Cardiolipin metabolism in Barth syndrome
Houtkooper, R.H.L.

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Chapter 6

The enigmatic role of tafazzin in cardiolipin metabolism

Houtkooper RH¹, Turkenburg M¹, Poll-The BT², Karall D³, Pérez-Cerdá C⁴, Wanders RJ¹, Kulik W¹ and Vaz FM¹

¹Laboratory Genetic Metabolic Diseases, Academic Medical Center, Amsterdam, The Netherlands
²Department of Pediatric Neurology, Academic Medical Center, Amsterdam, The Netherlands
³Neonatology, Neuropediatrics, and Inherited Metabolic Disorders, Innsbruck Medical University, Innsbruck, Austria
⁴Centro de diagnóstico de enfermedades moleculares, Facultad de Ciencias, CIBERER, Universidad Autónoma de Madrid, Spain

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Abstract
The mitochondrial phospholipid cardiolipin plays an important role in cellular metabolism as exemplified by its involvement in mitochondrial energy production and apoptosis. Following its biosynthesis, cardiolipin is actively remodeled to achieve its final acyl composition. An important cardiolipin remodeling enzyme is tafazzin, of which several mRNA splice variants exist. Mutations in the tafazzin gene cause the X-linked recessive disorder Barth syndrome. In addition to providing an overview of the current knowledge in literature about tafazzin, we present novel experimental data and use this to discuss the functional role of the different tafazzin variants in cardiolipin metabolism in relation to Barth syndrome. We developed and performed specific quantitative PCR analyses of different tafazzin mRNA splice variants in 16 human tissues and correlated this with the tissue cardiolipin profile. In BTHS fibroblasts we showed that mutations in the tafazzin gene affected both the level and distribution of tafazzin mRNA variants. Transient expression of selected human tafazzin variants in BTHS fibroblasts showed for the first time in a human cell system that tafazzin lacking exon5 indeed functions in cardiolipin remodeling.

Abbreviations
BTHS, Barth syndrome
CDP-DAG, cytidinediphosphate-diacylglycerol
CL, cardiolipin
MLCL, monolysocardiolipin
PC, phosphatidylcholine
PG, phosphatidylycerol
TAZ, tafazzin
Introduction
Cardiolipin (CL) is a phospholipid that, at least in eukaryotes, is primarily localized to the mitochondrial membranes. In contrast to other phospholipids CL has four acyl chains which are linked to a backbone of three glycerol moieties. Because of this unique composition CL can adopt a specific conical structure, which is important for membrane curvature. In the mitochondrial membrane CL has several specialized functions, especially in energy production and mitochondrial apoptosis. CL is synthesized from its precursors phosphatidylglycerol (PG) and cytidinediphosphatediacylglycerol (CDP-DAG) and after primary synthesis CL acyl chains are remodeled to achieve its final mature composition. To exert its function properly, it is essential that CL is present in this mature form, as illustrated by the mitochondrial disorder Barth syndrome, which is caused by mutations in the tafazzin gene encoding a CL remodeling enzyme. In this review we will discuss the composition of CL in relation to CL function, analytical methods for measurement of CL, and focus on Barth syndrome (BTHS) with emphasis on the function of the tafazzin. In addition to providing an overview of current knowledge in literature, we will provide experimental evidence to illustrate our views on the role of tafazzin in CL metabolism.

Cardiolipin
CL is an important constituent of the mitochondrial membrane of eukaryotes. It is primarily found in the inner mitochondrial membrane and to a lesser extent also in the outer mitochondrial membrane [1]. During certain processes, notably mitochondrial apoptosis, CL is translocated to the outer membrane and/or clusters at contact sites between the outer and inner membrane [2]. At the contact sites, CL is thought to serve as a platform for proteins involved in apoptosis, thereby bringing several components of this machinery together [3,4]. Besides this role in cell death, CL is also an important molecule required for the optimal functioning of the respiratory chain. Several of the proteins that constitute the respiratory chain have been reported to bind to CL or require CL for optimal activity (as reviewed in [5]). Furthermore, CL is reported to function in the stabilization of the individual respiratory chain complexes in a larger so-called supercomplex enabling efficient channeling of electrons through the complexes [6-8]. In a recent paper, Claypool et al have provided evidence to show that the mitochondrial ATP/ADP carrier, which is known to require CL for optimal activity [9], is also incorporated into a CL-dependent supercomplex containing complex III and IV in Saccharomyces cerevisiae [10]. The same group also showed that yeast tafazzin interacts with multiple proteins amongst which ATP synthase and the aforementioned ATP/ADP carrier [11]. Finally, the specific CL headgroup is thought to enable trapping of protons in the intermembrane space, thereby increasing the efficiency of the channeling of the protons back into the mitochondrial matrix by the ATP synthase complex [12]. For a more detailed review of CL function we recommend the following literature [5,13,14]. Recently, abnormal CL levels or composition have been linked to common diseases such as diabetes and heart failure. We will not focus on these aspects of CL and refer the reader to [5,15].
**CL synthesis and remodeling**

CL is synthesized in the mitochondrion from PG and CDP-DAG and this process has been described in detail elsewhere [5,14,16]. Briefly, CDP-DAG is converted to phosphatidylglycerolphosphate (PGP) by the enzyme PGP synthase, and PGP is dephosphorylated by a phosphatase yielding PG. In the final step of CL biosynthesis, condensation of one molecule of PG and one molecule of CDP-DAG by the enzyme CL synthase results in the formation of CL. Importantly, the mature acyl chain composition of CL is not explained by the substrate specificity of CL synthase [17-19], which implies that additional mechanisms are involved in achieving the final composition by acyl chain remodeling. Remodeling of phospholipids acyl chains can occur either by a deacylation-reacylation cycle, i.e. the Lands cycle [20], or by transacylation [21]. The Lands cycle for CL comprises a phospholipase-mediated deacylation forming monolyso-CL (MLCL) followed by a CoA-dependent reacylation. Several enzymes have been described that are capable of performing this reaction, and all these enzymes showed preference for acyl-CoA species that are typically found in mature CL [22-24]. CoA-independent transacylation of CL occurs by the transfer of an acyl chain from phosphatidylcholine (PC) to MLCL forming lyso-PC and CL [21]. This enzymatic activity was characterized in rat liver and human lymphoblasts and was shown to be specific for linoleoyl-containing PC [21]. The same group also demonstrated that *D. melanogaster* tafazzin, which in humans is the enzyme deficient in BTHS patients, also functions as a transacylase in CL metabolism by transferring a linoleic acid group from PC to MLCL, thereby enriching CL with this specific acyl chain [25]. A more detailed overview on the current state of knowledge on tafazzin will be provided below.

![Figure 1. Typical HPLC-MS spectra of (ML)CL in control and BTHS muscle.](image-url)

Control muscle

- CL
- MLCL
- CL i.s.

BTHS muscle

- CL i.s.

**Figure 1. Typical HPLC-MS spectra of (ML)CL in control and BTHS muscle.** CL in control muscle is highly enriched in linoleic acid acyl chains, as demonstrated by the high abundance of tetralinoleoyl-CL (m/z 723). MLCL levels in control muscle are very low. In BTHS muscle, CL levels are decreased and a more diverse pattern of CL species is observed (smaller clusters of CL around m/z 687, 700, 714). The MLCL levels are highly increased in BTHS muscle. CL i.s. = CL internal standard (tetramyristoyl-CL)
CL in Barth syndrome
BTHS (MIM 302060) is an X-linked recessive disorder, clinically characterized by the classical symptoms (cardio-)myopathy, neutropenia and growth delay [26,27]. BTHS is caused by mutations in the tafazzin gene [28], located at Xq28. As a result of the tafazzin mutations, the remodeling of CL is hampered and BTHS cells therefore show an abnormal CL profile [29-33]. CL levels in tafazzin-deficient (i.e. BTHS) cells are lower, MLCL levels are markedly elevated and the acyl chain composition is shifted towards less unsaturated species (figure 1). As a result of these abnormalities, mitochondria of tafazzin-deficient cells show abnormal cristae formation [26,34] and pathways which rely on properly formed CL are impaired [3,7,8]. How the reported functions of CL influence the pathophysiology of BTHS is matter of research in multiple laboratories, but remains unclear. It seems plausible that the impaired respiratory chain function could result in (cardio-)myopathy, since the energy demand of these tissues is high, which requires high levels of ATP synthesis. In marked contrast to what one would expect on the basis of this hypothesis, central nervous system pathology is not observed in BTHS patients, in contrast to (most) inherited respiratory chain disorders [35,36]. Furthermore, it is unlikely that the neutropenia of BTHS patients is caused by impaired respiratory chain function because neutrophils are mostly glycolytic [37]. Further studies are needed to clarify the pathological mechanisms in these specific tissues and/or cell types. It would also be very interesting to investigate which additional factors play a role in modifying the phenotype of BTHS patients, since mutations in the tafazzin gene do not correlate with the disease severity, even within one family [38].

CL composition in human tissues
As suggested by its name, CL is very abundant in heart, the organ where it was first isolated from [39]. In human heart, but also in other tissues, tetralinoleoyl-CL is the predominant species [40] (figure 2A). In other organisms, such as Saccharomyces cerevisiae or marine bivalves, other acyl chains, i.e. oleic acid and docosahexaenoic acid respectively, are predominantly incorporated in CL [41]. Although the specific species of CL are different between organisms, it appears that there always is a high degree of structural symmetry in CL [41], suggesting that throughout evolution this uniformity is important for CL function.

Because little information is available on the CL distribution in human tissues and on the variation between individuals, we set out to investigate the CL profile in different human tissues. In addition, we aimed to correlate these data with expression levels of the human tafazzin splice variants, as will be discussed below. We analyzed the CL profile by HPLC-MS, which gives a good indication of the acyl chain composition of CL. As shown in figure 2A, the heart is especially rich in CL. Tissues such as spleen, adipose tissue and pancreas showed lower CL levels and other tissues displayed considerable variation in CL levels between investigated individuals (kidney and brain). One of the main advantages of the HPLC-MS for the measurement of CL is that one can discriminate between the different species of CL. Therefore, we quantified the abundance of the various CL clusters (each cluster is represented by the number of carbon atoms in the combined acyl chains) in relation to the highest cluster (i.e. C72, most likely 4×C18). Heart and skeletal muscle, tissues that require high mitochondrial metabolic activity, contained mainly tetralinoleoyl-CL
Figure 2. Tissue distribution of CL. (A) CL abundance in various tissues. Each symbol represents the tissue of a single individual. (B) Distribution of various CL clusters. Clusters are indicated as the number of carbon atoms in the combined acyl chains (e.g. C70 represents all CL species which have 70 carbon atoms in the acyl chains, most likely 3×C18 and 1×C16). Individual clusters were quantified and the relative abundance to the highest cluster (C72) is shown. In brain, a more diverse pattern is observed with more CL clusters and a relatively high abundance. Data are means ± standard deviation. Number of samples is as shown in figure 2A. (C) Comparison of CL profile of human heart and brain. Human heart effectively contains only one CL species, whereas brain CL acyl chain composition is more diverse and also contains more polyunsaturated fatty acids such as arachidonic and docosohexaenoic acid.
Tafazzin function in cardiolipin metabolism

(part of the C72 cluster) whereas the other CL species were less abundant (figure 2B). Although other tissues showed more variation in cluster abundance, the C72 cluster was clearly the highest and only three other clusters could be detected (C68, C70 and C74). In brain, however, the CL pattern was completely different as compared to other tissues (figure 2C). There are at least seven CL clusters (C66 to C78) where the species distribution, as judged from the broadness of the clusters, is considerably greater than in other tissues. The high molecular weight clusters (C74 to C78) contain increasing amounts of polyunsaturated fatty acids like arachidonic and docosahexaenoic acid. A theory that is worth mentioning here is that the tissue-specific CL composition reflects the specific metabolic needs of a tissue, as suggested by the Han group [42] who also recently investigated the CL profile of (mouse) brain. According to this hypothesis one would expect that in tissues where mitochondria are mainly used for ATP production, e.g. heart, the CL species are specially equipped for this (i.e. tetralinoleoyl-CL), whereas in tissues that rely less on mitochondrial metabolism for energy production, e.g. brain, a specific CL composition is less important and the CL profile is more diverse. This phenomenon might also explain why BTHS patients do not suffer from brain abnormalities, since disturbances of the CL composition by tafazzin-deficiency are less severe for brain CL than for CL in tissues that predominantly have the highly symmetric (i.e. heavily remodeled) tetralinoleoyl-CL.

CL analysis

The available techniques for the identification and quantification of CL have been reviewed recently [43]. Beside the relatively insensitive, seldom used, $^{31}$P-NMR measurement, these techniques comprise of methods based on one/two dimensional TLC spot isolation followed by either colorimetric detection of total phosphate after hydrolysis or by detection of the fatty acid methyl esters of the methanolic HCl hydrolysate. Alternatively, HPLC fractionation of a phospholipid extract can be employed followed either by UV detection, fluorescence detection (after derivatization), scintillation counting (in case of radioactive species) or immunological detection of cardiolipin [43]. The main drawbacks of these methods are an extensive sample preparation and/or their limitations in specificity and sensitivity. The requirements of the study and the availability of equipment may direct the choice to one of these methods. At present, however, MS instrumentation can combine high sensitivity, high selectivity and structural information with a rapid sample preparation. For these reasons, it has become the analytical method of choice for many applications.

In general, the LC-MS methods have a sample preparation that is based on a simple methanol/chloroform extraction of the lipids [44,45] after which the crude sample is injected into the LC-MS system [46]. Electrospray ionization (ESI) is the most widely used ionization method, but to a lesser extent, without hyphenation (i.e. coupling to a pre-MS separation technique), matrix-assisted laser desorption/ionization (MALDI) and desorption electrospray ionization (DESI) are also used as ionization methods. Roughly, the MS-based methods for the determination of CL can be differentiated according to the extent of hyphenation, MS multiplicity (MS$^n$) and mass resolution. The potential of application of the MS approach without hyphenation has been clearly demonstrated by the Han group, elaborating on the so-called shotgun lipidomics technique [47]. By virtue of adjustment of solvent composition (mainly pH) to
enhance ‘intrasource’ separation either with or without pre-selected MS/MS scans, suppression effects and interference of isobaric components can be reduced. In addition, high mass resolution allows for m/z peak separation of the doubly charged CL species. Although the method is fast, the main drawback of the technique is the unpredictable amount of interfering components in the resulting composite spectra. For qualitative or semi-quantitative purposes, LC-MS techniques are available. HPLC-MS methods with reversed- or normal phase columns are used to separate CL from other efficiently ionizing (phospho)lipids. The power of HPLC to separate CL from other phospholipids results in the sensitive measurement of pure, characteristic CL profiles (as shown in figures 1, 2C and 5).

Quantitation of a large range of CL molecular species using an LC-MS approach, identifying CLs by the number of carbon atoms and the degree of unsaturation of the acyl chains, has been described previously [46,48]. Whether they use the singly [48] or doubly charged molecular species [46], the exact molecular structure of the CL (i.e., the exact location and identity of the four acyl chains) cannot be determined, although LC-MS/MS analysis of the precursor ions can provide a qualitative indication of the predominant fatty acyl chain composition within the mixture. In all cases the use of a set of synthetic CL standards, in combination with a mathematical correction for overlapping isotopic peaks of different molecular species, is a prerequisite for accurate quantitation [46]. Quantitation using an HPLC-MS setup is hampered, however, by the fact all CL species show a different analytical response (depending on the number of carbon atoms and double bonds in the acyl chain). Since the added synthetic standards (generally tetramyristoyl-CL) have a different composition compared to the analyzed CL species, pure quantitative data cannot be obtained although semi-quantitative analysis is possible.

Full characterization of the CL species in a biological mixture, including species that consist of various isomers can only be achieved in detail by (at least) MS³ using ion trap technology [49]. The differentiation of CL isomers and the identification of complex CL species consisting of multiple molecular structures, however, require highly specialized instruments which are seldom available for routine CL analyses.

**Tafazzin**

**Tafazzin gene, mRNA and protein**

As mentioned above, mutations in the *tafazzin* gene (*TAZ*, originally termed G4.5) result in the rare X-linked recessive disorder BTHS [28]. The Italian group that identified the *tafazzin* gene named it after a masochistic comic character from an Italian television sports show and the gene/protein has lived up to this masochistic character, as experienced by many researchers working on this subject. One of the complicating aspects of tafazzin research is the alternative splicing of the primary transcript resulting in different mRNAs and potentially different proteins [28]. As will be discussed below in more detail, expression of these different splice variants in tafazzin-deficient yeast (*tazΔ*) showed that only the human tafazzin variant lacking exon5 was able to fully restore the aberrant CL profile of the *tazΔ* mutant. The full-length tafazzin variant only partially restored the CL profile [31]. Protein sequence alignment suggested that exon5 in human tafazzin is actually introduced later in evolution since it is not found in tafazzin of lower organisms [31]. This idea was confirmed by tafazzin gene analysis which showed that the exon5 sequence was
present in all monkey species tested, but that the splice recognition site for exon5 was only present in primates yielding incorporation into the mRNA [50]. Because the CL composition in primates (i.e. which have mRNA transcripts containing exon5) and other mammals (i.e. which lack exon5) is identical and because the full-length human tafazzin was unable to fully restore the CL profile of taz\textsuperscript{Δ} yeast, it is important to determine the function of this full-length variant with respect to CL metabolism. It is relevant to note that recently BTHS patients have been found with a mutation in exon5 (“Human Tafazzin (TAZ) Gene Mutation & Variation Database”; www.barthsyndrome.org). Theoretically, these patients should have normal levels of the tafazzin variant lacking exon5 if the mutation does not lead to instability and/or degradation of the primary transcript. It would be very interesting to investigate the expression of the different tafazzin mRNA variants and more importantly the levels of CL and MLCL in these patients.

**Tafazzin mRNA expression – human tissue distribution**

One of the remaining questions is which of the tafazzin splice variants are functionally significant in vivo. The preferred method to check whether the variants are actually expressed in cells/tissues is obviously by protein detection using a tafazzin-specific antibody, however, until now it has proven to be impossible to detect endogenous levels of this protein. A second option is to investigate mRNA expression of the different variants. The original identification of the alternatively spliced tafazzin transcripts was based on regular PCR analysis on total RNA of fibroblasts, heart, skeletal muscle and leukocytes [28] and was confirmed in fibroblasts in a later study [50]. Because regular PCR is not suited for quantitative comparison of the different variants, especially when different primer sets are used, we used quantitative PCR on human tissue cDNA. Logically, one would expect that tafazzin expression is highest in tissues that require high levels of linoleic acid incorporation in CL and that are affected in BTHS patients, such as heart and skeletal muscle. According to this rationale, the expression of the proposed (semi-) active variants, i.e. full-length tafazzin and tafazzin lacking exon5, is expected to be highest in these tissues. Using variant-specific primers we analyzed expression levels of 6 different tafazzin splice variants in 16 different human tissues (figure 3). To our surprise, we found that in all investigated tissues all tafazzin variants were more or less equally expressed with the exception of tafazzin lacking exon6,7, which was very low. Unexpectedly, we found that the highest tafazzin expression was observed in pancreas and spleen, whereas expression levels of the different tafazzin variants in heart and muscle was in the same range as compared with other tissues. Interestingly, these data do not correlate with the observed CL profile of the various tissues (figure 2). The marked enrichment of heart, skeletal muscle and liver CL with linoleic acid is apparently not due to increased tafazzin mRNA expression. Concomitantly, higher tafazzin expression as observed in pancreas and spleen does not result in higher tetralinoleoyl-CL abundance. On the contrary, the CL composition of spleen was considerably more variable than that of heart. Obviously, this discrepancy could be caused by the fact that mRNA levels do not necessarily reflect protein levels, however, given the absence of an antibody that detects endogenous tafazzin protein(s), quantitative mRNA levels currently are the best available indicator of tafazzin expression. As will be discussed below, another important factor that likely influences the tafazzin-mediated CL remodeling
Figure 3. Tafazzin mRNA tissue distribution. mRNA abundance of tafazzin splice variants in human tissues. mRNA levels are in arbitrary units, however, can be directly compared because the PCR efficiency of the different primer sets was identical and the cDNA was normalized based on four housekeeping genes by the manufacturer. Data are mean ± standard deviation of three separate PCR reactions. He = heart, Br = brain, Pl = placenta, Lu = Lung, Li = liver, Mu = skeletal muscle, Ki = kidney, Pa = pancreas, Sp = spleen, Th = thymus, Pr = prostate, Te = testis, Ov = ovary, In = small intestine, Co = colon, Le = leukocytes.
towards mature CL is the nature and direct availability, i.e. the microenvironment, of substrates for tafazzin. For example, brain expression of tafazzin mRNA was among the lowest of the investigated tissues (figure 3). This could explain the diverse CL species distribution that was observed in this tissue (figure 2B and 2C), but the different CL profile may also be attributed to the specific microenvironment of the brain (i.e. the high levels of arachidonic and docosahexaenoic acid) and therefore could be relatively independent of tafazzin expression. How the final CL composition is achieved in vivo and which (genetic or environmental) factors play a role in this process is an important step towards the understanding of CL metabolism and is also an essential step for the development of therapeutic strategies for BTHS.

**Tafazzin mRNA expression – BTHS patients**

There is no apparent correlation between the genotype and clinical phenotype of BTHS patients [38]. Missing parameters in the genotype/phenotype comparison are tafazzin mRNA levels, protein levels, and enzymatic activities. Although some data are beginning to emerge, both quantitative protein analysis by immunoblotting and enzymatic activity measurements are still hampered by aforementioned experimental difficulties. As we developed a quantitative mRNA analysis for the different tafazzin splice variants we could now compare the mRNA levels in cells and/or tissues of control subjects and BTHS patients and at least determine whether such a correlation exists between the mRNA levels and, if known, the genotype. For instance, some tafazzin mutations may lead to increased transcription or (selective) degradation of instable mRNAs. As shown in figure 4A, tafazzin is equally expressed in all control fibroblasts. In BTHS fibroblasts, which were all diagnosed with BTHS based on an abnormal CL profile (see table 1 for available mutations), it appears that in some patients total tafazzin expression is higher. This increased expression in BTHS fibroblasts may be caused by transcriptional upregulation in order to compensate for the loss of functional enzyme due to the mutations in the TAZ gene. In two patients (TAZ005

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<th>Table 1. Tafazzin mutations of the BTHS fibroblasts used</th>
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<td><strong>BTHS patient</strong></td>
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*a All patients were diagnosed with BTHS on the basis of (ML)CL analysis

*b Tafazzin mutations were not determined for all BTHS fibroblasts
Figure 4. Tafazzin mRNA in control and BTHS fibroblasts. (A) total tafazzin mRNA expression in control and BTHS fibroblasts. The primer set was designed to detect all tafazzin variants irrespective of the alternatively spliced exons. (B) mRNA expression of the individual tafazzin splice variants in control fibroblasts. (C) mRNA expression of the tafazzin splice variants in BTHS fibroblasts. The mean ± standard deviation of the 5 controls is given for comparison. Levels of total tafazzin mRNA transcripts (A) as well as the variants lacking exon6,7 and exon5,6,7 (B) in BTHS patient TAZ002 (see #) is low. This is likely caused by the splice acceptor mutation of this patient. This mutation probably results in deletion of exon3 on which the respective forward primers anneal. Data are mean ± standard deviation of three separate PCR reactions.

and TAZ007) no tafazzin mRNA could be detected (unfortunately the genotype of these patients is unknown) and one patient showed an approximate reduction of 50% of tafazzin mRNA (TAZ013). This reduction is likely caused by reduced mRNA stability and/or increased degradation because of the aberrant splicing of exon2 due to the splice acceptor mutation of this patient. Interestingly, two patients with the same genotype (TAZ003 and TAZ006) displayed different total tafazzin mRNA levels and the patient having a nonsense mutation (TAZ001) in exon2 did not have reduced tafazzin mRNA levels, as frequently observed in case of nonsense mutations [51]. Tafazzin mRNA levels appeared to be severely reduced in patient TAZ002, but this is because the splice acceptor mutation of this patient leads to aberrant splicing of exon3, which is where part of the forward primer anneals.

Because several tafazzin mRNA variants have been described and it is still unknown which of these variants are relevant in vivo and whether tafazzin mutations in BTHS influence the variant mRNA distribution, we developed specific primer sets for each variant and analyzed the specific expression profile in control and BTHS fibroblasts.
All tafazzin variants were clearly detectable in the different control fibroblasts, except the variant lacking exon6,7, which was less abundant (figure 4B). The expression profile in control fibroblasts was highly similar to that found in human tissues (figure 3). In the fibroblasts of patients TAZ005 and TAZ007 (almost) no tafazzin splice variant mRNA was detected (figure 4C), confirming the lack of total tafazzin transcripts (figure 4A). Also, the 50% reduction in tafazzin mRNA of patient TAZ013 was confirmed by the splice variant analysis, which showed a 50% reduction of all variants. In patient TAZ002 (in which the total tafazzin mRNA was not detectable due to the specific mutation of this patient) most tafazzin mRNA variants were normally detected, except for the variants lacking exon6,7 and exon5,6,7, both of which also require exon3 for normal primer annealing.

Interestingly, it seems that the relative abundance of the TAZ transcripts in fibroblasts from some BTHS patients is different when compared to control fibroblasts. Like in human tissues (figure 3A), we observed that tafazzin lacking exon7 is the most abundant mRNA variant in control fibroblasts, followed by the other variants with the exception of tafazzin lacking exon6,7, which was very low. In contrast, fibroblasts from most BTHS patients showed expression levels of tafazzin lacking exon5 and full-length tafazzin that were identical or higher than the expression of tafazzin lacking exon7 (figure 4), which could be a result of preferential splicing and/or stabilization of these messages. The fact that these two variants have been shown to be functional with respect to CL metabolism suggests that they are selected to maximize residual CL remodeling activity. Although this analysis was not performed to investigate the genotype/clinical phenotype correlation, mRNA expression profiles may represent an additional piece in this complicated jig-saw puzzle if more patients are analyzed with known geno- and phenotypes. Obviously, additional investigations are needed using an antibody against human tafazzin or an enzymatic assay for human tafazzin to further establish the relevance of the increased expression of these variants.

Tafazzin functionality
Since the discovery in 1996 of tafazzin as the causative gene for BTHS [28], it has remained elusive what the role is of the various tafazzin splice variants. By expression of the different variants in the tafazzin deficient yeast mutant, we showed that only the variant lacking exon5 was able to fully restore the growth deficiency and CL profile of the mutant [31]. The human full-length tafazzin, however, partially restored the CL abnormalities but not the growth phenotype, and expression of the other variants resulted in no restoration at all [31]. Re-analyses of these samples using our new HPLC-MS system followed by closer examination of the data revealed some interesting novel aspects worth mentioning here, especially now that the enzymatic function of tafazzin is being unraveled. We performed a comparison between the yeast tafazzin deletion mutant tazΔ transformed with vehicle only, tazΔ transformed with full-length human tafazzin and tazΔ transformed with tafazzin lacking exon5 (figure 5). Expression of tafazzin lacking exon5 clearly led to restoration of CL species containing multiple C18:1 acyl chains (C70 (where C70 denotes the total number of carbon atoms of the combined acyl chains, with the most likely composition of 3×C18 + 1×C16 acyl chains) and C72 (most likely 4×C18) clusters), which were virtually absent in tazΔ transformed with full-length tafazzin. In contrast, full-length tafazzin fully restored the CL species containing multiple C16 acyl chains (C58 to
Chapter 6

Wild type + pYPGK18

\[ m/z \]

Relative to i.s. (%)

0 50 100 150 200 250

intensity relative to i.s. (%) 555 575 595 615 635 655 675 695 715 735

\[ m/z \]

Relative to i.s. (%) 40 0 20 40 0 20

\[ m/z \]

Relative to i.s. (%) 50 0 25 50 0 25

\[ m/z \]

Relative to i.s. (%) 50 0 25 50 0 25

\[ m/z \]

Relative to i.s. (%) 50 0 25 50 0 25

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Relative to i.s. (%) 50 0 25 50 0 25

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Relative to i.s. (%) 50 0 25 50 0 25

\[ m/z \]

Relative to i.s. (%) 50 0 25 50 0 25

\[ m/z \]

Relative to i.s. (%) 50 0 25 50 0 25
C64 clusters), which were only partially restored by tafazzin lacking exon5. Based on these observations it appears that tafazzin lacking exon5 preferentially incorporates C18:1 acyl chains, whereas full-length tafazzin incorporates C16 acyl chains, either because C16 is its preferred substrate or because it is unable to incorporate C18. Direct determination of the substrate specificity of these two tafazzins will hopefully clarify the matter. It should be noted that the partial restoration of the C16-enriched CL species was not sufficient to restore the growth phenotype of tazΔ [31] suggesting that these CL species are not functional and C18-enriched species are required in yeast.

Another somewhat surprising finding was that expression of tafazzin lacking exon5 also resulted in the formation of CL species containing short (C12 and C14) acyl chains (see inset, CL clusters C52 to C56). At this time we do not have an explanation for the presence of these small CL species, which in retrospect were also present in the previous expression studies [31].

Maybe the most remarkable aspect of the human tafazzin expression studies in the yeast mutant is that CL is restored to yeast CL (i.e. containing 18:1) and not human CL which primarily contains 18:2. The availability of substrates, i.e. the fatty acyl composition of available lipids, apparently dictates the formation of certain CL species whereas the selectivity of the tafazzin enzyme is less important or that tafazzin is not specific at all. Although the complementation experiments in yeast support the function of human tafazzin in CL remodeling, there is still no experimental evidence that directly shows that expression of human tafazzins in BTHS cells results in restoration of the CL profile. We therefore expressed the most relevant tafazzin splice variants in BTHS patient fibroblasts. Three days after transfection no effect was observed on CL levels and composition (data not shown). Because it has been described that the turnover of CL is very low [52], we hypothesized that the three days expression could be too short to remodel the CL pool. In order to verify this, we transfected the tafazzin variants in BTHS patient fibroblasts and used antibiotic selection for ten days to enrich the number of transfected cells and allow remodeling to occur, and we measured CL and MLCL in these cells. Expression of tafazzins was confirmed by Western blot analysis (figure 6, using one of the many antibodies that only detects overexpressed tafazzin). Expression of tafazzin lacking exon5 (the presumed active variant) resulted in a marked decrease in the MLCL/CL ratio (figure 6), which is used as a diagnostic marker for BTHS [53]. The MLCL/CL ratio was not restored to control levels indicating that ten days expression was still not sufficient.

Figure 5. Detailed HPLC-MS analysis of (ML)CL in yeast with and without expression of human tafazzin. Wild type and tazΔ yeast were transformed with pYPGK18 and the tazΔ mutant was transformed with pYPGK18 containing either full-length human tafazzin (HTAZfull) or tafazzin lacking exon5 (HTAZ-ex5). Clusters are indicated as the number of carbon atoms in the combined acyl chains (e.g. C70 represents all CL species which have 70 carbon atoms in the acyl chains, most like 3×C18 and 1×C16). The tazΔ yeast mutant showed increased MLCL levels and a shift towards more saturated acyl chains (i.e. the highest peak within a cluster is shifted to a higher m/z, an indication of less double bonds and hence a higher degree of saturation) when compared to wild type. HTAZ-full resulted in a partial restoration of the MLCL levels and a restoration of the C16 containing CL clusters C58-C66. The clusters that contain mainly C18 (C68-C72) are only partially or not restored at all. HTAZ-ex5 increased the incorporation of C18 in the CL clusters C68-C72, resulting in full restoration of these CL species. The relative abundance of the C16- and C18-containing clusters (e.g. compare cluster C62 to C64) suggests that HTAZ-full preferentially incorporates C16 in CL whereas HTAZ-ex5 preferentially incorporates C18. In HTAZ-ex5 we also observed short (likely C12- and C14-containing) CL species (C52-C56, see left inset).
which was also confirmed by the lack of restoration of the acyl chain composition. Expression of either full-length tafazzin (presumed semi-active variant) or tafazzin lacking exon7 (presumed inactive variant) also seemed to result in a small decrease in the MLCL/CL ratio (figure 6). Although this was significant within the experiment (when compared to the mock transfection) we doubt that this reduction of the MLCL/CL ratio truly reflects functionality of these two variants. Altogether, our data suggest that tafazzin lacking exon5 most likely represents the active tafazzin variant, but it is not excluded that the other variants also may play a role in CL remodeling. In addition, it is clear that transient expression of tafazzin is not sufficient for the complete remodeling of CL and that stable expression by means of viral transduction is necessary to establish the functionality of the variants in human BTHS cells.

**Tafazzin enzymatic function**

As mentioned above, tafazzin from *D. melanogaster* was reported to function in the transfer of a linoleoyl acyl chain from PC to MLCL, thereby forming CL [25]. By this means, tafazzin could function in the enrichment of this particular acyl chain in CL. The reverse reaction, however, in which PC is formed from lyso-PC and CL, is equally efficient [25]. This enzymatic activity could well be identical to that described by Testet and colleagues for *S. cerevisiae* tafazzin which was thought to function as a lyso-PC acyltransferase [54]. It should be noted that the tafazzins from both *D. melanogaster* and *S. cerevisiae* are most homologous to human tafazzin lacking exon5. Because of this, it might be expected that tafazzin lacking exon5 displays the same enzymatic activity, but the role of the other tafazzins, notably full-length tafazzin, remains to be elucidated as discussed above. Another interesting question that remains to be answered is how the direction of the enzymatic reaction is regulated. If PC indeed supplies the linoleic acid acyl chains for the tafazzin-mediated remodeling of CL, there has to be an additional mechanism that drives the maintenance of the linoleoyl-PC pool. Lyso-PC acyltransferases specific for linoleic acid could fulfill this role by functioning as an important regulator and it would be interesting to see whether such proteins are indeed involved in CL remodeling.
Discussion and future perspectives

Since the identification of BTHS as a primary CL remodeling disorder caused by mutations in the tafazzin gene, CL and tafazzin have received increasing attention. Despite the increased research effort many questions remain, especially in relation to BTHS. The most prominent gaps are the genotype-phenotype relation and the pathogenesis of BTHS, which are both still poorly understood. In order to answer these questions it will be of great value to know the exact function of the human tafazzin and its variants. The development of an enzymatic assay specific for tafazzin and the determination of its substrate preference is crucial and hopefully will provide an important link in resolving the relation between genotype and phenotype. Another important tool that is obviously needed to enhance the experimental possibilities is a proper tafazzin antibody which can detect endogenous tafazzin (and if appropriate; different specific variants). For instance, the interactions of yeast tafazzin that were recently reported could be verified in human cells [11]. Another key in understanding the genotype-phenotype relation and the pathogenesis of BTHS is the identification of so-called modifying factors. Obvious candidate proteins influencing the genotype-phenotype relation and the diverse pathology of BTHS patients include proteins involved in oxidative phosphorylation, apoptosis and oxidative stress defense, as was recently suggested [55]. Proteins involved in mitochondrial import also could play a role in these processes as stressed by the identification of a BTHS-like disorder, which is caused by mutations in the mitochondrial import protein TIM14 [56].

Another interesting avenue for future research is the regulation of CL metabolism, notably the tafazzin-mediated CL remodeling. Although some studies were performed that showed that CL metabolism is transcriptionally regulated [57-59], not much is known about the regulation of CL synthesis and remodeling and whether this could be used as a potential treatment target for BTHS patients. Currently used model systems for BTHS include yeast, fly and zebrafish. Although all these models have proven to be (and still are) very useful, one obvious missing model system is the tafazzin-deficient mouse. The generation of a mammalian model for BTHS is crucial in order to resolve the questions mentioned above but also to test possible therapeutic options for this frequently lethal disorder.

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Materials and methods

CL analysis in human tissues

Anonymized human postmortem frozen tissue samples were obtained from the NICHD Brain and Tissue bank for Developmental Disorders at the University of Maryland. Tissues were homogenized in PBS using an Ultra-Turrax homogenizer and sonicated for 20 seconds using a tip sonicator. The protein concentration was determined according to the Bradford protocol [60]. Phospholipids were extracted using a 2-phase extraction [31]. The HPLC-MS system was operated as described previously [18]. For quantification of CL and MLCL levels, the area under the curve of the HPLC profile corresponding to the mass spectra of CL and MLCL species was integrated, as well as that of the internal standard. CL concentration was calculated based on the internal standard, assuming identical response.

Tafazzin mRNA tissue distribution

Tissue distribution of human tafazzin splice variants was analyzed in a MTC human tissue cDNA panel (Clontech). Specific primer sets were developed for the different human tafazzin splice variants by using specific combinations of exon-exon boundaries (for primer sequences see table 2). For the quantitative real-time PCR the LightCycler 480 SYBR Green I Master kit (Roche) was used. The amount of cDNA in the individual samples of the cDNA panel was normalized for a combination of four housekeeping genes (α-tubulin, β-actin, glyceraldehyde-3-phosphate dehydrogenase, phospholipase A2), as described by the manufacturer. All samples were analyzed in triplicate. The data were analyzed using linear regression calculations as described previously [61].

Tafazzin mRNA analysis in control and BTHS fibroblasts

Total RNA was isolated from control and BTHS fibroblasts using Trizol extraction. cDNA was produced using a first-strand cDNA synthesis kit. Real-time PCR and linear regression calculations were performed as described above. Specific primer sets were used for “total tafazzin” (i.e. all variants amplified together) and for all the distinct tafazzin variants (see table 2). Cyclophilin was used as a housekeeping gene to adjust for variations in the amount of input RNA. Sequence analysis of the amplified PCR product confirmed that the primer sets were specific for the particular mRNA variants. As an additional control for PCR efficiency, we amplified the various tafazzin variants from a pool of linearized plasmids containing an equal amount of each of the different variants and showed that PCR efficiency was virtually identical for all variants. All samples were analyzed in triplicate.

Yeast complementation analysis

Expression of tafazzin variants in wild type (w303) and tazΔ yeast was performed as described previously [31]. The improved HPLC-MS analysis was performed as described in [18].

Transient tafazzin expression

The various tafazzin cDNAs were cloned in pcDNA3 and transfected to control and BTHS fibroblasts using the Amaxa electroporation procedure (Amaxa). In short, 2 µg of plasmid was transfected to 500,000 cells, for each condition we performed
10 transfections in parallel, which were pooled after the electroporation. After 48 hours culture on normal culture medium (DMEM+10% FCS), cells were shifted to medium containing resistance selection marker (100 µg/mL G-418 in DMEM+10% FCS) and were cultured for additional 8 days with regular changing of the selective medium and shift to larger culture flasks when necessary. Ten days post-transfection cells were harvested by trypsinization. The resulting cell pellet was homogenized in PBS, sonicated for 20 seconds using a tip sonicator and protein concentration was determined using the Bradford protocol. The equivalent of 40 µg of protein was used for SDS-PAGE followed by immunoblotting using the crude serum of a rabbit that was immunized with C-terminal 100 amino acids of human tafazzin (a kind gift of Dr. A. Metzenberg). The remaining cell suspension (~300 µg) was used for CL analysis as described previously [29].

References

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Tafazzin function in cardiolipin metabolism