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Defining functional classes of Barth syndrome mutation in humans

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ABSTRACT

The X-linked disease Barth syndrome (BTHS) is caused by mutations in *TAZ*; *TAZ* is the main determinant of the final acyl chain composition of the mitochondrial-specific phospholipid, cardiolipin. To date, a detailed characterization of endogenous *TAZ* has only been performed in yeast. Further, why a given BTHS-associated missense mutation impairs *TAZ* function has only been determined in a yeast model of this human disease. Presently, the detailed characterization of yeast tafazzin harboring individual BTHS mutations at evolutionarily conserved residues has identified seven distinct loss-of-function mechanisms caused by patient-associated missense alleles. However, whether the biochemical consequences associated with individual mutations also occur in the context of human *TAZ* in a validated mammalian model has not been demonstrated. Here, utilizing newly established monoclonal antibodies capable of detecting endogenous *TAZ*, we demonstrate that mammalian *TAZ*, like its yeast counterpart, is localized to the mitochondrion where it adopts an extremely protease-resistant fold, associates non-integrally with intermembrane space-facing membranes and assembles in a range of complexes. Even though multiple isoforms are expressed at the mRNA level, only a single polypeptide that co-migrates with the human isoform lacking exon 5 is expressed in human skin fibroblasts, HEK293 cells, and murine heart and liver mitochondria. Finally, using a new genome-edited mammalian BTHS cell culture model, we

demonstrate that the loss-of-function mechanisms for two BTHS alleles that represent two of the seven functional classes of BTHS mutation as originally defined in yeast, are the same when modeled in human TAZ.

INTRODUCTION

The boundaries of cells and organelles are marked by membranes that differ in their phospholipid and protein compositions. The collection of phospholipids, including their associated fatty acyl chains, that establish a defined membrane-bound compartment confer unique membrane properties (curvature and rigidity) and contribute to numerous functions that occur at/across the membrane surface (transport and signaling) or within the space that it confines. As such, changes in membrane lipid composition can directly impact numerous fundamental cellular processes. A particularly salient example of the importance of proper lipid composition for organelle function is provided by the mitochondrion, the powerhouse of the cell, and its signature phospholipid, cardiolipin (CL).

CL is required for a diversity of basic processes that are central to mitochondrial biology (1). In fact, CL is of such fundamental importance that mammalian development fails *in utero* in its absence (2). CL is a dimer of two phosphatidic acids linked together by a glycerol bridge. CL associates with numerous mitochondrial proteins, including every oxidative phosphorylation (OXPHOS) complex/component, many solute carriers, and translocases in both the outer and inner mitochondrial membranes (3-5). Collectively, these interactions with CL stabilize individual proteins and multisubunit complexes, and can stimulate enzyme activity either directly (e.g. serve a catalytic function (6)) or

secondarily (e.g. promote the assembly of individual respiratory complexes into more efficient supercomplexes (7-9)). Finally, CL serves as a signal that is detected by CL-binding proteins with downstream consequences that range from mitochondrial fission and inner mitochondrial membrane (IMM) fusion (10, 11) to apoptosis and mitophagy (12, 13).

The biosynthesis of CL occurs through a reaction series that is highly conserved from yeast to humans (14). With respect to its acyl chain composition, the CL that is newly produced is not what accumulates at steady state, which is highly homogeneous and characterized by increased acyl chain unsaturation and molecular symmetry (15). The steady state form of CL is established by an acyl chain remodeling process that begins with the removal of a fatty acyl chain from CL by a deacylase(s), generating monolysocardiolipin (MLCL), which is then re-acylated by the evolutionarily conserved MLCL transacylase, tafazzin (TAZ) (16). Mutations in *TAZ* result in the X-linked cardio- and skeletal myopathy, Barth syndrome (BTHS) (17, 18). In the absence of TAZ, there is less CL, the remodeling precursor and product, and more MLCL, the remodeling intermediate (19). Further, the CL that remains has an abnormal acyl chain profile (15, 20). These BTHS-associated changes in mitochondrial phospholipid composition destabilize respiratory supercomplexes, reduce OXPHOS efficiency, increase reactive oxygen species production, and impair sarcomere organization (21-24).

BTHS is the founding member of a new class of mitochondrial disorder that results from nuclear defects that impact mitochondrial phospholipid metabolism (25). Notably, some of the disorders, including dilated cardiomyopathy with ataxia (DCMA), Sengers, and MEGDEL syndromes, in this new class of mitochondrial disease share some

biochemical phenotypes with BTHS. In each case, changes in the acyl chain pattern, abundance, and or submitochondrial distribution of CL are postulated to contribute to disease pathogenesis (26-29). However, their exact pathogenic mechanisms have not been established and thus the basis for the shared and unique phenotypes of this cohort of syndromes is presently unclear.

Currently, a detailed topological map of tafazzin-based CL remodeling has only been drawn in the yeast *Saccharomyces cerevisiae*. In yeast, all of the steps up to and including the initiation of the remodeling process occur on the matrix side of the IMM (14). In contrast, yeast Taz1 is anchored to both the IMM and outer mitochondrial membrane (OMM) facing the intermembrane space (IMS) by virtue of a membrane anchor that protrudes into but not completely through the lipid bilayer (30). Thus, in yeast, CL remodeling involves an obligate trafficking step of MLCL from one side of the IMM to the other. Further, a yeast BTHS model has been exploited to determine the disease pathogenicity of a multitude of patient-associated missense alleles. To date, seven distinct pathogenic loss-of-function (LOF) mechanisms have been identified (30-32). Thus at present, our understanding of the basic cell biology of this clinically important remodeling pathway and the biochemical abnormalities associated with any genetic lesion linked to BTHS is based solely on work in yeast.

Here, we show that mammalian TAZ is localized to the mitochondrion where it adopts an extremely protease-resistant fold, associates non-integrally with IMS-facing membranes, and assembles in a range of complexes. Even though multiple isoforms are expressed at the transcript level, only a single TAZ polypeptide that co-migrates with the human isoform lacking exon 5 is detected in mitochondria isolated from human skin

fibroblasts, HEK293 cells, and murine heart and liver. Utilizing a newly established mammalian BTHS cell model, we determined that the two pathogenic mutations tested result in the same biochemical consequences when modeled in human TAZ as originally defined in yeast. Interestingly, overexpression of the unstable R57L mutant which lacks transacylase activity *in vitro*, partially rescues the mitochondrial CL and MLCL abnormalities of the *taz* deficient cells *in vivo*, suggesting that strategies aimed at stabilizing this mutant allele could be of therapeutic benefit.

RESULTS

Mammalian TAZ is localized to the mitochondrion

The absence of antibodies capable of detecting endogenous TAZ has significantly impeded progress in our understanding of BTHS pathogenesis. To overcome this obstacle, three TAZ-specific mouse monoclonal antibodies (mAb; 2G3F7, 3D7F11, and 2C2C9) were generated. Critically, each mAb detected endogenous TAZ in fibroblast cell extracts from healthy controls (C109) but not two BTHS patients (TAZ001 and TAZ003) (Fig. 1A). TAZ001 encodes a truncated 50 amino acid protein while TAZ003 has a single R57L missense allele in TAZ (20). Using these new reagents, endogenous TAZ co-fractionated with mitochondria (Fig. 1B) and was detected in both human and murine mitochondria (Fig. 1C).

Human TAZ mRNA is present as a number of splice variants (20, 33). To determine the capacity of the three mAbs to detect different TAZ isoforms, the epitope recognized by each mAb was mapped; each of the three mAbs binds an epitope that is present in a different exon (Fig. 1D). Moreover, they each have the capacity to recognize

every predicted isoform based on mRNA analyses (20, 33). Following immunoprecipitation of TAZ with one mAb (3D7F11), only a single TAZ band was detected by immunoblot using a second mAb (2C2C9) in control but not BTBS cell extracts (Fig. 1E). Finally, based on co-migration with assorted human TAZ isoforms expressed in *taz1*Δ yeast (34), all of the cells and tissues surveyed only express TAZ lacking exon 5 (TAZ-ex5; exon 5 is missing in rodents so full-length murine TAZ already lacks exon 5) (Fig. 1F). Thus, TAZ-ex5 is likely the major isoform expressed at the protein level *in vivo*.

Generation of a mammalian BTBS cell model

To establish a new mammalian BTBS cell culture model, we utilized TALEN-mediated genome editing in 293 Flp-In cells to introduce genetic lesions within *TAZ* exon 1. Two *taz*^{TALEN} clones were identified that had undetectable levels of TAZ protein (Fig. 2B) and which contained genomic deletions in the targeted locus (Fig. 2A). In both *taz*^{TALEN}.2 and 19, a proline that is highly conserved in mammals but not in fungi, is deleted (Fig. S1); in addition, there was a missense mutation (V12A) identified in *taz*^{TALEN}.19 and five additional amino acids removed (amino acids 11-16) in *taz*^{TALEN}.2. Surprisingly, neither deletion altered the overall reading frame of TAZ. Based on *in silico* analyses (Table SI), we hypothesize that Pro13 is either a critical determinant of a non-bilayer spanning membrane anchor and/or essential for TAZ folding and stability. Consistent with these possibilities, proteosomal inhibition with MG132 did not stabilize TAZ in either *taz*^{TALEN} cell line (Fig. 2C). The lack of protein expression did not reflect differences in TAZ mRNA levels (Fig. 2D). Moreover, the expression of representative proteins of each mitochondrial subcompartment and every OXPHOS complex was the

same in mitochondria isolated from each *taz*^{TALEN} cell line as WT (Fig. 2E and F). Of note, the expression of PHB2 and DNAJC19, proteins functionally linked to TAZ (28), was also not altered in either *taz*^{TALEN} cell line. Finally, as matrix-targeted Su9-DHFR was imported into mitochondria isolated from both *taz*^{TALEN} cells at rates similar to that of WT mitochondria, the membrane potential across the IMM is not grossly impaired in the *taz*^{TALEN} cells (Fig. 2G and H).

Next, the phospholipid composition of mitochondria isolated from WT and *taz*^{TALEN} cells was determined by mass spectrometry. An increased MLCL:CL ratio is a biochemical hallmark of TAZ deficiency (35). Importantly, mitochondria from each *taz*^{TALEN} cell contained a significantly increased MLCL:CL ratio (Fig. 3C) that was largely driven by an accumulation of MLCL (Fig. 3B). The amount of CL was not significantly changed (Fig. 3A). TAZ-based CL remodeling is a major determinant of the final acyl chain composition of CL at the steady state, which is typified by the inclusion of more unsaturated acyl chains compared to pre-remodeled CL. While there were only modest changes in CL acyl chain length in the *taz*^{TALEN} cells (Fig. 3E), the amount of CL containing fewer double bonds was significantly increased (Fig. 3F). Moreover, the relative abundance of unsaturated acyl chains for CL molecules of a given length was consistently lower in *taz*^{TALEN} than in WT mitochondria (Fig. 3G-K). In addition to its dramatically increased abundance, there were also notable changes in the acyl chain profile of the remodeling intermediate MLCL in mitochondria isolated from both *taz*^{TALEN} cells (Fig. 3L-O). Specifically, MLCL with longer acyl chains that contained fewer double bonds accumulated in the absence of TAZ activity. Other alterations in the mitochondrial lipidome that were consistent features of mitochondria from both *taz*^{TALEN}

cells included an increased abundance of phosphatidylglycerol (PG, Fig. 3D), bis-monoacylglycerol phosphate, reduced levels of phosphatidylinositol, and subtle changes in the acyl chain length and saturation of phosphatidylcholine (PC) and phosphatidylethanolamine, two lipid classes that can be used by TAZ as acyl chain donors for MLCL re-acylation (16) (Fig. S2-S4). Collectively, these results validate the *taz*^{TALEN} cells as new cellular BTHS models.

Mammalian TAZ is a membrane protein that assembles in high molecular weight complexes

Yeast Taz1 associates with the IMS-side of the IMM and OMM by virtue of a membrane anchor that extends into but not through the lipid bilayer (30). To determine if human TAZ is similarly membrane-associated, mitochondrial extracts from 293 Flp-In or C109 fibroblasts were sonicated and centrifuged to obtain the pellet, containing membrane-associated proteins, and the supernatant, containing soluble proteins. TAZ, like TIM23 and TOM20, was detected in the pellet fraction (Fig. 4A). Next, alkali extraction was performed. As expected, the integral proteins TIM23 and TOM20 were largely retained in the pellet whereas the peripheral cytochrome c (CYT C) was quantitatively released into the supernatant (Fig. 4B). Like yeast Taz1, human TAZ was steadily released into the supernatant as the pH increased, suggesting a similar mode of membrane association (Fig. 4C). Alternatively, analogous to succinate dehydrogenase subunit A (SDHA), TAZ membrane association may be determined by an interaction with a yet-to-be identified integral membrane protein.

As yeast Taz1 engages in a range of macromolecular complexes (30, 36), the assembly of mammalian TAZ was examined by 2D blue native (BN)/SDS-PAGE. In

both human and murine mitochondria, TAZ assembled in a range of complexes (Fig. 4D). In sum, these results demonstrate that mammalian TAZ associates with mitochondrial membranes and engages in macromolecular complexes in a manner that is highly reminiscent of its yeast ortholog.

Mammalian TAZ is highly protease resistant and localized to IMS-facing leaflets

Next, we determined the submitochondrial localization of TAZ. First, mitochondria from 293 Flp-In cells were titrated with increasing amounts of digitonin, causing the sequential release of soluble IMS proteins, soluble matrix proteins, membrane proteins of the OMM, and finally IMM-embedded proteins into the supernatant (Fig. 5A and B). The fractionation profile of human TAZ overlapped with both IMM and OMM proteins (Fig. 5B) suggesting that like yeast Taz1, human TAZ may associate with both the IMM and OMM (4, 30, 37).

Second, a protease-protection assay was performed to determine if human TAZ is associated with IMS-facing membranes as predicted based on results in yeast. In brief, human and murine mitochondria were incubated in iso-osmotic buffer, low osmotic buffer, or low osmotic buffer supplemented with detergent, with each permutation performed in the absence or presence of the non-specific protease mixture, pronase E (Fig. 5C). Accordingly, OMM proteins facing the cytosol (e.g. TOM20) are accessible to added protease in each condition, residents of the IMS (TIM23; TIM23 is an IMM protein with a large domain exposed to the IMS) are degraded by added protease following osmotic rupture of the OMM (low osmotic buffer) and detergent solubilization, and matrix proteins (GRP75) are only degraded upon addition of detergent with protease. Like the matrix marker GRP75, in every source of mitochondria tested, mammalian TAZ

was only degraded by pronase after detergent solubilization. These results thus suggest that mammalian TAZ is localized to the matrix-facing leaflet of the IMM, the opposite side of the IMM as yeast Taz1.

This conclusion, however, is predicated on TAZ being sensitive to protease. Thus, another potential explanation for the observed pattern is that perhaps TAZ adopts a highly protease-resistant fold that becomes accessible to proteolytic cleavage only in the presence of detergent. To test the idea that TAZ is protease-resistant, we compared its sensitivity to proteases upon addition of detergent with or without prior heat denaturation (Fig. 5D). When solubilized with SDS, heat denaturation increased the sensitivity of TAZ to degradation by each protease. One potential explanation for the resistance of TAZ to proteolytic degradation is that it forms aggregates. However, using a sequential detergent solubility assay where TAZ was readily solubilized with digitonin (protein aggregates resist solubilization even when re-extracted with TX-100), TAZ does not form protein aggregates (Fig. 5E).

In light of TAZ's protease resistance, the assays performed cannot conclusively establish the topology of membrane-associated TAZ. Therefore, we performed an *in organello* import assay in which radiolabeled TAZ precursor was incubated with WT mitochondria in the presence or absence of a membrane potential and non-imported precursor removed with trypsin (Fig. 5F). Import of TAZ was time-dependent but membrane potential-independent. The TAZ precursor was not processed upon import consistent with the failure of most *in silico* programs (Table SII) to predict a mitochondrial presequence cleavage site. Critically, ³⁵S-TAZ precursor was readily degraded by trypsin following incubation with mitochondria on ice and disruption of the

OMM (compare lanes 7 and 8). Finally, following import in the presence of a membrane potential, disruption of the OMM in the presence of trypsin drastically decreased the ³⁵S-TAZ detected (lane 6). Thus, the protease resistance of TAZ observed at steady state occurs after it is imported into the mitochondrial IMS.

To gain further insight into the topology of TAZ, epitope tags were added to either termini of TAZ (CNAP-TAZ and TAZ-3XFLAG for NH₂- or COOH-terminal tagged TAZ, respectively) (Fig. 6A). Upon transfection into the *taz*^{TALEN}.19 cell line, each construct was expressed similar to untagged WT TAZ (Fig. 6B) and importantly, rescued the altered MLCL:CL ratio of this TAZ-deficient model (Fig. 6C and D). The sensitivity of the appended epitope tags to proteolytic degradation was determined in intact mitochondria, following OMM rupture, or in the presence of detergent. Like the IMS marker TIM23, which is anchored to the IMM but exposes a large domain in the IMS, the NH₂-terminal epitope of CNAP-TAZ became sensitive to added protease when the OMM was disrupted (Fig. 6F). In contrast, 3XFLAG added to the COOH terminus of TAZ was resistant to proteolytic degradation unless detergent was included (Fig. 6F). Thus, either the COOH terminus of TAZ is in the matrix, implying that TAZ has a membrane-spanning domain contrary to expectations based on its alkali extraction profile (Fig. 4B and C), or alternatively, the added tag is incorporated in the core protease-resistant structure of TAZ. To distinguish between these two possibilities, we transfected the *taz*^{TALEN}.19 cell line with TAZ harboring a COOH-terminal APEX2 tag (38, 39). APEX2 encodes an engineered ascorbate peroxidase that can be used in electron microscopy studies to localize a tagged protein (38, 39). TAZ-APEX2 was expressed normally and functional based on the restored MLCL:CL ratio (Fig. 6B-D). When

taz^{TALEN} cells expressing TAZ-APEX2 were reacted with 3,3'-diaminobenzidine (DAB), in the presence of H₂O₂, electron micrographs revealed reaction product in and around the mitochondrial IMS (Fig. 6G). Conversely, DAB staining of *taz*^{TALEN} cells expressing untagged TAZ (control) revealed no staining of the IMS. Our combined results indicate that mammalian TAZ adopts a highly protease-resistant structure following its import into the mitochondrial IMS where it non-integrally associates with mitochondrial membranes with both termini exposed to the IMS.

The R57L allele encodes an unstable protein

TAZ003 fibroblasts harbor an R57L missense mutation that was previously classified in yeast (K65L) as a temperature-sensitive allele (Fig. 7A) (20, 32). As such, the inability to detect TAZ in TAZ003 cells could provide evidence that like in yeast, the R57L allele is intrinsically unstable. To determine if the R57L allele encodes an unstable polypeptide, we re-introduced into the two *taz*^{TALEN} cell lines WT TAZ, R57L, and an H69Q TAZ mutant that in yeast encodes a catalytically-null polypeptide (32). The 5-6 subclones that were isolated per construct per parental cell line displayed little variability in TAZ expression for a given TAZ allele (Fig. 7B). Consistent with the possibility that the R57L allele is intrinsically unstable, its steady state expression in clones from both *taz*^{TALEN} cells was dramatically reduced compared to WT TAZ-expressing cells. The reduced expression was not reflected at the transcript level (Fig. 7C), did not result from impaired import into the mitochondrial IMS (Fig. 7D), and was not restored upon proteasomal inhibition (Fig. 7E and S5A). Moreover, when cytosolic protein synthesis was inhibited with cycloheximide, R57L had a much shorter half-life ($t_{1/2}$) than WT TAZ

(Fig. 7F and S5B). Thus, as previously characterized in a yeast BTHS model (32), the R57L BTHS variant encodes an unstable protein.

The H69Q allele is catalytically-null

The H69Q mutation disrupts a highly conserved HXXXXD acyltransferase motif. When modeled in yeast, the equivalent H77Q mutation results in a protein that behaves biochemically and cell biologically like WT Taz1 but that is completely devoid of transacylase activity (32). As expected, expression of H69Q in whole cell extracts (Fig. 7B) and isolated mitochondria (Fig. 8A) was similar to WT TAZ. The H69Q allele was imported into the mitochondrial IMS (Fig. 8B) and assembled in complexes (Fig. 8C and S5C) just like WT TAZ. The preserved complex formation of the H69Q mutant indicates that TAZ assembly is insensitive to increased MLCL or changes in CL acylation. To directly measure the transacylase activity of WT and mutant TAZ, isolated mitochondria were incubated with ¹⁴C-labeled lyso-PC and its conversion to PC monitored (Fig. 8D). Overexpression of WT TAZ resulted in dramatically increased transacylase activity. In stark contrast, mitochondria overexpressing H69Q (or R57L) lacked any detectable transacylase activity above background. Finally, while WT TAZ restored the MLCL:CL ratio of both *taz*^{TALEN} cell lines to normal, overexpression of the H69Q mutant did not (Fig. 8E-G). Therefore, the H69Q variant encodes a catalytically inactive polypeptide in both mammals and yeast. Intriguingly, overexpression of the R57L mutant, which was devoid of transacylase activity *in vitro* (Fig. 8D) did partially improve the MLCL:CL ratio of both *taz*^{TALEN} cell lines *in vivo* (Fig. 8E-G) indicating that prior to its degradation, it retained some residual catalytic activity. Consistently, the R57L allele was still assembled normally (Fig. S5C). Collectively, these results suggest that the LOF

mechanisms that underlie certain missense mutations are conserved in yeast and mammalian BTHS models.

DISCUSSION

We have determined that CL remodeling is organized in topologically similar ways in mammals and yeast. Like in yeast, mammalian TAZ is localized to the IMS-facing leaflets of the OMM and IMM. Thus, the main determinant of the final CL acyl chain pattern, TAZ, resides on the opposite side of the IMM as the CL biosynthetic machinery. In yeast, the lipase that functions upstream of Taz1, Cld1, is peripherally associated with the matrix side of the IMM (40). At present, the identity and detailed localization of the mammalian lipase(s) that functions upstream of human TAZ to initiate CL remodeling has not been defined. The rationale for the localization and topology of TAZ-based CL remodeling in yeast and mammals is presently unclear but is presumed to be important for it to function properly. Notably, besides dictating the final acyl chain pattern of CL, the physiological functions of this conserved remodeling pathway remain elusive (41).

Overall, our work suggests that human and yeast TAZ, whose polypeptides are only 18% identical and 41% conserved, are remarkably similar biochemically. Not only are their submitochondrial localizations the same, yeast and mammalian TAZ also interact with mitochondrial membranes in a similar manner which in yeast involves a membrane anchor that extends into but not completely through the lipid bilayer (30).

Further, both yeast and mammalian TAZ assemble in a range of complexes that appear qualitatively quite comparable (36). Previously, we demonstrated that yeast Taz1 does not associate with itself but does interact with the ATP synthase as well as the major mitochondrial ADP/ATP carrier, Aac2 (36). Thus, it is possible that these interactions will be evolutionarily conserved. However, it is important to mention that the vast majority of the yeast Taz1 interactome remains undocumented and the noted interactions between yeast Taz1 and the ATP synthase or Aac2 are clearly sub-stoichiometric. The composition of all of the mammalian TAZ-associated complexes is similarly unknown but is something we are actively investigating.

Critically, another feature shared between yeast and mammalian TAZ are the LOF mechanisms associated with distinct pathogenic mutations. Using a novel BTHS cell model, the LOF mechanism for the two tested pathogenic mutations were conserved as modeled in yeast or human TAZ. In patient-derived TAZ003 cells, the protein product of the R57L allele is undetectable. When overexpressed in *taz*^{TALEN} cells, the R57L mutant localized properly and assembled normally (Fig. S5A-C), but accumulated to a much lesser extent and had a significantly shorter half-life than WT TAZ. The corresponding yeast mutant, K65L, is a temperature-sensitive allele that becomes unstable and loses activity at non-permissive temperature (32). Interestingly, even though the R57L allele lacked *in vitro* activity, its overexpression did improve the MLCL:CL ratio in both *taz*^{TALEN} cells (Fig. 7G). Therefore, small molecules capable of stabilizing the R57L mutant could increase its activity and thus represent viable therapeutic options. Importantly, dysfunctional conservation extends to the H69Q mutation. In yeast, the corresponding H77Q mutant is identical to WT Taz1 in every way tested except that it is

devoid of transacylase activity (32). As predicted from yeast, the H69Q mutant allele closely resembled WT TAZ except that it was catalytically-null.

TAZ is a promiscuous transacylase that can utilize a range of lyso-lipid substrates and phospholipid acyl chain donors. Moreover, TAZ itself lacks intrinsic acyl chain specificity which raises the obvious question of how a non-specific enzyme can generate CL that typically has a specific collection of acyl chains. Recent experiments have suggested that the physical state of phospholipid membranes can affect the substrate specificity of TAZ. Under experimental conditions that mimic high membrane curvature, have an increased proportion of non-bilayer lipids, or perturbed bilayer states, TAZ gains the capacity to generate the physiologically-relevant CL molecular form that contains four attached 18:2 linoleic acids (42). In mitochondria, such privileged lipid domains may be delineated by the recently described prohibitin/DNAJC19 complexes (28). The ring-shaped prohibitin complexes, composed of PHB1 and PHB2 subunits and including DNAJC19 (28), form protein and lipid scaffolds within the IMM that contribute to cristae morphogenesis and cell proliferation (43). Surprisingly, knockdown of either PHB2 or DNAJC19, which do not interact with TAZ, changes the acyl chain pattern of CL similar to TAZ depletion (28). Depletion of either protein with TAZ does not prevent the accumulation of MLCL. These findings suggest that PHB/DNAJC19 complexes participate in CL remodeling after the process is initiated. Since MLCL does not accumulate, TAZ is still active when PHB2 or DNAJC19 expression is reduced. Although both completely traverse the IMM bilayer, the majority of the DNAJC19 polypeptide resides in the matrix. Thus, how PHB/DNAJC19 complexes could modulate TAZ specificity remains unclear based on the submitochondrial localization and

interfacial membrane association of TAZ as defined previously in yeast and here in mammals. Given our placement of mammalian TAZ on the IMS-leaflet of the IMM, it is tempting to speculate that PHB/DNAJC19 complexes sequester TAZ within membrane domains with physical properties (high membrane curvature) that confer acyl chain specificity to TAZ. While the exact details of how PHB/DNAJC19 complexes modulate the specificity of TAZ-mediated CL remodeling is undefined, the functional relationship between PHB/DNAJC19 complexes and TAZ is underscored by the fact that mutations in DNAJC19 result in DCMA, a disease that shares many clinical features with BTHS (25, 26). Our results thus establish a framework to dissect the functional relationship between PHB/DNAJC19 complexes and TAZ which may in turn reveal the basis for the shared and distinct phenotypes of BTHS and DCMA.

MATERIALS AND METHODS

Molecular Biology — The human TAZ ORF lacking exon 5 was amplified by PCR using cDNA from HeLa cells and subcloned first into pBSK (Stratagene) and then into pcDNA5/FRT (Invitrogen). The R57L and H69Q mutations were introduced into the parental pcDNA5/FRT_{hTAZ} plasmid *via* overlap extension (44). TAZ with an NH₂ terminal CNAP tag (45) or COOH terminal 3XFLAG or APEX2 tags were introduced into the parental pcDNA5/FRT_{hTAZ} plasmid *via* overlap extension (44). For TAZAPEX2, pcDNA3APEX2-NES (39), a gift from Alice Ting (Addgene plasmid # 49386), was used as template. For *in vitro* transcription/translation, WT TAZ and the R57L and H69Q alleles were subcloned into pSP64. The sequence of every construct was

verified by DNA sequencing. The TALEN binding pair recognizes sequences in *TAZ* exon1 that are separated by 16 nucleotides and immediately downstream of the start site (Life Technologies). The two constructs, which are fused to the truncated FokI nuclease, were subcloned into pcDNA3.1 and pEF6/V5-HisA (Life Technologies) generating pcDNA3TAZ1-R and pEF6ATAZ1-L, respectively.

Cell Culture — All cells were grown at 37°C, 5% CO₂. Control (C109) and BTHS patient fibroblasts (TAZ001 and TAZ003) (20) were maintained in DMEM (Cellgro) containing 10% fetal bovine serum (FBS, Hyclone), 2 mM L-glutamine (Gibco), and 50 units/L penicillin-streptomycin (Gibco). HEK293 Flp-In cells (Invitrogen) were grown in DMEM containing 10% FBS, 2 mM L-glutamine, and 100 µg/ml zeocin (Invitrogen). To establish a BTHS cell model, 293 Flp-In cells grown in medium lacking antibiotic but supplemented with 50 µg/ml uridine, were transfected with a 1:1 ratio of pcDNA3TAZ1-R and pEF6ATAZ1-L using FuGENE 6 (Promega), selected with 0.5 mg/ml G418 and 5 µg/ml blasticidinS (Cellgro) for 1 week, and single colonies isolated by ring cloning. Individual clones were maintained in the same medium as used for the parental 293 Flp-In cells and the inclusion of uridine found to not be essential. Whole cell extracts derived from candidate clones were harvested and analyzed by SDS-PAGE and immunoblot. Stable *taz*^{TALEN} rescue cell lines were generated by co-transfecting the two *taz*^{TALEN} cell lines with pOG44 (expressing the Flp-recombinase) and the relevant pcDNA5/FRT plasmid (WT, CNAP-TAZ, TAZ-3XFLAG, TAZ-APEX2, R57L, H69Q) at a ratio of 9:1 using FuGENE 6. Transfected cells were selected using 200 µg/ml hygromycin B (Invitrogen), individual clones recovered by ring cloning, expanded, and screened by immunoblot. Equal numbers of individual clones per construct were combined to

establish a pooled clonal population that was then used in all subsequent analyses. Each pooled population was derived from at least 5 individual clones except for *taz*^{TALEN}.¹⁹ overexpressing CNAP-TAZ which was established from 3 clones. Mycoplasma contamination was routinely monitored and not detected.

Purification of recombinant human TAZ — The entire open reading frame of *TAZ* lacking exon5 was cloned into the pET28a vector (Novagen) downstream of the 6xHis tag, induced at 37°C for 4 hours with 1 mM IPTG in C41 (DE3) *Escherichia coli*, and inclusion bodies separated from native protein extracts by centrifugation at 22,000 ×g for 1 hour at 4°C. Lipids and debris were removed from the inclusion bodies by resuspending the pellet with 7-10 mls of wash buffer (50 mM Tris-HCl, pH 7.0, 2.5 mM EDTA, 1 M urea, 1% (v/v) Triton X-100) per gram of cells using a dounce homogenizer followed by centrifugation at 22,000 ×g for 30 minutes at 4°C. This wash step was repeated three times until the supernatant was clear. A final wash was performed using wash buffer lacking urea and Triton X-100. The final recovered protein pellet was resuspended using a dounce homogenizer with 4 ml of extraction buffer (50 mM HEPES-KOH, pH 7.5, 5 mM EDTA, 8 M guanidine-HCl, 2 mM β-mercaptoethanol) per gram of cells and clarified by centrifugation at 100,000 ×g for 1 hour at 4°C. The solubilized inclusion bodies were mixed 1:1 with ddH₂O, His₆-TAZ purified using Ni²⁺ agarose (Qiagen), and bound material recovered under denaturing conditions.

Antibodies — Custom monoclonal antibodies against human TAZ-ex5 were produced by Genscript using purified recombinant protein as antigen. Three hybridomas – 2C2C9, 3D7F11, and 2G3F7 – that secrete TAZ-reactive antibodies were established. Antibodies against the following proteins were: GRP75 (Antibodies Inc. 75-127); β-actin (A5441)

and TIMM9 (WH0026520M1) (Sigma); topoisomerase I (Developmental Studies Hybridoma Bank CPTC-TOP1-1-S); calreticulin 3 (sc-134295) and TOM20 (sc-11415) (Santa Cruz); HLA-B (Epitomics 2389-1); TIM23 (611332), CYCS (556433), SMAC/DIABLO (612246), and cyclin D1 (556470) (BD); UQCRC2 (ab14745), ACO2 (ab129069), VDAC1 (ab15895), and COX4 (ab16056) (Abcam); SDHA (Invitrogen 4592000); PHB2 (BioLegend 611802); DNAJC19 (ProteinTech 12096-1-AP); and NDUFB6 (Molecular Probes, discontinued). F1 β was a kind gift from Dr. Peter Pedersen (Johns Hopkins School of Medicine) and the ANT2/5H7 (46) antibody was raised against the yeast ADP/ATP carrier, Aac2, but conveniently cross-reacts with human and murine ANT2. Goat anti-rabbit or mouse secondary antibodies conjugated to horseradish peroxidase were also used (31460 and 62-6520; Pierce).

Whole cell extraction — Confluent culture dishes were washed twice with ice-cold PBS and lysed with RIPA lysis buffer (1% (v/v) Triton X-100, 20 mM HEPES-KOH, pH 7.4, 50 mM NaCl, 1 mM EDTA, 2.5 mM MgCl₂, 0.1% (w/v) SDS) spiked with 1 mM PMSF for 30 minutes at 4°C with rotation. Insoluble material was removed by centrifugation for 30 minutes at 21,000 \times g at 4°C, the supernatant collected, and protein quantified using a bichichronic acid (BCA) assay (Pierce).

Immunoblotting — SDS-PAGE and immunoblotting was performed as previously described (47). Images were captured with a Fluorchem Q (Cell Biosciences, Inc) quantitative digital imaging system and quantitation performed using the indicated software. Images were processed in Adobe Photoshop with only linear adjustments in contrast and brightness and assembled in Adobe Illustrator.

Preparation of subcellular fractions — Fibroblasts and 293 Flp-In cells were seeded onto four 150 mm×25 mm tissue-culture dishes and allowed to expand to confluency. Two days prior to mitochondrial isolation, the cells were switched from dextrose- to galactose-based media (DMEM without glucose, Gibco; supplemented with 10 mM galactose, Sigma). Mitochondria were isolated on ice (with ice-cold buffers) using a protocol adapted from (48). Briefly, after aspirating spent media from dishes, cells were scraped off in PBS and centrifuged at 600 ×g for 10 minutes. After washing the cells once more with PBS, the pelleted cells were resuspended in 3 ml isolation buffer (IB_c, 10 mM Tris-MOPS, 1 mM EGTA/Tris, pH 7.4, 200 mM sucrose) and homogenized with 40-45 strokes in a motor-driven tightly fitting Teflon Potter-Elvehjem at 1,600 rpm. Cell lysates were then centrifuged twice at 600 ×g for 10 minutes; the resulting pellet is the nuclear fraction. Mitochondria were recovered from the supernatant by centrifugation at 7,000 ×g for 10 minutes. The mitochondrial pellet was resuspended to a volume that fits a 1.5 ml microcentrifuge tube; after an additional 7,000 ×g spin, a 10,000 ×g clarifying spin was performed to obtain crude mitochondria. The supernatant from the prior 7,000 ×g spin contains cytosolic, lysosomal, and ER fractions which were further sub-fractionated as follows. Lysosomes were pelleted after a 20,000 ×g spin for 30 minutes and resuspended in a residual amount of IB_c. The remaining supernatant was centrifuged at 40,000 ×g for another 30 minutes to obtain plasma membranes. Further centrifugation of the obtained S40 (100,000 ×g for 1 hour) resulted in the isolation of ER (pellet) and cytosolic fractions (supernatant). Heart and liver mitochondria from 10-12 week old female FVB mice were isolated on ice using a protocol adapted from (49, 50). Briefly, tissues were rinsed with ice-cold CP1 (100 mM KCl, 50 mM MOPS, pH 7.4, 5 mM

MgSO₄, 1 mM EGTA, 1 mM ATP), minced with scissors and washed several times with CP1 before resuspension in CP2 (CP1 supplemented with 2 mg/ml BSA). Tissues were then homogenized using a Polytron (three 2.5 second pulses on medium setting; Fisher PowerGen 1000 large bore homogenizer). The homogenate was centrifuged at 580 ×g for 7 minutes at 4°C and the resultant supernatant contained either liver mitochondria or in the case of heart tissue, subsarcolemmal mitochondria (SSM), while the pellet contained heart interfibrillar mitochondria (IFM). Liver and SSM supernatants were repeatedly centrifuged at 580 ×g until there were no visible cell/debris pelleting. A final clarifying spin at 3,000 ×g for 7 minutes was performed before resuspending the mitochondrial pellets in KME (100 mM KCl, 50 mM MOPS, 1 mM EGTA). To extract IFM mitochondria, the pellet was resuspended in CP1 containing 5 mg trypsin per gram tissue with mixing, for 10 minutes on ice. The trypsin was inactivated with an equivalent volume of CP2 before centrifugation at 7,500 ×g for 7 minutes at 4°C. For our purposes (to maximize heart mitochondrial yield) the resultant pellet was resuspended in CP2, clarified with a 3,000 ×g spin before being resuspended in KME and combined with the SSM mitochondria. Organellar and mitochondrial fractions were quantitated using a BCA assay. Mitochondria were resuspended to 10 mg/ml in IB_c (cells) or KME (tissues) and if not used immediately, aliquoted, snap frozen with liquid N₂, and stored at -80°C for downstream analyses.

Epitope-Mapping — A peptide scan consisting of 15mer peptides with an 11 amino acid overlap between adjacent peptides that spans the entire 262 amino acid TAZ protein was generated by JPT Peptide Technologies. The membrane was moistened with methanol prior to immunoblotting and the membrane subsequently regenerated with 100 mM β-

mercaptoethanol, 1% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.7, two more times to map epitopes bound by the different TAZ monoclonal antibodies. The epitopes recognized by each of the three monoclonal antibodies were determined by densitometry analysis of the immunoreactive PepSpots.

Immunoprecipitation — The TAZ monoclonal antibody, 3D7F11, or normal mouse serum was conjugated to protein G-sepharose using the Seize X Protein A Immunoprecipitation kit and protocol (Pierce). Confluent T75 cm² flasks of the indicated cells were lysed with 1 ml RIPA lysis buffer and protein extracts quantified as described above. 1.5 mg cellular extract per cell line was diluted to 1 ml with protease inhibitor-spiked lysis buffer and rotated with 90 µl normal mouse serum-conjugated beads for 1 hour at 4°C. After centrifugation at 376 ×g for 5 minutes at 4°C, the pre-cleared lysates were transferred to tubes containing 90 µl 3D7F11-conjugated beads and rotated at 4°C for 4 hours. Following low speed centrifugation as before, unbound extract was collected for analysis and the bound material (both the pre-clear and IP beads) was sequentially washed (10 minutes rotating per wash) twice with Wash Buffer (0.1% (v/v) Triton X-100, 20 mM HEPES-KOH, pH 7.4, 50 mM NaCl, 1 mM EDTA, 2.5 mM MgCl₂, 0.1% (w/v) SDS; 1 ml per wash), twice with High Salt Wash Buffer (0.1% (v/v) Triton X-100, 20 mM HEPES-KOH, pH 7.4, 500 mM NaCl, 1 mM EDTA, 2.5 mM MgCl₂, 0.05% (w/v) deoxycholate; 1 ml per wash) and once with Low Salt Wash Buffer (0.1% (v/v) Triton X-100, 20 mM HEPES-KOH, pH 7.4, 1 mM EDTA, 2.5 mM MgCl₂, 0.05% (w/v) deoxycholate; 1 ml per wash). To elute bound material, three sequential elutions were performed using 0.1 M glycine, pH 2.8, and the pooled eluates were neutralized with 50 µl 1 M Tris-Cl, pH 8.0. Eluates were transferred to a spin column (Pierce 1824826) and

centrifuged for 2 minutes at $376 \times g$ to reduce resin carry-over. Finally, the unbound extracts (pre-clear and IP beads) and eluates were TCA-precipitated (20% (v/v) TCA with 0.07% (v/v) deoxycholate) for 1 hour on ice, the pellet fractions resuspended in a 1:1 mix of 2X reducing sample buffer and 0.1 M NaOH, and boiled for 5 minutes.

Genotyping *taz*^{TALEN} cells — Genomic DNA was extracted using the Genra Puregene Cell Kit (Qiagen) and the genomic region surrounding the target site (exon1 extending into exon2, encompassing the ATG) was PCR amplified, digested with XbaI/HindIII (NEB), and ligated into pBSK(-). Transformants were analyzed by Sanger sequencing. As 293 cells are hypotriploid, sequences of at least 10 individual clones for each of the two *taz*^{TALEN} cell lines were obtained, revealing disruption of TAZ downstream of the initiation codon.

Quantitative RT-PCR — Total RNA was isolated from WT, *taz*^{TALEN}, and *taz*^{TALEN} overexpressing *TAZ* alleles using the PureLink RNA Mini Kit with DNase treatment (Invitrogen). 25 ng of RNA was analyzed in 20 μ l reactions using the EXPRESS One-Step SYBR GreenER qRT-PCR Kit (Life Technologies) with 7500 Real-Time PCR Systems (Applied Biosystems) according to the manufacturer's instructions. Each reaction, including non-template controls, was performed at least in duplicate with a minimum of three different biological replicates and contained 50 nM ROX and 200 nM each of forward and reverse gene-specific primers designed with Primer3 (51). The reaction conditions were as follows: 5 minutes at 50°C, 2 minutes at 95°C, followed by 40 two-temperature cycles (15 seconds at 95°C and 1 minute at 60°C) and melt curve profiling. Expression of *TAZ* was analyzed by the comparative C_T ($\Delta\Delta C_T$) method ($X_{Test}/X_{GAPDH}=2^{-\Delta\Delta C_T}$) with *GAPDH* as an endogenous reference gene. Values were

represented as mean $\Delta\Delta C_T \pm SEM$ or fold change ($2^{\Delta\Delta C_T}$) $\pm SD$ relative to *TAZ* expression in 293 Flp-In cells (Prism 6).

Import — Radiolabeled precursors were produced using an SP6 Quick Coupled Transcription/Translation system (Promega) spiked with Easy-Tag L-³⁵S-methionine (PerkinElmer). Radiolabeled precursors were incubated in import buffer (20 mM HEPES-KOH, pH 7.4, 250 mM sucrose, 80 mM KOAc, 5 mM MgOAc, 10 mM succinate, and 5 mM methionine) with freshly isolated mitochondria for the indicated amount of time at 37°C or 4°C, as specified. To dissipate the mitochondrial proton-motive force, mitochondria were pre-incubated with 1 μ M valinomycin and 5 μ M carbonyl cyanide *m*-chlorophenyl hydrazine for 5 minutes at 37°C. Import was stopped with an equal volume of ice-cold import buffer containing 20 μ g/ml trypsin. Where indicated, the OMM was ruptured as already described in the presence of 20 μ g/ml trypsin. Following 30 minutes on ice, 100 μ g/ml soybean trypsin inhibitor was added and mitochondria were re-isolated by centrifugation at 21,000 $\times g$ for 5 minutes at 4°C. 100% of each time point and 5% of imported precursors were resolved on 12% SDS-PAGE gels and analyzed by phosphoimaging.

Lipidomics — A defined amount of internal standards (0.1 nmol of CL(14:0)₄, 0.2 nmol of BMP(14:0)₂, 2.0 nmol of PC(14:0)₂, 0.1 nmol of PG(14:0)₂, 5.0 nmol of PS(14:0)₂, 0.5 nmol of PE(14:0)₂, 1.0 nmol of PA(14:0)₂, 2.0 nmol of SM(14:0)₂, 0.02 nmol of LPG(14:0), 0.1 nmol of LPE(14:0), 0.5 nmol of LPC(14:0), 0.1 nmol of LPA(14:0) (Avanti Polar Lipids) dissolved in 120 μ l of chloroform/methanol (1:1, v/v)) and 1.5 ml of chloroform/methanol (1:1, v/v) were added to 1 mg mitochondria. Subsequently, the mixture was sonicated in a water bath for 5 minutes, followed by centrifugation at 15,000

$\times g$ for 5 minutes. The organic layer was transferred to a glass vial and dried under a nitrogen stream at 60°C. Subsequently, the residue was dissolved in 200 μ l of chloroform/methanol/water (50:45:5, v/v/v) containing 0.01% of NH_4OH , and 10 μ l of the solution was injected into the liquid chromatography-mass spectrometry (LC/MS) system. LC/MS analysis was performed as described in (35). In negative mode, mass spectra of phospholipid molecular species were obtained by continuous scanning from m/z 380 to 1500 with a scan time of 2 seconds. In positive mode, mass spectra of PC, LPC and SM were obtained by continuous scanning for product ions with m/z 184 from m/z 400 to 1000. Mass spectra for PE, LPE and PE plasmalogens were obtained by scanning on neutral losses of 141 from m/z 400 to 1000. The raw LC/MS data were converted to mzXML format using msConvert (52) for the negative scan data and ReAdW for the positive specific scan data. Details on the metabolic data pre-processing pipeline will be described elsewhere (manuscript in preparation). Briefly, the dataset was processed using a semi-automated xcms-based metabolomics workflow (53) written in the R language and statistical environment (<http://www.r-project.org/>). The workflow comprises pre-processing (peak-finding and quantification), identification of metabolites, isotope correction, normalization/scaling based on internal added standards and statistical analysis of the results.

Membrane association assays — Sonication experiments utilized 0.2 mg mitochondria and were performed in the absence of KCl as described (40). The supernatant and membrane fractions were separated by ultracentrifugation at 175,000 $\times g$ using a TLA120.1 rotor for 30 minutes at 4°C. Carbonate extraction was performed as described (30) except that 0.5 ml of 0.1 M Na_2CO_3 at the indicated pH was added to 0.1 mg

mitochondria, and following 30 minutes on ice, the pellet and supernatant fractions were separated by centrifugation at $175,000 \times g$ for 15 minutes at 4°C using a TLA120.1 rotor.

Blue native (BN) gel electrophoresis — Mitochondria were solubilized for 30 minutes on ice in 20 mM HEPES-KOH, 10% glycerol, 50 mM NaCl, 1 mM EDTA, 2.5 mM MgCl_2 , pH 7.4 supplemented with 1% (w/v) (mouse tissues) or 1.25 % (w/v) (human cells) digitonin (Biosynth International, Inc) and protease inhibitors. Extracts were clarified by centrifugation for 30 minutes at $21,000 \times g$ at 4°C and analyzed by 2D BN/SDS-PAGE exactly as described (45).

Digitonin fractionation assay — Digitonin-based submitochondrial localization was adapted from (40). In brief, 0.1 mg mitochondria were resuspended in 50 μl SEHK buffer (250 mM sucrose, 5 mM EDTA, pH 7.0, 10 mM HEPES-KOH, pH 7.4, 200 mM KCl) supplemented with 0-0.5% digitonin and 1 mM PMSF. Following a brief vortex on level 1, samples were incubated for 2 minutes on ice and solubilization stopped by adding 450 μl cold SEHK buffer. Solubilized proteins were separated from the membrane pellets by centrifugation at $100,000 \times g$ for 10 minutes at 4°C . After the supernatant was TCA-precipitated, the membrane and supernatant-derived pellets were resuspended in equal volumes of reducing sample buffer.

Protease-based assays — Freshly isolated mammalian mitochondria (0.2 mg) from cells or tissues were resuspended in iso-osmotic IB_c or KME buffers, respectively. To rupture the OMM, mitochondria (0.2 mg) were resuspended in 5 mM Tris-Cl, pH 7.5 for 30 minutes on ice; 0.5% (v/v) deoxycholate was added to further solubilize the IMM. Each of these conditions was performed in the absence and presence of 125 $\mu\text{g}/\text{ml}$ pronase E. Pronase was inhibited with 5 mM PMSF and samples precipitated with 20% (v/v) TCA

and 0.07% (v/v) deoxycholate, incubated at 60°C for 5 minutes followed by an hour on ice. Samples were centrifuged at 21,000 ×g for 10 minutes at 4°C, washed with 1 ml of cold acetone, resuspended in a 1:1 mix of 2X reducing sample buffer:0.1M NaOH and boiled for 5 minutes. To determine sensitivity to assorted proteases following heat denaturation, mitochondria (0.2 mg) were resuspended in 5 mM Tris-Cl, pH 7.5 containing 0.1% (v/v) SDS, kept on ice or incubated at 95°C for 5 minutes, and then 5 mM Tris-Cl, pH 7.5 with 0.1% SDS containing 250 µg/ml pronase E, 200 µg/ml proteinase K, or 40 µg/ml trypsin added. Following a 30 minute incubation on ice, pronase E and proteinase K were inhibited with 5 mM PMSF and trypsin with 100 µg/ml soybean trypsin inhibitor. Every sample was precipitated with 20% (v/v) TCA and 0.07% (v/v) deoxycholate, incubated at 60°C for 5 minutes followed by an hour on ice. Samples were centrifuged at 21,000 ×g for 10 minutes at 4°C, washed with 1 ml of cold acetone, resuspended in a 1:1 mix of 2X reducing sample buffer:0.1M NaOH and boiled for 5 minutes.

Protein aggregation assay — Protein aggregation was determined in mitochondria (0.2 mg) isolated from 293 Flp-In cells exactly as previously described (31).

Phospholipid labeling and analyses — Cells were seeded onto 60 mm dishes or 6 well plates and allowed to grow to 50-70% confluency at 37°C, 5% CO₂. Following aspiration, regular media spiked with 2.5 µCi/ml ³²P_i was added and the cells incubated at 37°C, 5% CO₂ for 24 hrs. Cells were washed twice with ice cold 1X PBS, detached in 0.8 ml IB_c buffer using a cell scraper, transferred to a microfuge tube, collected at 600 ×g 5 min at 4°C, and resuspended in 0.1 ml IB_c buffer. Phospholipids were extracted from equal amounts of labeled cells, determined by liquid scintillation, as described (30) except that

0.9% (w/v) NaCl was used to initiate phase separation instead of ddH₂O. Samples were resuspended in chloroform, loaded onto ADAMANT TLC plates (Machery-Nagel), resolved once in chloroform:ethanol:H₂O:trimethylamine (30:35:7:35, v/v/v/v), and phospholipids visualized using a K-screen and FX-Imager (Bio-Rad Laboratories).

Electron microscopy — Cells were seeded onto 100 mm dishes and allowed to grow to ~70% confluence before fixing with 3.0% formaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate, 5 mM CaCl₂, 2.5% (w/v) sucrose, pH 7.4, for 1 hour at RT. Cells were washed three times for 15 minutes each with 0.1 M sodium cacodylate, 2.5% (w/v) sucrose, pH 7.4, and another three times for 5 minutes each with 50 mM Tris-HCl, 7.5% (w/v) sucrose, pH 7.4, prior to labeling with 0.5 mg/mL DAB and 0.015% H₂O₂ in 50 mM Tris-HCl, 7.5% (w/v) sucrose, pH 7.4 for 20 to 60 minutes. The labeling reaction was stopped by rinsing cells twice with 50 mM Tris-HCl, 7.5% (w/v) sucrose, pH 7.4, and once with 0.1 M sodium cacodylate, pH 7.4, 5 mM CaCl₂, 2.5% (w/v) sucrose, before harvesting the cells with 1 ml of 0.1 mM cacodylate buffer, pH 7.4; and pelleting the cells in 1.5 ml Eppendorf tube. Cells were pelleted progressively by spinning from 3,000 rpm to 12,000 rpm over the course of 10 minutes and the pellet was post-fixed in reduced osmium tetroxide (1% OsO₄, 1% potassium ferrocyanide in 0.1 M sodium cacodylate, 5 mM CaCl₂, pH 7.4) for 45 minutes at RT. The pellets were washed thoroughly with 0.1 M sodium cacodylate, pH 7.4, 5 mM CaCl₂, 2.5% (w/v) sucrose several times until solution becomes clear, dehydrated through a graded series of ice-cold ethanol, and subsequently embedded in Epon resin. Sections were cut on a Leica UCT ultramicrotome and observed on an FEI Tecnai 12 transmission electron microscope at 100 kV. Images were captured with a Soft Imaging System Megaview III digital camera

and figures were assembled in Adobe Photoshop and Illustrator with only linear adjustments in contrast and brightness.

Cycloheximide chase — Cell culture medium lacking antibiotics but containing 100 µg/ml cycloheximide was added to confluent 6-well plates and incubated at 37°C, 5% CO₂. At the designated times, cells were solubilized with 100 µl 1% (w/v) SDS and diluted to 0.5% SDS, 1% β-mercaptoethanol with 2% (v/v) β-mercaptoethanol. After denaturing the lysates at 100°C for 10 minutes, protein was quantified using the Bradford method (Bio-Rad).

Transacylation assay — The transacylase assays were performed as previously described (16, 32) using 1 nmol of 1-[¹⁴C]palmitoyl-2-lyso-PC (0.05 mCi; PerkinElmerLife Sciences) per reaction in 0.2 ml reaction buffer (10 mM β-mercaptoethanol, 0.5 mM EDTA, 50 mM Tris, pH 7.4). To initiate the reactions, ~125 µg mitochondria (the exact amount was determined by the BCA assay) were added, the reactions vortexed on high for 1 second, and incubated with shaking at 220 rpm for 5 minutes at 37°C. Lipid extraction, thin layer chromatography separation, image acquisition and analyses were executed as described (32).

Data analysis — Band densitometry analyses were performed using Quantity One (Bio-Rad) or Image J (54). With the exception of the data in Fig 2D and 7D, statistical analyses were performed using SigmaPlot 11 software (Systat Software) and all comparisons performed by t test or one-way analysis of variance with Holm-Sidak pairwise comparisons. For Fig 2D and 7D, comparisons were performed using one-way analysis of variance with Geisser-Greenhouse correction Tukey's multiple comparison test available in the Prism 6 software (GraphPad). All graphs report the mean ± SEM.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest to declare.

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LEGENDS TO FIGURES

Figure 1. One isoform of TAZ is expressed and localized to mitochondria in mammalian cells and tissues. (A) Whole cell extracts (50 μ g) from healthy control (C109) and BTHS patient fibroblasts (TAZ001 and TAZ003) were immunoblotted with three different mAbs generated against recombinant human TAZ. (B) Subcellular fractions (50 μ g) derived from C109 cells immunoblotted as indicated. (C) Mitochondrial extracts (50 μ g) from the indicated source immunoblotted with the TAZ mAbs. (D) The epitope recognized by each TAZ mAb was determined by peptide array. The exon that harbors each epitope is indicated. (E) TAZ was immunoprecipitated with 3D7F11 from 1.5 mg of the indicated cell extracts. 50 μ g starting material and non-binding flow through and 100% of bound material (Preclear and IP) were immunoblotted using 2C2C9. (F) Whole cell extracts from WT or *taz1* Δ yeast transformed as indicated and mitochondrial extracts from the designated source immunoblotted for TAZ. EV, empty vector. *, nonspecific band.

Figure 2. Generation of a BTHS cell culture model using TALEN-mediated genome editing. (A) TALEN-mediated disruption of TAZ in 293 Flp-In cells introduces nucleotide deletions that result in an in-frame deletion in *taz*^{TALEN}.2 and a missense and in-frame deletion in *taz*^{TALEN}.19. (B) Whole cell extracts from the indicated cells grown in dextrose or galactose-based media were resolved by SDS-PAGE and immunoblotted. (C) WT 293 Flp-In and *taz*^{TALEN} cells were incubated for 4 hours with 20 μ M of the proteasome inhibitor, MG132. 40 μ g of whole cell lysates were resolved by SDS-PAGE and immunoblotted for the indicated proteins. Cyclin D1, a target of the ubiquitin-

proteasome pathway (55), is used as a positive control. (D) Relative mRNA level of *TAZ* in WT (293 Flp-In) and each *taz*^{TALEN} cell. Data was analyzed by the comparative C_T ($\Delta\Delta C_T$) method, represented as mean $\Delta\Delta C_T \pm \text{SEM}$ $n = 8$ relative to WT expression level, which was set to 0. (E) Mitochondria (50 μg) from the designated source were immunoblotted as listed. (F) Protein expression in (E) was quantified by densitometry and normalized to WT levels (mean \pm SEM; $n = 3$). Significant differences are indicated. (G) *In organello* import of radiolabeled [³⁵S]-Su9-DHFR into WT 293 Flp-In and *taz*^{TALEN} mitochondria in the presence or absence of a membrane potential. Following import, mitochondria were osmotically ruptured, treated with trypsin, resolved by SDS-PAGE, and bands detected by phosphoimaging. P, precursor (5% of precursor protein + 100 μg mitochondria); p, precursor; m, mature. (H) The percentage of precursor imported at each time point was calculated (mean \pm SEM; $n = 3$). See also Fig. S1 and Table SI.

Figure 3. *taz*^{TALEN} cells have altered mitochondrial CL and MLCL profiles as determined by high-performance liquid chromatography-mass spectrometry. The relative amounts of CL (A), MLCL (B), and PG (D) was determined by high-performance liquid chromatography-mass spectrometry. (C) The ratio of MLCL:CL as determined from (A and B). The abundance of CL (E) and MLCL (L) of a given acyl chain length (E,L) or containing the indicated number of double bonds (F, M), was determined as a percentage of the total amount of CL or MLCL. The distribution of double bonds per acyl chain length for CL (G-K) and MLCL (N-O) expressed as a percent of the sum of all species of the indicated length. All data are the mean \pm SEM ($n = 3$). Significant differences are indicated. See also Figures S2-S4.

Figure 4. Membrane association and assembly of TAZ. (A) Mitochondria were sonicated and equal volumes of the pellet (P) and TCA-precipitated supernatant (S) fractions were immunoblotted as indicated. SM, starting material. (B) Mitochondria were incubated in 0.1 M Na₂CO₃ of the listed pH. Integral proteins (P) were separated from released proteins (S) by ultracentrifugation and equal volumes of each were immunoblotted as indicated. (C) Quantified band intensities of the P and S fractions were plotted as the percentage of total protein released into the supernatant for each pH (mean \pm SEM; $n = 3$ (C109) or 4 (293)). (D) Mitochondria from control and BTHS fibroblasts (450 μ g), 293 Flp-In cells (200 μ g), and mouse heart and liver (200 μ g) were solubilized with digitonin, separated by 2D BN/SDS-PAGE, and immunoblotted for TAZ.

Figure 5. Mammalian TAZ is protease resistant and associates with IMS-facing leaflets. (A) Mitochondria were titrated with increasing amounts of digitonin to differentially solubilize mitochondrial compartments. After centrifugation, equal volumes of the pellet (P) and supernatant (S) fractions were resolved by SDS-PAGE and immunoblotted. (B) Quantified band intensities for two markers per compartment were combined and plotted as the percent detected in supernatant (mean \pm SEM; $n = 7$). (C) Mitochondria, osmotically ruptured mitoplasts, or detergent-solubilized mitochondria from the indicated source were incubated without or with protease, resolved by SDS-PAGE, and immunoblotted. (D) Mitochondria were solubilized with 0.1% (v/v) SDS and where indicated, heated at 95°C for 5 minutes prior to adding pronase E, proteinase K, or trypsin. Equal volumes of each sample were resolved by SDS-PAGE and immunoblotted. (E) Mitochondria were solubilized with digitonin and fractionated into a supernatant (S1) and pellet (P1) by centrifugation. Material not extracted by digitonin (P1) was further

solubilized with TX-100 and separated into a supernatant (S2) and pellet (P2) by centrifugation. Fractions were resolved by SDS-PAGE and immunoblotted as indicated. (F) *In organello* import of radiolabeled [³⁵S]-TAZ into 293 Flp-In mitochondria at the indicated temperature in the presence or absence of a membrane potential. Following import, mitochondria were treated with trypsin, resolved by SDS-PAGE, and bands detected by phosphoimaging. Where indicated, mitochondria were osmotically ruptured in the presence or absence of trypsin. P, precursor (5% of precursor protein + 100 μg mitochondria).

Figure 6. Both termini of TAZ are in the IMS. (A) Cartoon of epitope tagged TAZ constructs. (B) Whole cell extracts (30 μg) were immunoblotted for the indicated proteins. (C) Phospholipids from the indicated cells were labeled with ³²P_i and separated by TLC. (D) Quantification of the MLCL:CL ratio (mean ± SEM; *n*=3). Significant differences compared to WT 293 Flp-In cells were determined by one-way ANOVA. n.s. = differences not significant. (E and F) The protease accessibility of epitope tags added to the NH₂ (E) or COOH (F) termini of TAZ was determined as in Fig. 5C. (G) *taz*^{TALEN}.19 cells overexpressing WT or TAZ-APEX2 were labeled *in vivo* with H₂O₂ and DAB followed by OsO₄ and EM imaging. Scale bars are provided in each panel.

Figure 7. The R57L allele is highly unstable. (A) Sequence alignment of TAZ from the indicated species encompassing human Arg57 and His69 (*white* face on black background) and the HXXXXD motif (underlined). (B) Whole cell extracts (20 μg) from 5-6 clones transfected with the indicated TAZ alleles were immunoblotted. (C) Relative mRNA level of TAZ analyzed by the comparative C_T (ΔΔC_T) method, represented as mean fold change (2^{ΔΔC_T}) ± SD *n* ≥ 4 relative to 293 Flp-In TAZ expression. (D) *In*

organello import of radiolabeled precursor R57L TAZ into freshly isolated 293 Flp-In mitochondria as described in Fig. 5F. (E) *taz*^{TALEN} cells overexpressing WT, R57L and H69Q alleles were incubated for 4 hours with 20 μ M of the proteasome inhibitor, MG132. 40 μ g of whole cell lysates were resolved by SDS-PAGE and immunoblotted for the indicated proteins. (F) Whole cell extracts (20 μ g) were collected after incubation with cycloheximide for the indicated times, and the amount of TAZ remaining assessed by immunoblot. The half-lives of the indicated TAZ alleles were calculated using the mean of five individual repetitions. See also Fig. S5.

Figure 8. The H69Q mutant is catalytically dead. (A) Mitochondria (20 μ g) from 293 Flp-In, *taz*^{TALEN}, and *taz*^{TALEN} cells overexpressing WT, R57L and H69Q alleles were immunoblotted as indicated. (B) *In organello* import of radiolabeled precursor H69Q TAZ into freshly isolated 293 Flp-In mitochondria as described in Fig. 5F. (C) Assembly of H69Q as determined by 2D BN/SDS-PAGE. (D) Lyso-PC to PC transacylase activities were determined in mitochondria isolated from 293 Flp-In, *taz*^{TALEN}, and *taz*^{TALEN} cells overexpressing the indicated TAZ alleles (mean \pm SEM, $n = 3$). Significant differences are indicated. n.s., not significant. The relative amounts of CL (E) and MLCL (F), and the subsequent MLCL:CL ratio (G), in *taz*^{TALEN} cells overexpressing WT, R57L, and H69Q were determined as previously described. See also Fig. S5.

ABBREVIATIONS

BTHS, Barth syndrome; CL, cardiolipin; IMM, inner mitochondrial membrane; IMS, intermembrane space; MLCL, monolysocardiolipin; LOF, loss-of-function; mAb, monoclonal antibody; OMM, outer mitochondrial membrane; OXPHOS, oxidative phosphorylation; PC, phosphatidylcholine; PG, phosphatidylglycerol.

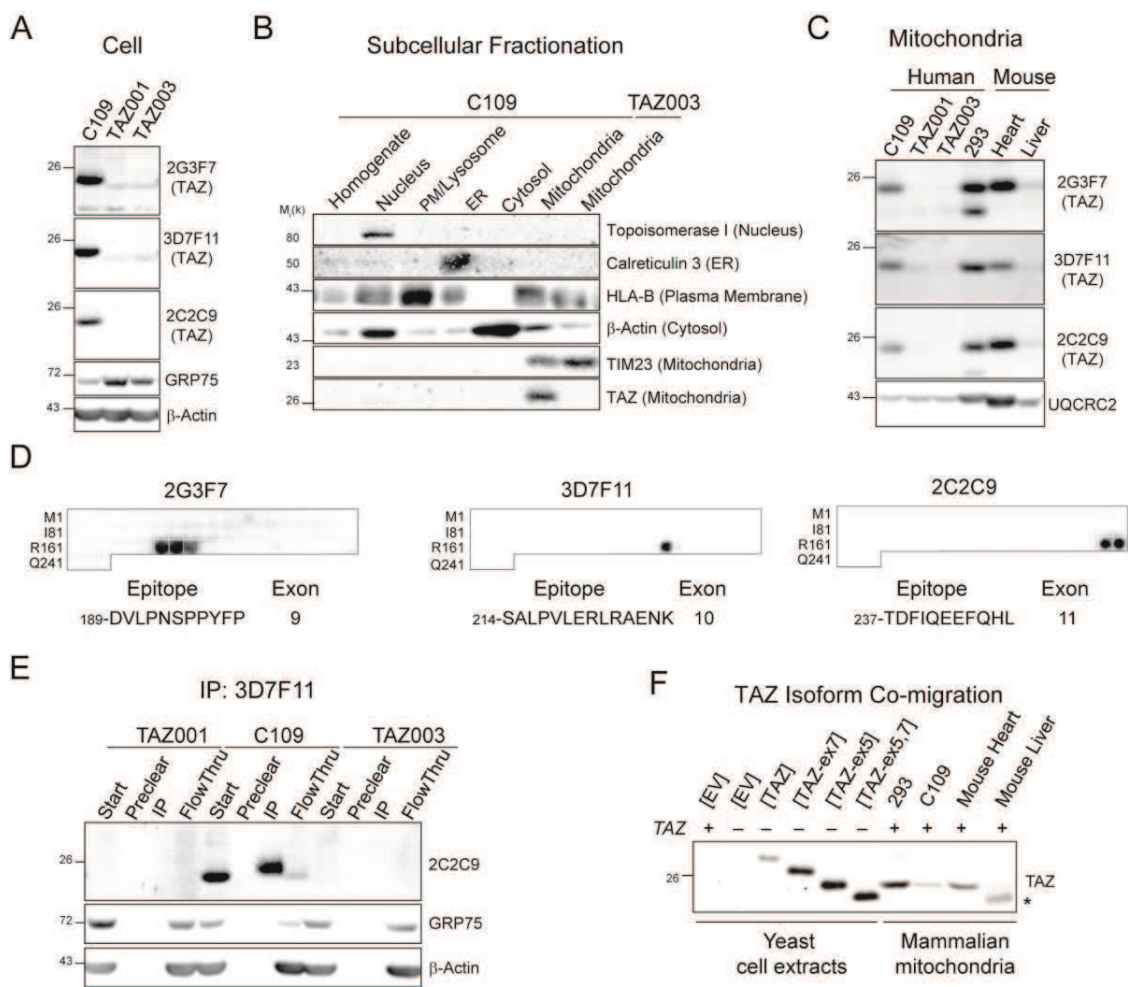


Figure 1

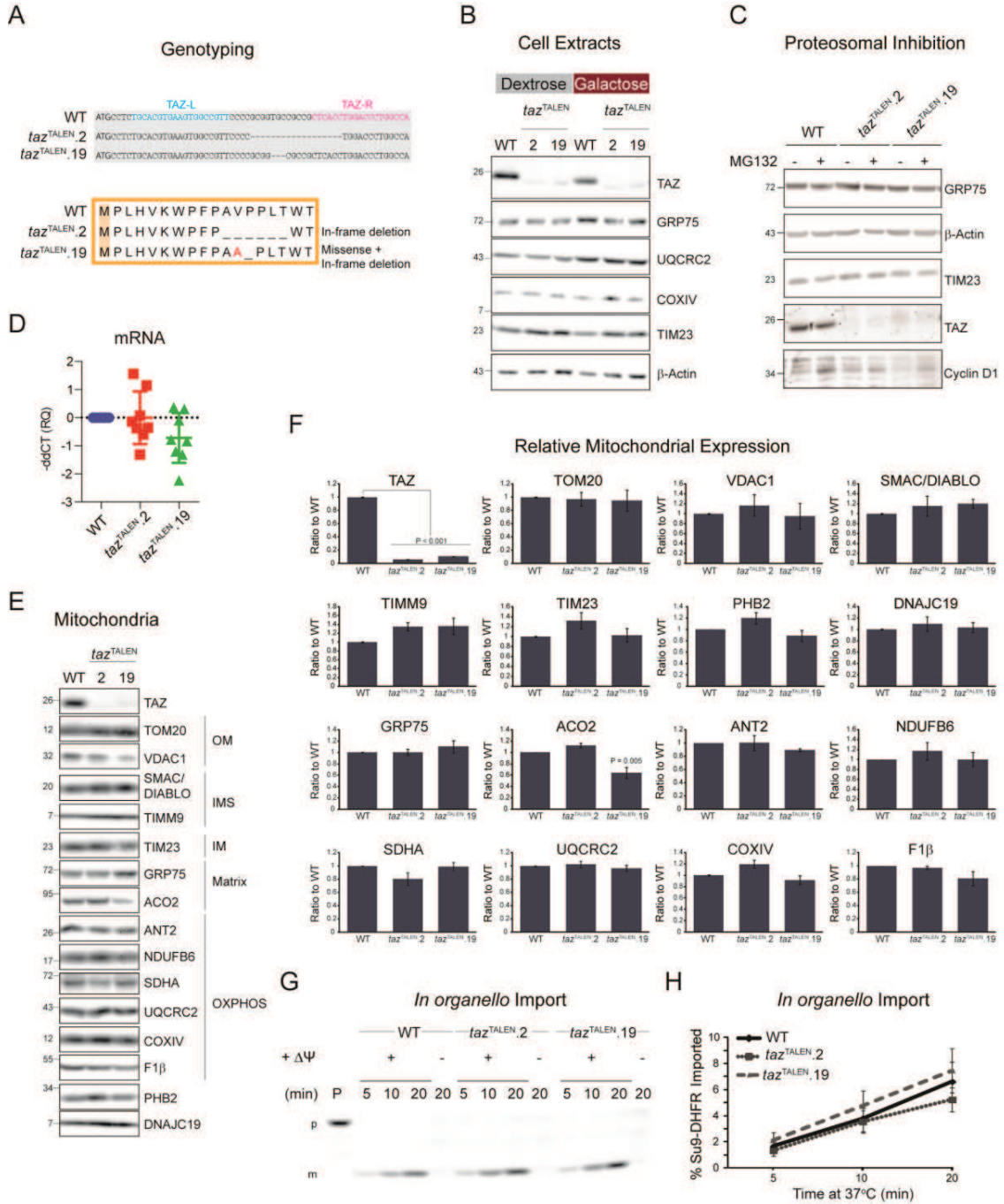


Figure 2

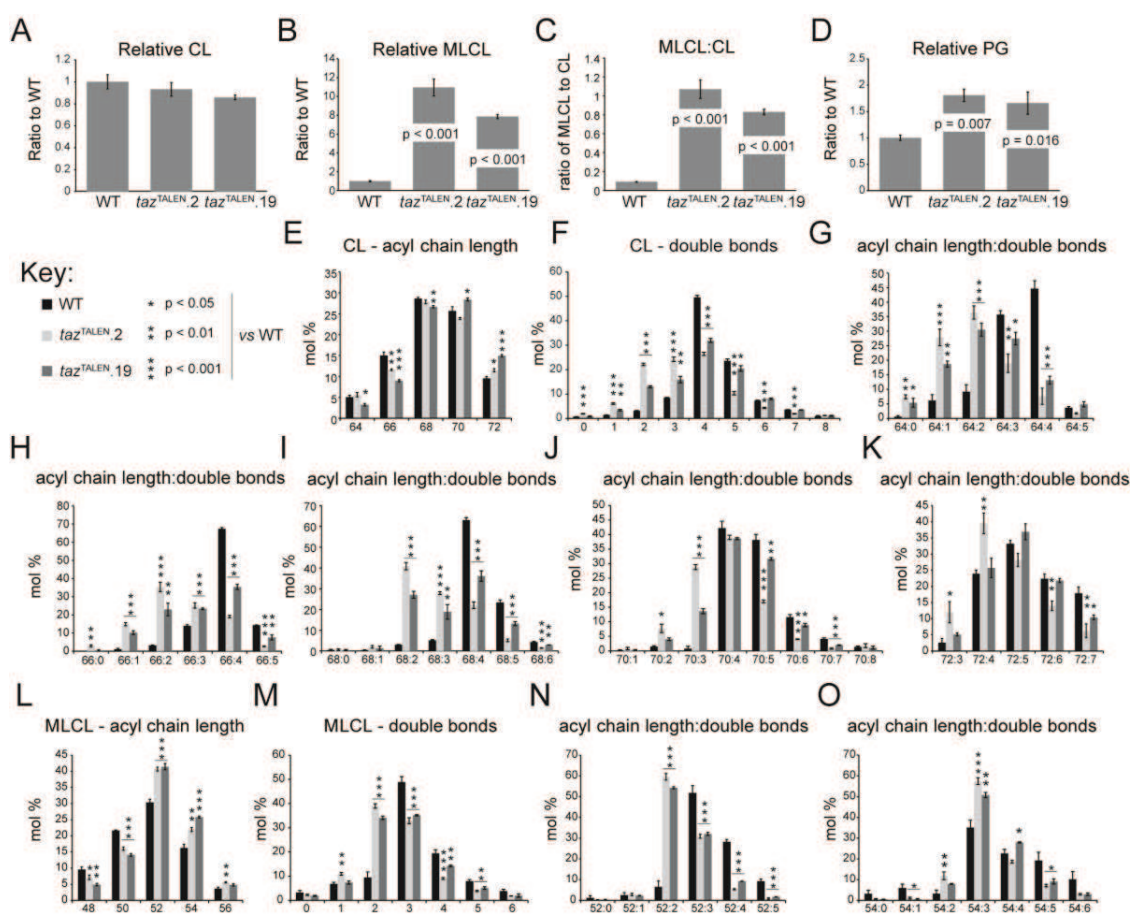


Figure 3

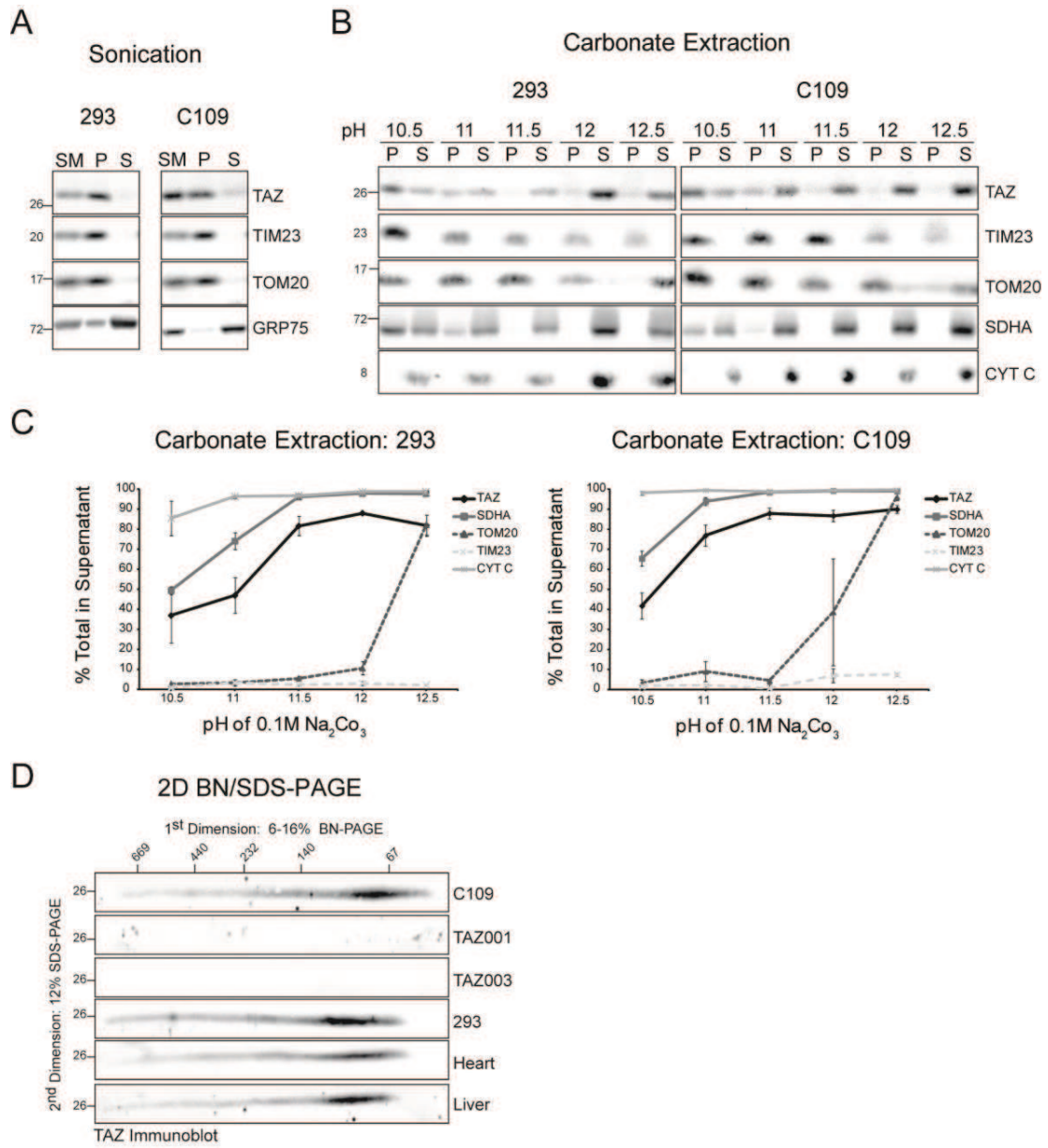


Figure 4

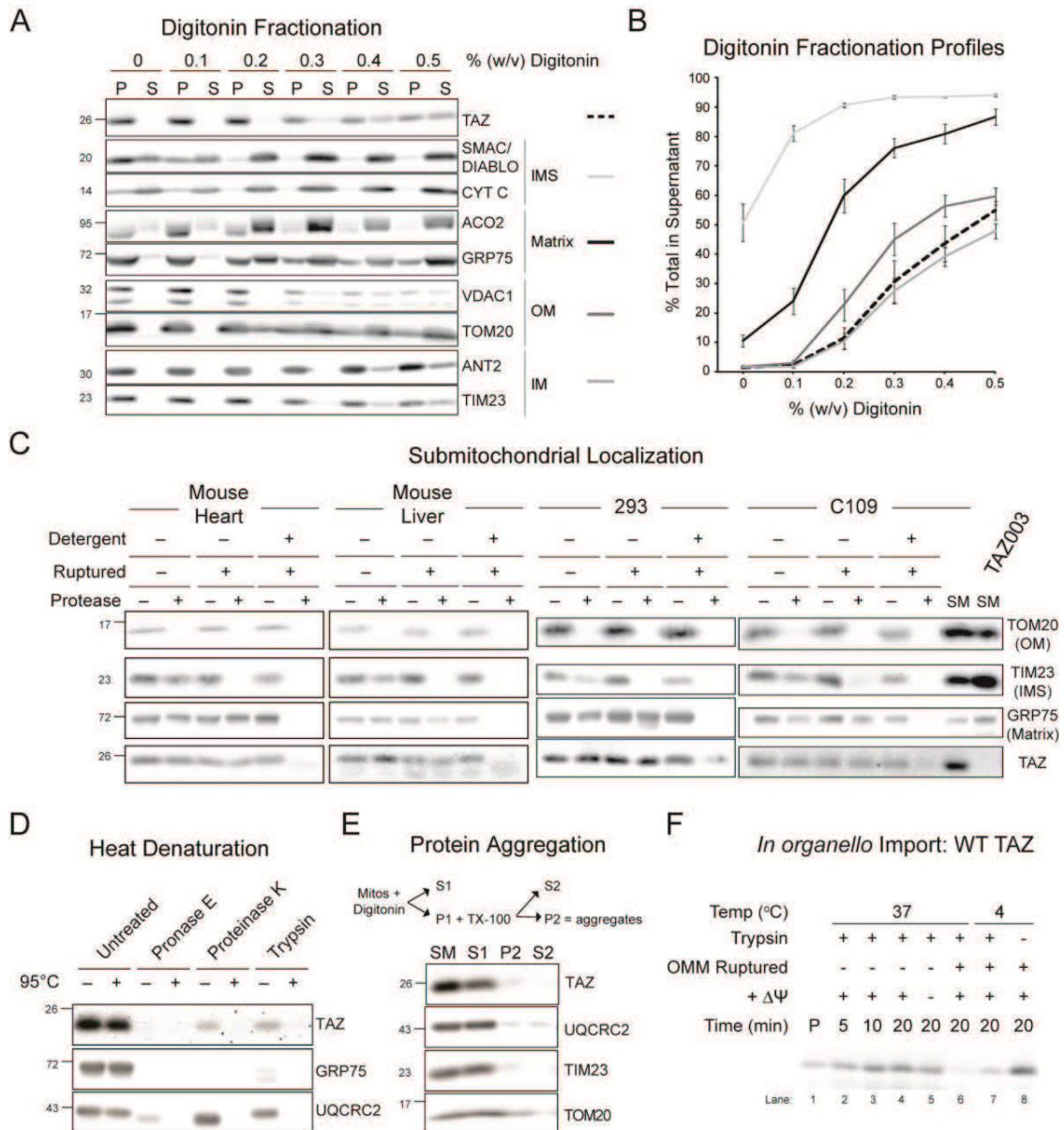


Figure 5

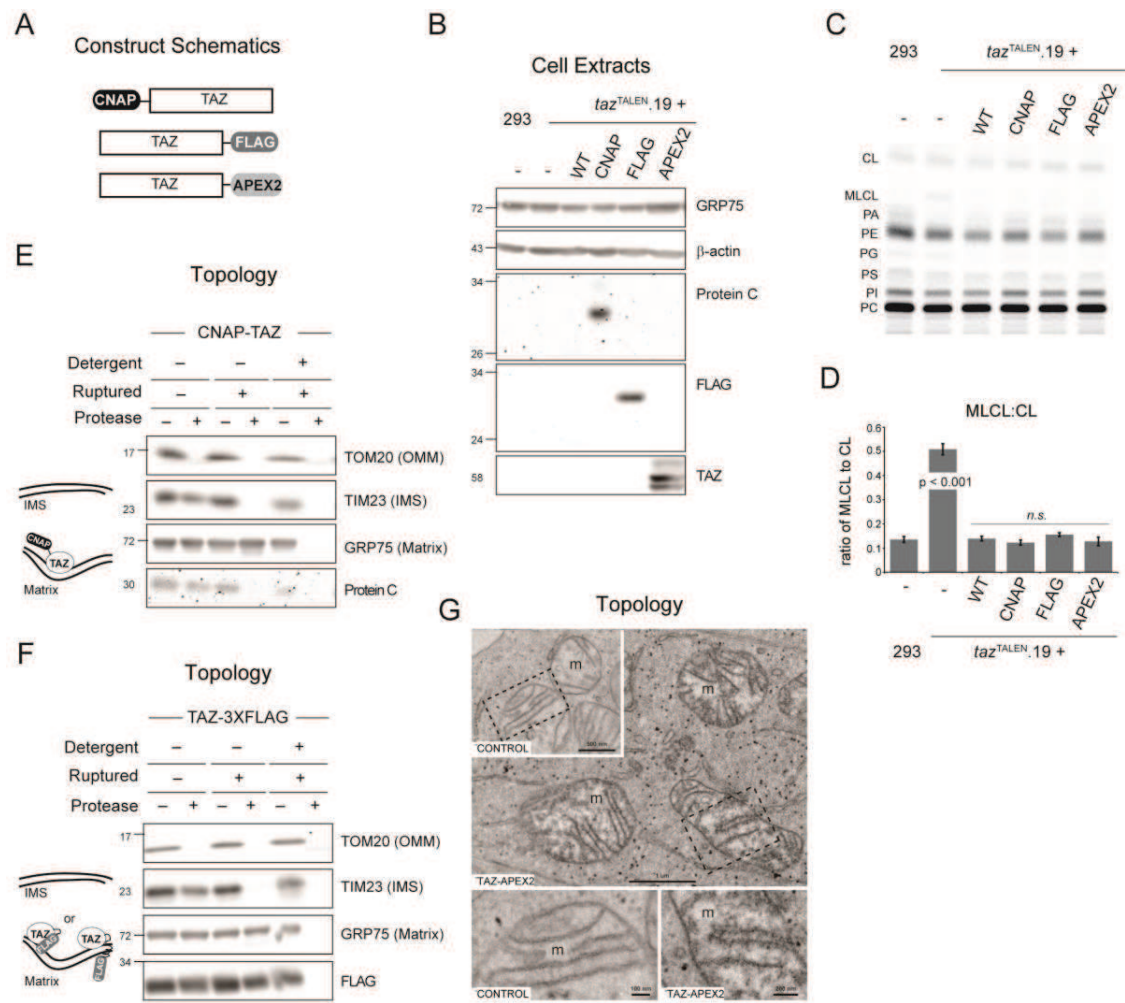


Figure 6

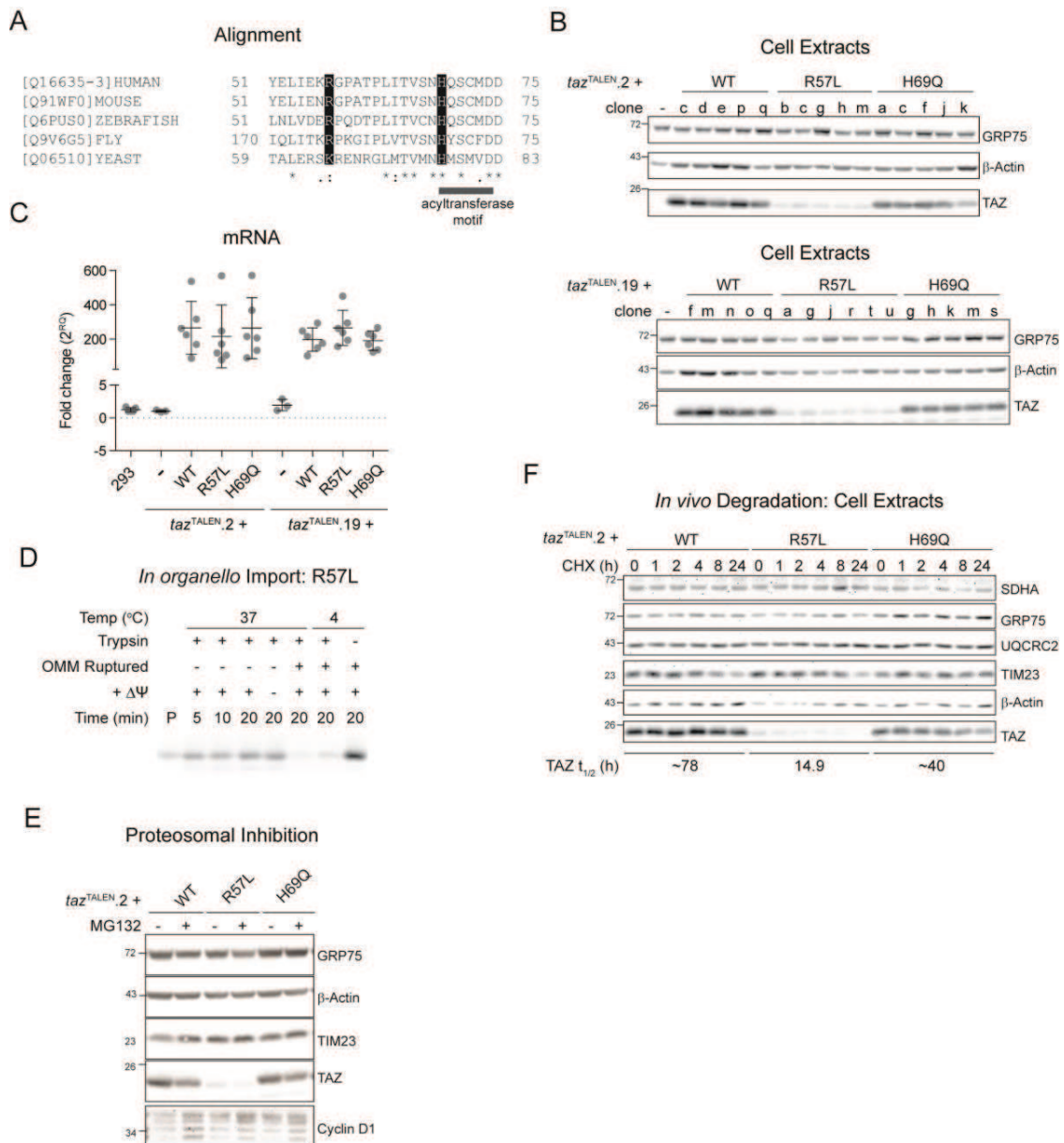


Figure 7

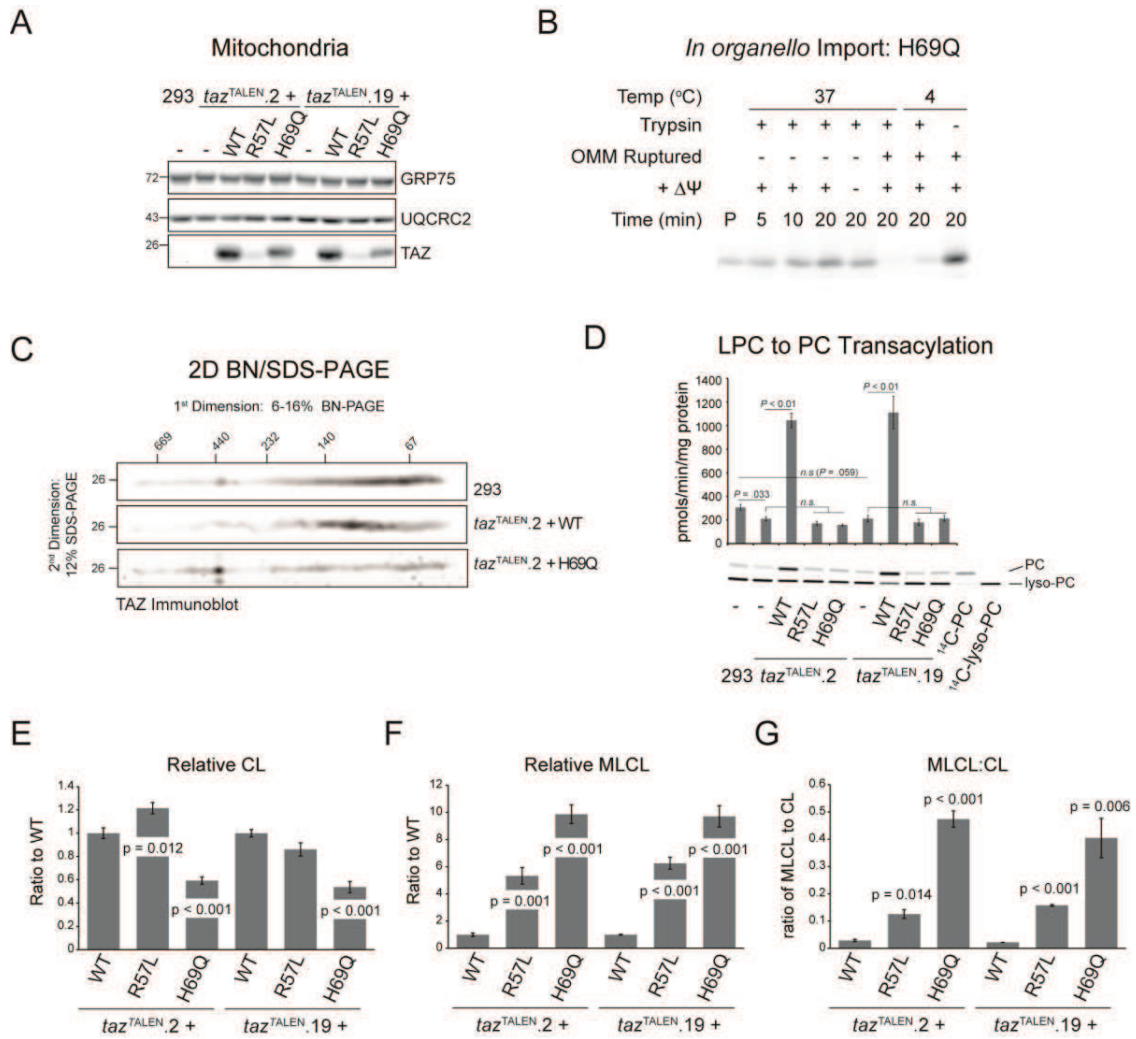


Figure 8