Role of calcium-independent phospholipase A_2 in the pathogenesis of Barth syndrome

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Quantitative and qualitative alterations of mitochondrial cardiolipin have been implicated in the pathogenesis of Barth syndrome, an X-linked cardioskeletal myopathy caused by a deficiency in tafazzin, an enzyme in the cardiolipin remodeling pathway. We have generated and previously reported a tafazzin-deficient *Drosophila* model of Barth syndrome that is characterized by low cardiolipin concentration, abnormal cardiolipin fatty acyl composition, abnormal mitochondria, and poor motor function. Here, we first show that tafazzin deficiency in *Drosophila* disrupts the final stage of spermatogenesis, spermatid individualization, and causes male sterility. This phenotype can be genetically suppressed by inactivation of the gene encoding a calcium-independent phospholipase A_2, iPLA2-VIA, which also prevents cardiolipin depletion/monolysocardiolipin accumulation, although in wild-type flies inactivation of the iPLA2-VIA does not affect the molecular composition of cardiolipin. Furthermore, we show that treatment of Barth syndrome patients' lymphoblasts in tissue culture with the iPLA$_2$ inhibitor, bromoenol lactone, partially restores their cardiolipin homeostasis. Taken together, these findings establish a causal role of cardiolipin deficiency in the pathogenesis of Barth syndrome and identify iPLA2-VIA as an important enzyme in cardiolipin deacylation, and as a potential target for therapeutic intervention.

cardiolipin | *Drosophila* | iPLA2 | tafazzin

Barth syndrome (MIM 302060) is caused by mutations in the X-linked tafazzin gene (TAZ) (1), which encodes a mitochondrial phospholipid-lysophospholipid transacylase required for cardiolipin (CL) homeostasis (2). In eukaryotes, CL is present exclusively in the membranes of mitochondria, where it interacts with a number of mitochondrial proteins and is essential for optimal mitochondrial functions (3–6). Defective tafazzin results in CL deficiency (7–10) and, not surprisingly, patients with Barth syndrome present symptoms, such as cardioskeletal myopathy and exercise intolerance, which are commonly associated with mitochondrial diseases (11).

In mitochondria, CL is synthesized de novo through condensation of phosphatidylglycerol (PG) with cytidine diphosphate-diacylglycerol (CDP-DAG) catalyzed by cardiolipin synthase (CLS) (3). Newly synthesized CL molecules are remodeled by transacylation catalyzed by tafazzin to yield the characteristic steady-state acyl composition of the specific cells and tissues (6). In cells from Barth syndrome patients, because of defective tafazzin, CL molecules fail to reach the normal fatty acyl composition (9, 10, 12). In addition, the level of total CL decreases despite normal de novo synthesis of CL molecules, and CL degradation intermediates, monolyso-CLs, accumulate (13, 14). These features of tafazzin-deficient cells suggest that normal steady-state levels of CL are maintained by at least 3 enzymatic reactions: (*i*) de novo synthesis catalyzed by cardiolipin synthase, (*ii*) deacylation of CL to monolyso-CL catalyzed by a mitochondrial phospholipase A, and (*iii*) reacylation of monolyso-CL catalyzed by tafazzin. Thus, the transacylase activity of tafazzin is required for maintaining not only the normal CL fatty acyl composition, but also normal CL levels.

Although it has been established that tafazzin deficiency causes both Barth syndrome and a derangement of CL metabolism, evidence that it is, in fact, the CL deficiency that contributes to Barth syndrome has been circumstantial (15). To elucidate the pathogenic mechanism of Barth syndrome and to identify potential targets for therapeutic intervention, we have created a *Drosophila* model of Barth syndrome (7) by knocking out the tafazzin gene and have asked whether the resulting phenotypic changes can be suppressed by partially restoring CL homeostasis without correcting the tafazzin defect. Because reacylation of monolyso-CL is reduced due to tafazzin deficiency in Barth syndrome and a new steady state of low CL and high monolyso-CL is reached, we reasoned that an inhibition of mitochondrial phospholipase A_2 activity could reverse these phenotypic effects, even though CL molecules would still have an abnormal fatty acyl chain composition. Here, we identify a calcium-independent phospholipase A_2, iPLA2-VIA, as an important enzyme in CL deacylation and monolyso-CL accumulation in Barth syndrome, and show that genetic inactivation of iPLA2-VIA suppresses the phenotype caused by tafazzin deficiency in *Drosophila*.

Results

Tafazzin Deficiency Causes Male Sterility in *Drosophila*. Like Barth syndrome patients, the *Drosophila* model flies that we generated (7) have CL deficiency, abnormal mitochondria, and poor motor function. We now report that, in addition, *Drosophila* males homozygous for TAZ deletion mutations are also sterile (Fig. 1*A*). We ruled out the possibility that the male sterility might be caused by other mutations present on the same chromosome with the TAZ mutation, because the males compoundly heterozygous for those deletions were also sterile. These observations strongly suggested that the inactivation of the defective tafazzin gene is the cause of male sterility. We proved that this is indeed the case, because transgenic expression of full-length *Drosophila* tafazzin, driven by GAL4 under the ubiquitously active *daughterless* (*da*) promoter, rescued both the male sterile phenotype (Fig. 1*A*) and the CL deficiency in TAZ deletion mutants (Fig. 1*B*). The easily identifiable male sterility phenotype allowed us to examine the effect of second gene mutations in the Barth model flies.

Spermatid Individualization Is Disrupted in Tafazzin-Deficient *Drosophila* Males. The most common cause of male sterility in *Drosophila* is defective spermatogenesis (16). Electron micro-

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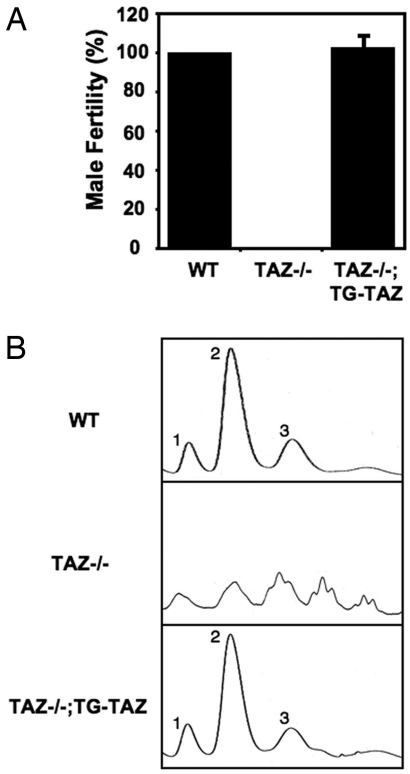

Fig. 1. Transgenic expression of full-length tafazzin restores male fertility and the normal cardiolipin profile of TAZ-deletion mutant flies. (*A*) Male fertility of the tafazzin mutant flies without (TAZ−/−) or with (TAZ−/−; TG-TAZ) the transgenic expression of tafazzin gene driven by GAL4 relative to the fertility of wild-type flies (WT). Data from 3 independent experiments. (*B*) Fluorescence HPLC cardiolipin profiles from WT, TAZ−/−, and (TAZ−/−; TG-TAZ) flies. The fluorescence yield (ordinate) is plotted against retention time (abscissa). In normal *Drosophila*, because of cardiolipin remodeling, peak 2, which contains a mixture of cardiolipin molecules with palmitoleoyl (16:1) and linoleoyl (18:2) residues (see ref. 25), is the dominant component. This peak is markedly reduced in the TAZ−/− mutant and is restored to nearly normal levels in the transgenic flies.

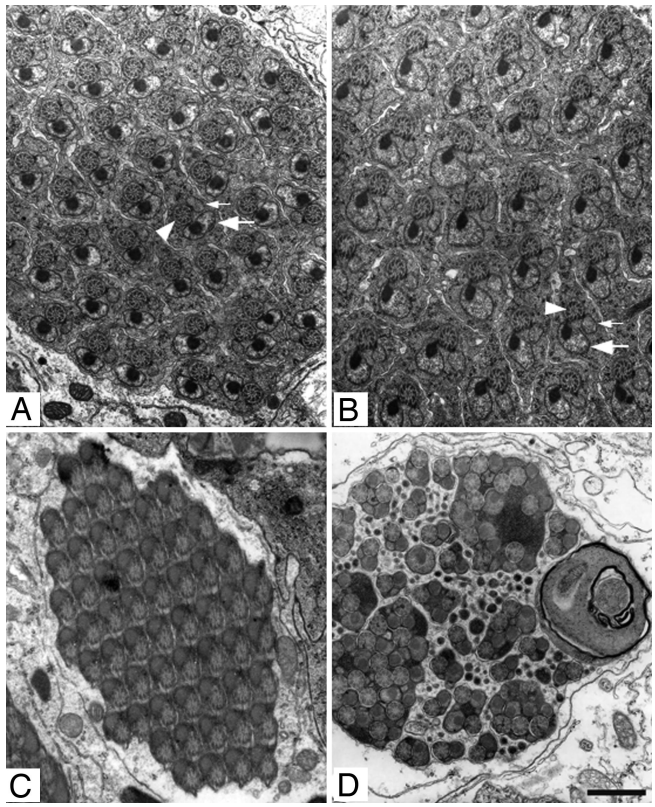

Fig. 2. Cross-section electron micrographs through preindividualized (*A* and *B*) and mature (*C* and *D*) portions of spermatogenic cysts from wild-type (*A* and *C*) and TAZ deletion mutant (*B* and *D*) testes. Preindividualized spermatogenic cysts are similar in TAZ−/− (*B*) and wild-type (*A*) testes. These cysts are identifiable by their light-staining major mitochondrial derivatives (big arrow), in which an electron-dense material accumulates, enlarged minor mitochondrial derivatives (small arrow), and the presence of extensive cytoplasm between the axonemes (arrow head). After individualization, wild-type cysts contain highly ordered arrays of elongated spermatids that have undergone a significant reduction in the amount of cytoplasm that can be seen between the sperm tails (*C*). TAZ mutant cysts fail to undergo proper individualization and in them spermatid arrangements are grossly disorganized (*D*). (Scale bar, 1 μm.)

scopic examination of the testes of tafazzin-deficient males (Fig. 2) revealed no abnormalities in spermatogenesis until the stage of spermatid individualization, the terminal differentiation process that transforms elongated syncytial spermatids within spermatogenic cysts into individual spermatozoa (17, 18). In the testes of tafazzin-deficient males, elongated syncytial spermatids within the cysts contained normal axonemes and mitochondrial derivatives (Fig. 2*B*), but no highly ordered bundles of individual spermatids, like those in wild-type testes (Fig. 2*C*), were found. Instead, the terminally differentiated cysts in the TAZ mutant testis appear grossly disorganized (Fig. 2*D*). Taken together, these findings suggest that the male sterility in tafazzin-deficient flies is caused by defective spermatid individualization.

The Male-Sterile Phenotype of Tafazzin-Deficient Flies Is Suppressed by a Loss-of-Function Mutation in the Gene Encoding the *Drosophila* Calcium-Independent Phospholipase A_2. The finding that in cells from Barth syndrome patients, CL depletion is accompanied by monolyso-CL accumulation suggests that the disease phenotype results at least in part from the fact that, in the absence of tafazzin, cells cannot counter by transacylation the effects of a mitochondrial phospholipase A_2 that deacylates CL. The superfamily of phospholipase A_2 (PLA2) comprises a number of very different proteins that belong to 5 principal classes of enzymes: secretory sPLA2s, cytosolic cPLA2s, calcium-independent iPLA2s, platelet-activating factor acetylhydrolases (PAF-AH), and lysosomal PLA2s (19). The most likely candidate for cardiolipin deacylation in vivo would be iPLA2-VIA, a calcium-independent phospholipase A_2 that has been localized to mitochondria (20, 21) and has a well-known function in phospholipid remodeling (19). The *Drosophila* ortholog of the mammalian iPLA2-VIA is the gene CG6718 (FlyBase).

To test whether inactivation of iPLA2-VIA suppresses the *Drosophila* TAZ mutant phenotype, we obtained from the Bloomington Drosophila Stock Center a fly strain that carries a P element insertion (EY05103) in the gene CG6718 (Fig. 3*A*), and demonstrated by RT-PCR that CG6718 expression was knocked out in this strain (Fig. 3*B*). We then generated double-mutant flies that contain both the TAZ deletion and the EY05103 insertion and found that the homozygous double-mutant males are fertile (Fig. 4*A*). To prove that the suppression of the male-sterile phenotype is, indeed, due to the EY05103 insertion, we generated flies with precise excisions of the P element by introducing a transposase in an appropriate cross and found that this restored the male-sterile phenotype of the TAZ deletion mutant (Fig. 4*A*). It can, therefore, be concluded that genetic inactivation of iPLA2-VIA suppresses the male-sterile phenotype of tafazzin deficiency in *Drosophila*.

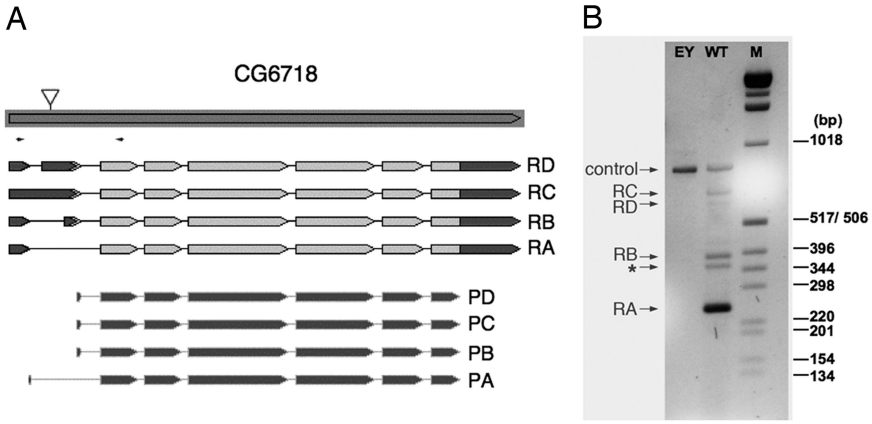

Fig. 3. The P element insertion in EY05103 inactivates the gene CG6718 encoding the calcium-independent phospholipase A2, iPLA2VIA. (*A*) The *Drosophila* genomic region containing the gene (top bar, adapted from Flybase). The Flybase annotation indicates that CG6718, through alternative splicing, produces 4 mRNA transcripts (RA, RB, RC, and RD) that encode 2 protein products with different N termini (PA and that of PB, PC, or PD). The position of the EY05103 P element insertion is indicated by an open triangle above the genomic region. Two arrowheads below the genomic region and above the mRNAs schematics indicate the positions of the primers used for RT-PCR. (*B*) Agarose gel electrophoresis of the products generated by RT-PCR from the CG6718-mRNAs in wild type (WT) and the EY05103 (EY) fly strain. Lane EY, homozygous EY05103 insertion strain; lane WT, w^{1118} strain; lane M, DNA size marker. RA, RB, RC, and RD correspond to the CG6718 transcripts annotated in the Flybase. The asterisk indicates a new alternatively spliced transcript not yet annotated in the Flybase. Control indicates the amplified transcript fragment of an irrelevant gene (CG34133) as an internal control. The identity of each fragment was confirmed by DNA sequencing.

Genetic Inactivation of the *Drosophila* Calcium-Independent Phospholipase A_2 also Partially Prevents CL Depletion and Monolyso-CL Accumulation in the Tafazzin-Deficient Flies. To determine whether the male sterility associated with tafazzin deficiency correlates with changes in CL metabolism, we measured the CL/MLCL profiles in wild-type, iPLA2-VIA mutants, TAZ mutants, and iPLA2-TAZ double-mutant flies by using an HPLC fluorescence detection technique capable of quantifying both monolyso-CL (MLCL) and CL. In wild-type flies and flies with genetic inactivation of the calcium-independent phospholipase A_2 very little MLCL is detected and CL profiles are not significantly different (Fig. 4*B Top* and *Top Middle*). In contrast, the TAZ mutation causes not only a decrease in CL levels and a diversification of the CL species present (compare Fig. 4*B*, TAZ−/− with wild type), as we previously reported, but also a major increase in MLCL/CL ratio (Fig. 4 *B* and *C*), similar to that found in cells from Barth syndrome patients (13, 14). Strikingly, this ratio is significantly reduced in the double mutant (Fig. 4 *B* and *C*). These results suggest that although iPLA2-VIA is not required for CL remodeling, in the Barth syndrome model, in which tafazzin is absent, the iPLA2-VIA activity is responsible for the CL depletion and the concomitant monolyso-CL accumulation. The chromatograms show that the diversification of CL species associated with the TAZ mutation (indicating incomplete remodeling of fatty acyl composition) remains present in the double mutant (Fig. 4*B*). Thus, the male sterility of tafazzin-deficient flies is caused by either CL depletion or monolyso-CL accumulation, or both, but not by abnormal CL composition due to lack of remodeling.

Treatment of Lymphoblasts from Barth Patients in Tissue Culture with an $iPLA_2$ Inhibitor Almost Completely Normalizes MLCL/CL Ratio. The observations made with the *Drosophila* Barth syndrome model led us to investigate whether the abnormal MLCL/CL ratio observed in Barth patients' lymphoblasts can also be normalized by reducing the activity of the calcium-independent phospholipase A_2. To this effect, lymphoblast cultures from 2 Barth patients (BTHS) and 2 normal controls (NC) were treated with bromoenol lactone (BEL), an iPLA2-VI-specific inhibitor. HPLC analysis of the cultures after incubation with 2.5 μM BEL for 48 h showed that BEL treatment of Barth patients' lymphoblasts greatly reduced their MLCL/CL ratios to values close to normal (Fig. 4*D*), suggesting that $iPLA_2$ activity substantially contributes to the accumulation of MLCL in BTHS lymphoblasts, as it does in the *Drosophila* model.

Discussion

The cardiolipin metabolism defect associated with Barth syndrome is manifested by the triad of CL depletion, monolyso-CL accumulation, and CL species diversification, i.e., the generation of CL molecules with different fatty acyl compositions (15). It has not been clear whether the abnormal CL homeostasis actually plays a role in the pathogenesis of Barth syndrome, and if so, which aspect is the key factor. In the present study, we have addressed this issue in a *Drosophila* model of Barth syndrome.

Cardiolipin and *Drosophila* Spermatid Individualization. We found that tafazzin deficiency in *Drosophila*, which alters CL homeostasis and reduces CL levels, also disrupts spermatid individualization during spermatogenesis, resulting in male sterility, and that this male-sterile phenotype can be suppressed by inactivation of the CL-degrading enzyme iPLA2-VIA, which partially restores CL homeostasis in double-mutant flies. These observations suggest that CL content, or at least the MLCL/CL ratio, plays a critical role in *Drosophila* spermatid individualization. It was recently shown that the final stage of spermatid differentiation in *Drosophila* involves an apoptosis-like mechanism, in which the cytochrome *c*-dependent caspase activation is required for the elimination of excess cytoplasm (22). Cardiolipin has been shown to play important roles in mitochondria-dependent apoptosis (23) and a recent report demonstrated that CL deficiency increases cells' resistance to apoptosis (24). Therefore, CL deficiency in *Drosophila* testes may prevent the syncytial spermatids from initiating the apoptosis-like mechanism required for normal spermatid individualization.

Cardiolipin Deficiency and Barth Syndrome. The cardinal characteristics of Barth syndrome are cardioskeletal myopathy, exercise intolerance, neutropenia, abnormal mitochondria, and altered CL metabolism (11). Because in eukaryotes CL is localized exclusively in mitochondria and is required for optimal mitochondrial function, it has been generally assumed that the

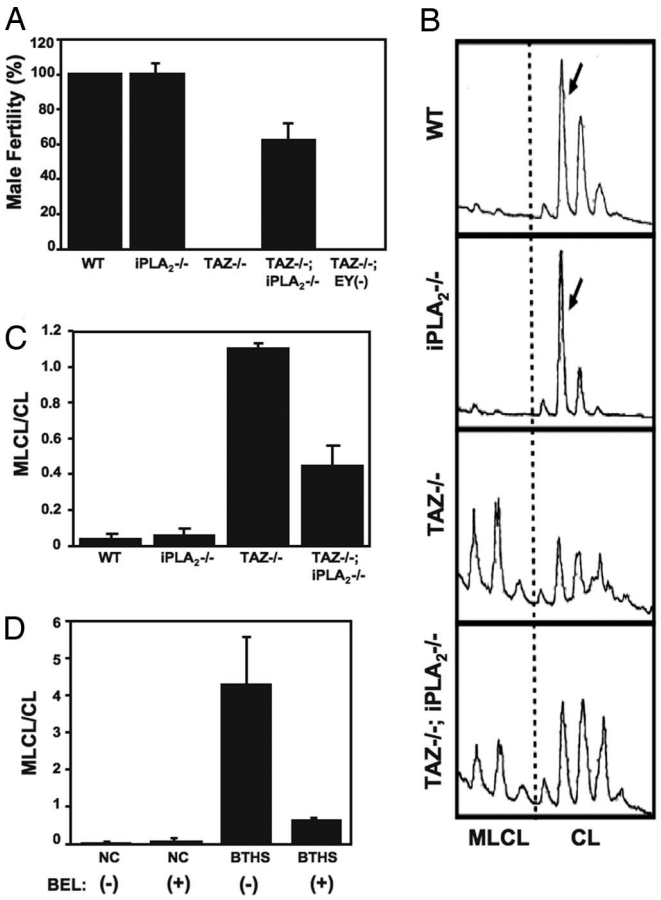

Fig. 4. Inactivation of $iPLA_2$ suppresses the male-sterile phenotype of tafazzin-deficient flies and partially restores the MLCL/CL ratio. (*A*) Relative male fertilities of w^{1118} (WT), tafazzin mutant (TAZ−/−), EY05103 P element insertion strain ($iPLA_2$−/−), double mutant (TAZ−/−; $iPLA_2$−/−) and the precise P element excision strain [TAZ−/−; EY(−)]. That the excision in the TAZ−/−; EY(−) strain was precise and restored the normal $iPLA_2$ gene was determined by PCR amplification of the relevant chromosomal region and DNA sequencing through the previous insertion site. The male fertility of WT flies is defined as 100%. Data from 3 independent experiments were obtained. (*B*) Fluorescence HPLC profiles of MLCL and CL isolated from WT, $iPLA_2$−/−, TAZ−/−, and the double-mutant flies. The fluorescence yield (ordinate) is plotted against retention time (abscissa). The vertical dotted lines separate the regions with peaks representing CL (to the right) from those with peaks corresponding to monolyso-CL species (to the left). The arrows indicate the dominant CL peak containing mature cardiolipin molecules with palmitoleoyl (16:1) and linoleoyl (18:2) residues due to remodeling (25). (*C*) Quantification of the MLCL/CL ratio in WT, $iPLA_2$−/−, TAZ−/−, and double-mutant flies. Data are from the profiles obtained from 4 independent experiments. (*D*) Treatment of BTHS lymphoblasts with an iPLA2 inhibitor (BEL) partially restores their MLCL/CL ratio. The lymphoblast cultures were treated with 2.5 μM bromoenol lactone (BEL) for 48 h and their MLCL and CL contents were determined by fluorescence HPLC. The data are presented as the average obtained from 2 BTHS lymphoblast cell lines and 2 normal control (NC) lymphoblast cell lines.

defective CL metabolism causes the pathophysiology of Barth syndrome (15). In the present study, we have tested this hypothesis by genetically manipulating CL metabolism in the *Drosophila* model. Our finding that partial restoration of CL homeostasis through genetic inactivation of iPLA2-VIA suppresses the male-sterile phenotype of tafazzin-deficient flies provides the direct evidence that altered CL metabolism is a major contributing factor in Barth syndrome.

The most abundant CL molecular species from various organisms and tissues contain only 1 or 2 types of fatty acids (25). In many mammalian tissues, the predominant fatty acyl moiety in CL is linoleic acid (C18:2) (3). For example, 80% of CL molecules in heart and skeletal muscle are tetralinoleoyl CL (12, 26). However, the role of CL molecular species in vivo remains speculative.

The characteristic fatty acyl composition of CL in vivo is achieved through tafazzin-dependent remodeling of nascent CL. However, tafazzin deficiency, such as in Barth syndrome, results not only in abnormal CL acyl composition, but also in CL depletion and monolyso-CL accumulation. Thus, it has been unclear which aspect of the CL metabolic disorder contributes to the pathogenesis of Barth syndrome (15). Our finding that the male-sterile phenotype of tafazzin-deficient flies can be suppressed by genetic inactivation of iPLA2-VIA, which prevents CL depletion and monolyso-CL accumulation without correcting the abnormal CL acyl composition, suggests that the abnormal levels of CL and/or monolyso-CL are important pathogenetic factors. Because a cardiolipin synthase mutant of yeast exhibits abnormal mitochondrial function (27), it is likely that the low CL content is critical in the molecular mechanism of Barth syndrome. Nevertheless, because Barth syndrome is a multisystem disorder, involvement of monolyso-CL accumulation and abnormal CL acyl composition may also play a role in certain tissues and organs.

Cardiolipin Remodeling and iPLA2-VIA. The mature acyl chain composition of CL is achieved through a remodeling process, which requires the action of tafazzin. We showed previously that tafazzin catalyzes phospholipid-lysophospholipid transacylation that involves both deacylation of a phospholipid such as CL and reacylation of a monolyso-phospholipid, such as monolyso-CL (2). Unlike the CoA-dependent deacylation-reacylation cycle (Lands cycle), in which a nascent phospholipid is deacylated by a phospholipase A to yield a free fatty acid and a lysophospholipid that is then reacylated by an acyl-CoA-dependent acyltransferase, transacylation does not require acyl-CoA, and proceeds directly by transferring a fatty acyl chain from a phospholipid to a lysophospholipid; no phospholipase is involved and no free fatty acid is generated in the process (6). Here, we found that, although the calcium-independent phospholipase A_2, iPLA2-VIA, is not required for CL remodeling, in the absence of tafazzin, i.e., in the Barth syndrome model, the enzyme plays a major role in the depletion of CL and the accumulation of monolyso-CL.

Our finding that the phenotypic features of tafazzin deficiency can be suppressed by inhibiting iPLA2-VIA activity identifies this enzyme as a potential target for therapeutic intervention in Barth syndrome. Indeed, we found that treatment of cultured lymphoblasts from Barth patients with the iPLA2 inhibitor BEL partially restores CL homeostasis. The calcium-independent iPLA2-VIA has been implicated in a variety of biological processes, including phospholipid remodeling, arachidonic acid release, apoptosis, and store-operated calcium entry (19). In addition, iPLA2-VIA knockout mice develop age-dependent neurological impairment (28, 29) and mutations in the iPLA2-VIA gene have been identified in patients with infantile neuroaxonal dystrophy and neurodegeneration with iron accumulation in the brain (30, 31). Therefore, a therapeutic approach to Barth syndrome based on the inhibition of iPLA2-VIA is likely to require either careful titration of the phospholipase inhibitor, or even its tissue-specific targeting.

Materials and Methods

***Drosophila* Strains and Human Lymphoblast Cell Lines.** The tafazzin-deficient mutant flies were described in ref. 7. The fly strain, y[1] w[67c23]; P{w[+mC] y[+mDint2] = EPgy2}CG6718[EY05103], that carries a P element insertion (EY05103) in the gene CG6718 and the 3rd chromosome *daughterless* (*da*)-GAL4 driver line were obtained from the Bloomington Drosophila Stock

Center. Lymphoblast cell lines of Barth syndrome patients and control subjects were described in ref. 32.

Molecular Biology. For transgenic rescue experiments, we subcloned the cDNA encoding N-terminally hemagglutinin (HA) epitope-tagged full-length tafazzin (PA) into the P element transformation vector pUAST. To identify precise excisions of the P element in EY05103, genomic DNAs were isolated from excision strains and the DNA segments including the site of the previous P element insertion were amplified by using the Expand Long Template PCR System from Roche, purified by agarose-gel electrophoresis, and analyzed by DNA sequencing. The relative amount of the CG6718 mRNA in flies was determined by RT-PCR with an internal control as follows. The total RNAs were purified from wild-type (w^{1118}) and homozygous EY05103 flies by using the High Pure RNA Isolation Kit from Roche and used to generate the oligo(dT)$_{18}$ primed first-strand cDNAs by using the Transcriptor First Strand cDNA Synthesis Kit from Roche. The first-strand cDNAs were used as PCR templates and 2 pairs of primers were present in each PCR: one (5′-TAC TGG AAT TGT GCG ATA AGG-3′/5′-GAT GTG GTA TTG GAA TCC GAG-3′) for amplifying the CG6718 transcripts and the other (5′-CAA CGA AGA CAC TGA TAG TG-3′/5′-GAG GTC AGC TTA AGG ATG TG-3′) for amplifying the transcript of an irrelevant gene (CG34133) as an internal control. The amounts of amplified CG6718 cDNA fragments (RA, 253 bp; RB, 391 bp; RC, 669 bp; RD, 576 bp) were normalized to the internal control fragment (819 bp) by staining with ethidium bromide after agarose-gel electrophoresis. The identities of the amplified cDNA fragments were confirmed by sequencing each fragment extracted from the agarose gel after electrophoresis.

***Drosophila* Genetics.** By using the standard *Drosophila* germ-line P element transformation technique, we generated multiple UAS-tafazzin transgenic lines, and those with the transgene mapped to the 3rd chromosome were used in the transgenic rescue experiments. For transgenic rescue of the tafazzin mutant phenotype, both UAS-tafazzin transgenic lines and the 3rd chromosome *da*-GAL4 driver line were further crossed into the tafazzin mutant background, respectively, by using standard *Drosophila* genetic techniques and appropriate fly strains. The precise P element (EY05103) excision strains were generated by using standard *Drosophila* genetic techniques and appropriate fly strains as described in ref. 7. Male fertility was assessed by placing 5 males with 5 virgin w^{1118} females in a culture vial for 6 days at 20 °C and then counting the F_1 progeny. At least 3 vials were set up and scored for each genotype tested.

CL and MLCL Analysis. To measure actual concentrations of CL and MLCL, we used HPLC with fluorescence detection. Lipids were extracted, derivatized with 1-naphthylacetic anhydride, and purified by solid-phase extraction as described (26, 33). The subsequent HPLC method was modified to include monolysocardiolipin in the analysis. Derivatized samples were injected via a 20-μL loop into a Hypersil ODS column (150 mm $\times$ 4.6 mm; particle size, 5 μm). A linear gradient was run from 100% acetonitrile to 100% 2-propanol in 100 min at a flow rate of 2 mL/min. Fluorescence was recorded at an excitation wavelength of 280 nm and an emission wavelength of 360 nm. We showed that MLCL gave the same fluorescence yield per nanomole as CL. Quantification was performed with the internal standard oleoyl-tristearoyl-CL, which was produced from bovine heart cardiolipin by catalytic hydrogenation (33).

Electron Microscopy. *Drosophila* testes were dissected and fixed in 2% glutaraldehyde followed by 2% osmium tetroxide, and processed for embedding in epoxy resin. Thin sections were examined in a JEOL LEM1200EXII transmission electron microscope operated at 80 kV.

BEL Treatment of Lymphoblast Cultures. The lymphoblast cell lines were cultured in RPMI medium 1640 containing heat-inactivated FBS (10%), penicillin (50 IU/mL), and streptomycin (50 μg/mL) at 37 °C under 5% CO_2 atmosphere. To inhibit $iPLA_2$ activity, bromoenol lactone (BEL, catalog no. B1552-5MG, Sigma, 10 mM stock solution in DMSO) was added to proliferating lymphoblast cultures (1×10^6 cells per mL) to the final concentration of 2.5 μM and the culture medium was changed every day with fresh medium containing BEL. The cells were harvested 48 h later by centrifugation and the cell pellets were stored at −70 °C for MLCL/CL analysis.

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