and Δtaz1Δcrd1[CNAPTaz] mitochondria were resolved by 2D BN/SDS-PAGE, and Taz1p complexes were detected by immunoblot. n = 3.
and immunoblotting (Figure 5C). Taz1p, the ATP synthase, and AAC2 were not detected in samples derived from extracts containing untagged Taz1p and CL (top three panels), demonstrating the specificity of this approach. In marked contrast, a range of Taz1p-containing complexes were observed when extracts contained CNAPTaz and CL, including the largest Taz1p-containing complex (middle three panels). This largest Taz1p complex was not detected when CNAPTaz was affinity-purified from extracts lacking CL (bottom three panels). In addition, CNAPTaz copurified from CL-containing membranes the ATP synthase in a complex of ~600 kDa (red arrow) and AAC2 complexes that range from ~500–300 kDa (green arrows). When expressed in membranes devoid of CL, CNAPTaz still copurified a small amount of ~600-kDa ATP synthase; however, no AAC2-containing complexes were detected. The failure to detect AAC2-containing complexes in the 2D analysis was seemingly at odds with the co-IP results (Figure 5A); this most likely reflects a further destabilization of the already weakened Taz1p–AAC2 interaction during the BN-PAGE in the absence of CL. Based on the size of the ATP synthase–Taz1p complex and the fact that 5 of the 13 ATP synthase subunits were identified by LC-MS/MS, we conclude that Taz1p physically associates with the assembled ATP synthase. Thus, Taz1p participates in at least two distinct protein complexes, one consisting of assembled ATP synthase and another that includes AAC2. Further, based on the co-IP data (Figure 5, A and B), both associations are stabilized by, but do not absolutely require, CL.

Probing the Functional Relevance of the Defined Taz1p Interactome

To begin to dissect the functional significance of the established Taz1p interactome, we analyzed the expression level (Figure 6A) and assembly status (Figure 6B) of the ATP synthase and AAC2 in assorted yeast mutants. Because both AAC2 and the ATP synthase are required for growth on respiratory media, the mitochondria used in this analysis were isolated from strains grown with dextrose as the carbon source. The expression level (Figure 6A) and assembly status (Figure 6B) of the ATP synthase was not altered by the absence of Taz1p, Crd1p, or AAC2. Similarly, the expression level and complex assembly of AAC2 was not changed when mitochondria lack either Taz1p or the ATP synthase (bottom panels). As previously demonstrated, AAC2 complex formation was drastically dependent on CL and the largest AAC2 complex, representing AAC2 associated with respiratory supercomplexes, was not detected in rho– mitochondria (Claypool et al., 2008). Thus, Taz1p is not required for the proper expression and/or assembly of the ATP synthase or the range of AAC2-containing complexes.

Taz1p expression was the same in wt mitochondria as in mitochondria lacking CL, AAC2, Atp2p (F1β), or the mitochondrial genome (rho–; Figure 6A). Intriguingly, in Δatp2 and rho– mitochondria, both of which fail to assemble ATP synthase dimers or monomers (Figure 6B, top panels), but not in Δatp2 mitochondria, the largest Taz1p complex was not detected (Figure 7A). Although this is consistent with the interpretation that the largest Taz1p-containing complex represents the Taz1p–ATP synthase complex, this conclusion is clouded by the observation that the largest Taz1p
complex was only very weakly detected in mitochondrial extracts derived from wt yeast grown in dextrose. To determine if the ability to detect the largest Taz1p complex was influenced by the carbon source used to grow the wt yeast, we directly compared Taz1p complexes in dextrose- and lactate-derived wt extracts. Oligomers of the ATP synthase have been demonstrated to be critical in establishing and maintaining normal cristae morphology (Paumard et al., 2002; Goyon et al., 2008; Strauss et al., 2008). To gain insight into the functional relevance of the Taz1p–ATP synthase association, we analyzed wt and Δtaz1 yeast by electron microscopy (Figure 8). Unlike the wt strain, which always had normal mitochondrial profiles with well-defined cristae (Figure 8, A and B), Δtaz1 mitochondria often had dramatic alterations in IM structure, with profiles containing circular arrays or elongated cristae (Figure 8, C–F). The aberrant IM structures were often greater than 1 μm in length. Strikingly, the altered mitochondrial morphologies observed in the Δtaz1 yeast were similar to those reported for ATP synthase oligomer mutants (Paumard et al., 2002; Goyon et al., 2008) even though ATP synthase oligomerization was normal in the absence of Taz1p (Figure 6B).

**DISCUSSION**

Whereas mutations in TAZ1 result in BTHS, why either the absence of Taz1p or the expression of BTHS mutant tafazzins result in the numerous pathologies associated with BTHS remains largely a mystery. To begin to address this critical issue, we focused our efforts on defining the Taz1p interactome. The importance of CL in the defined interac-
Figure 6. ATP synthase and AAC2 expression and assembly do not require Taz1p. (A) Steady-state expression of assorted proteins (5, 10, and 20 µg protein) in mitochondria isolated from the indicated strains as assessed by immunoblot. n = 3. (B) Mitochondrial extracts from the indicated strains were resolved by 2D BN/SDS-PAGE and immunoblotted for ATP synthase (top) and AAC2 (bottom). n = 3.

The Taz1p Interactome

The interactome was additionally addressed because we demonstrated that in the absence of CL, the membrane association of Taz1p is subtly but significantly altered and a very large Taz1p complex is missing. A prerequisite to identifying Taz1p-interacting proteins was to address the possibility that, as suggested (Brandner et al., 2005), Taz1p associates with itself. In fact, we demonstrated using two distinct strategies that yeast Taz1p does not form homodimers. Instead, Taz1p participates in at least two distinctly sized complexes of different subunit composition. Specifically, Taz1p associates with assembled ATP synthase and separately with AAC2 in complexes that range in size from ~500 to 300 kDa. The conclusion that the Taz1p–ATP synthase and Taz1p–AAC2 complexes are distinct is based on the following observations: 1) endogenous Taz1p was co-IPed with AAC2 in a strain lacking assembled ATP synthase; 2) the ATP synthase was not co-IPed with AAC2 from wt extracts; 3) AAC2 was not co-IPed with the ATP synthase even though CNAPTaz was; 4) 2D BN/SDS-PAGE analysis of CNAPed samples demonstrated that CNAPTaz copurified an ATP synthase complex of ~600 kDa, likely representing the monomeric form, and several AAC2-containing complexes that range from ~500 to 300 kDa; and 5) the largest Taz1p complex was not detected in mitochondrial extracts lacking assembled ATP synthase but was detected in the absence of AAC2. Although this last point is obscured to some degree because of the decreased ability to detect the largest Taz1p complex in mitochondria isolated from dextrose-grown wt yeast relative to lactate-grown wt yeast, the bulk of the data are consistent with the conclusion that the largest Taz1p complex corresponds to Taz1p-ATP synthase.

Worth noting, the vast majority of Taz1p was resolved by 2D BN/SDS-PAGE from ~67 to 232 kDa and thus clearly represents Taz1p not associated with either AAC2 or the ATP synthase. There are at least two possible explanations for our failure to identify the nature of these most abundant Taz1p complexes. First, the unidentified proteins that participate in these low-molecular-weight Taz1p complexes may not be detected by the used proteomic strategy (SDS-PAGE followed by LC-MS/MS). It is well documented that different proteomic strategies provide distinct information (Reinders et al., 2006); thus complimentary downstream analyses after CNAP may facilitate identification of these partner proteins. Second, the low-molecular-weight Taz1p complexes may represent Taz1p in association with phospholipids and/or their derivatives. Phospholipid analyses after CNAP should provide insight into this possibility.

AAC2 and the ATP synthase are functionally interrelated. AAC2 mediates the 1:1 exchange of ADP\textsubscript{in} and ATP\textsubscript{out} across the mitochondrial IM. Thus, AAC2 provides the substrate, ADP that is utilized by the ATP synthase to produce ATP. Once produced, ATP is transported across the IM by AAC2 in exchange for ADP. Both AAC2 and the ATP synthase are structurally associated with CL (Beyer and Klingenberg, 1985; Eble et al., 1990; Nury et al., 2005) as opposed to simply embedded in membranes that happen to contain CL. Very little is known about how so-called structural phospholipids are incorporated into the final tertiary and/or quaternary structure of membrane proteins. Is this a passive process that occurs coincident with nascent protein folding/assembly or instead does it represent an active process mediated by a protein that loads phospholipids into the correct final destination? If it is the latter, then in the absence of this activity, proteins that require it for their proper folding and assembly might display altered expression and/or aberrant formation of complexes. As a phospholipid transacylase, we hypothesized that Taz1p might facilitate the proper insertion of phospholipids into the final assembled ATP synthase and assembled AAC2 complexes. As the steady-state expression and complex assembly of the ATP synthase and AAC2 did not depend on Taz1p expression, this hypothesis is likely not correct, a conclusion further supported by the stability of the Taz1p, AAC2, and ATP synthase complexes even when protein synthesis is blocked.

So what is Taz1p doing in the context of the two defined interactions? In stark contrast to the ATP synthase that seems undeterred by the absence of CL, assembly of AAC2 into normal complexes is drastically altered when mitochon-
The Taz1p complexes represent stable interactions, and the largest Taz1p complex requires assembled ATP synthase and is influenced by mitochondrial respiratory capacity. (A) Mitochondrial extracts from the indicated strains were resolved by 2D BN/SDS-PAGE and immunoblotted for Taz1p. n = 3. (B) Mitochondrial extracts from the wt strain grown in dextrose- or lactate-based media were resolved by 2D BN/SDS-PAGE, and Taz1p complexes were identified by immunoblot. n = 3. (C) Steady-state 32P labeling and analyses of wt yeast grown in dextrose or lactate media were performed as described in Figure 2. The relative abundance of MLCL and CL is expressed as a % of the total phospholipid in each strain. The difference in CL abundance is statistically significant as calculated by the Student’s t test, p = 0.003 (mean ± SEM, n = 5–6). (D) WT yeast were grown in lactate medium and where indicated, 200 µg/ml cycloheximide (+CHX) was added for the final 4 h of growth before mitochondrial isolation. Mitochondrial extracts were resolved by 2D BN/SDS-PAGE and immunoblotted for Taz1p (top panels), ATP synthase (middle panels), and AAC2 (bottom panels). n = 3.

Drial membranes lack CL (Figure 6B; Claypool et al., 2008). In addition, AAC function depends on CL, both in vitro and in vivo (Hoffmann et al., 1994; Jiang et al., 2000; Claypool et al., 2008). Moreover, CL peroxidation inactivates mammalian AAC resulting in apoptosis (Imai et al., 2003). The electron transport chain is known to be the major source of reactive oxygen species, and CL, because of its high degree of unsaturation, is particularly sensitive to oxidative damage. Therefore, it is tempting to speculate that Taz1p might preserve AAC function subsequent to peroxidation of CL structurally associated with AAC. If true, deficits in AAC function could underlie the variable oxidative phosphorylation defects observed in BTHS patients. Additionally, this would indicate that Taz1p not only establishes, but also maintains, the normal fatty acyl chain profile of CL. Unfortunately, this is a difficult issue to address at present, a fact that primarily reflects the three aforementioned hallmarks of the complete loss of Taz1p function. In the absence of Taz1p, CL levels are reduced (variable 1), the remaining CL contains random more saturated fatty acyl chains (decreased sensitivity to oxidative damage, variable 2), and MLCL accumulates (variable 3). Acquisition of a temperature-sensitive Taz1p mutant would remove these variables from the equation and allow this potential function of Taz1p to be addressed.

Early studies on patient biopsies (Barth et al., 1983), as well as more recent analyses using electron tomography (Acechan et al., 2007), indicate that mitochondrial ultrastructure is abnormal in BTHS tissues and cells. Specifically, mitochondrial size is more variable, giant mitochondria more abundant, and, similar to our observations (Figure 8, C–F), cristae morphology is altered in the absence of Taz1p activity. CL is a so-called structural phospholipid capable of adopting lamellar or inverted hexagonal structures depending on the presence of divalent cations (Ortiz et al., 1999). Taz1p, as a transacylase, can actually perform this function in either direction, i.e., it can transfer an acyl chain from PC to MLCL and from CL to lyso-PC (Xu et al., 2006b). Although the PC–CL transacylase activity is the highest, Taz1p can catalyze reversible transacylations between PE and CL, PC and PE, and PC and PA (Xu et al., 2006b). PE and CL are both cone-shaped phospholipids that exhibit negative spontaneous curvature; lyso-PC, an inverted cone-shaped lipid, exhibits positive spontaneous curvature (Chernomordik and Kozlov, 2005). Thus, Taz1p has the ability to modify multiple classes of phospholipid that, because of their inherent structure, have been implicated in membrane curving and fusion events (Chernomordik et al., 2006). Our prior localization of Taz1p to all IMS-facing membranes is consistent with the hypothesis that Taz1p participates in enzymatic cascades that promote the establishment and maintenance of a mechanistically unexplained morphological feature of mitochondria, namely contact sites between the IM and OM.

Our identification that Taz1p interacts with assembled ATP synthase offers additional insight into how Taz1p may participate in shaping mitochondria. Work in yeast and mammalian systems has demonstrated a critical role for
ATP synthase oligomers in establishing and maintaining normal cristae morphology (Paumard et al., 2002; Goyon et al., 2008; Strauss et al., 2008). A very exciting possible function for Taz1p-ATP synthase is that Taz1p, because of its lipid modifying capabilities, is involved in the ATP synthase–dependent establishment and maintenance of normal cristae morphology, perhaps by lowering the energy barrier required to create invaginated membrane structures. Consistent with this hypothesized function, even though ATP synthase oligomerization is like wt in the absence of Taz1p activity, cristae morphology is altered. Collectively, this indicates that ATP synthase oligomerization is required but not sufficient for normal cristae morphology. Alternatively, the reduced steady-state levels of CL and/or the increased abundance of MLCL independent of the association of Taz1p with the ATP synthase may result in the altered mitochondrial morphology observed in the absence of Taz1p. However, similar to the AAC2 interaction, a concrete definition of the functional relevance of the association of Taz1p with the ATP synthase is prevented by the trifecta of alterations observed in mitochondrial membranes lacking Taz1p activity.

As CL is known to increase the efficiency of oxidative phosphorylation at least in part through its ability to promote the association of respiratory complexes into higher order supercomplexes (Boumans et al., 1998; Zhang et al., 2002; Pfeiffer et al., 2003; Zhang et al., 2005; Claypool et al., 2008), the majority of studies performed to date to characterize Barth syndrome have focused on determining the importance of CL remodeling, and thus Taz1p activity, for full CL function. Our present demonstration that Taz1p physically associates with two distinct respiratory components suggests that the variable respiratory defects observed in BTHS patients may be caused by absent physical associations of Taz1p with either AAC2 and/or the ATP synthase and not secondary to alterations in the CL profile in BTHS patient mitochondria. As such, unexpected insight into many of the observed abnormalities associated with BTHS mitochondria, including variable defects in oxidative phosphorylation and altered mitochondrial ultrastructure, was provided by the presently defined yeast Taz1p interactome.

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