A Zebrafish Model of Human Barth Syndrome Reveals the Essential Role of Tafazzin in Cardiac Development and Function

Zaza Khuchua, Zou Yue, Lorene Batts, Arnold W. Strauss

Abstract—Barth syndrome is an X-linked disorder characterized by cardiomyopathy, skeletal myopathy, neutropenia, organic aciduria, and growth retardation caused by mutations in tafazzin. The sequence similarity of tafazzin to acyltransferases suggests a role in mitochondrial phospholipid metabolism. To study the role of tafazzin in heart function and development, we created a knockdown zebrafish model. Zebrafish tafazzin mRNA is first evident at 7 hours post-fertilization (hpf). At 10 and 24 hpf, tafazzin mRNA is ubiquitous, with highest levels in the head. By 51 hpf, expression becomes cardiac restricted. The tafazzin knockdown created by antisense morpholino yolk injection resulted in dose-dependent lethality, severe developmental and growth retardation, marked bradycardia and pericardial effusions, and generalized edema, signs that resemble human Barth syndrome heart failure. This knockdown phenotype was rescued by concomitant injection of normal tafazzin mRNA. Abnormal cardiac development, with a linear, nonlooped heart, and hypomorphic tail and eye development proves that tafazzin is essential for overall zebrafish development, especially of the heart. The tafazzin knockdown zebrafish provides an animal model similar to Barth syndrome to analyze the severity of human mutants and to test potential treatments. (Circ Res. 2006;99:201-208.)

Key Words: cardiac development ■ cardiomyopathy ■ membrane lipids ■ Barth syndrome ■ tafazzin ■ zebrafish

Mitochondria play an essential role in energy production, ion homeostasis, and apoptosis, processes mediated through the distinctive “double” phospholipid cardiolipin, which is abundant in highly oxidative tissues, including the heart. The importance of cardiolipin is emphasized by the recognition that tafazzin (Taz) shares sequence similarity to acyltransferases1–3 and that mitochondrial cardiolipin is deficient in patients with Barth syndrome (Mendelian Inheritance in Man [MIM] no. 302060).4 Mutations in Taz cause this X-linked genetic disorder characterized by infantile-onset skeletal myopathy, diminished linear growth, chronic fatigue, and organic aciduria.5–7 Mitochondrial abnormalities in Barth patients include aberrant cristae, formation of crystalline structures,8 diminished concentrations of cytochrome c, b, and aa3,9 and severely reduced activities of respiratory chain complexes,10,11 suggesting that Barth syndrome is a disorder of mitochondrial function. Recently, dramatic reductions in cardiolipin and accumulation of unusual medium chain and fully saturated acyl-cardiolipin species in Barth syndrome mitochondria were demonstrated, suggesting the hypothesis that Taz is required for cardiolipin remodeling.4,10,11

Cardiolipin is a dimeric phospholipid with 4 fatty acyl chains carrying 2 negative charges. It is uniquely localized to its site of synthesis in the mitochondrial inner membrane; interacts with a large number of mitochondrial enzyme complexes, including cytochrome c oxidase, the ATP/ADP transporter, and mitochondrial creatine kinase; is crucial for the localization, tertiary structure, and optimal activity of these complexes;12,13; and is required for permeability transition pore formation. Permutations of the 4 fatty acyl chains of cardiolipin produce many different molecular types,14 with species ranging from 14 to 20 carbons and various degrees of unsaturation. Following synthesis of cardiolipin, the fatty acyl chains are altered by deacylation and reacylation reactions, putatively catalyzed by a family of acyltransferases,2,15 a process termed remodeling. In eukaryotes, cardiolipin remodeling results in the unique and symmetric species linoleoyl (C18:2), carboxyl16 Cardiolipin remodeling biochemistry studies are just beginning.

This Taz–cardiolipin remodeling hypothesis is elegantly supported by data obtained from deletion of the Taz homologue in Saccharomyces cerevisiae,17,18 because similar alterations in mitochondrial function and cardiolipin biochemistry to those present in human Barth syndrome result. However, the precise biological role of Taz remains poorly understood.

Because no animal model of tafazzin deficiency was available, we created a knockdown of Taz in the zebrafish and report the phenotype, which does resemble human Barth syndrome.
Materials and Methods

Reagents
Reagents were purchased from Sigma, unless otherwise noted.

Fish Care and Maintenance
Zebrafish were raised and handled as described.19 5'/H11032-RACE (5'/H11032-rapid amplification of cDNA ends) was performed using 1/H9262 g of total RNA isolated from 24-hour zebrafish embryos19 and a SMART RACE cDNA Amplification kit, (BD Biosciences). The 5'/H11032-ends were amplified from first-strand cDNA by PCR (5 cycles: 94°C/30 seconds, 72°C/120 seconds; 5 cycles: 94°C/30 seconds, 70°C/30 seconds, 72°C/120 seconds; 25 cycles: 94°C/30 seconds, 68°C/30 seconds, 72°C/120 seconds), using an exon 9 Taz-specific antisense primer (5'/H11032-TCGAGGAATGTATGGAGTCTCATTTGG-3') and Universal Primer Mix. PCR products were subcloned (pCRII vector [Invitrogen]) and sequenced.

Taz Antisense Morpholino Design and Injection
We designed 3 different 25-mer morpholino oligonucleotides (Gene Tools LLC) complementary to the 5'/H11032-most translational start codon and flanking sequences of zebrafish Taz mRNA (Taz mRNA antisense MO [asMO]: 5'/H11032-CATGTCACCTCCAAGGGCATCGCCA-3'); the exon 3/intron 3 splice donor site of the fish Taz gene (E3 asMO: 5'/H11032-CCTTCACTAACTCACCATCTCATCT-3'); or the exon 4/intron 4 splice donor site (E4 asMO: 5'/H11032-TAGATAAAGTTACCTCTAACAACAACAG-3') (Figure 1c). As a negative control, embryos were injected with control asMO (5'/H11032-CCTCTTACCTCAGTTACAATTTATA). 20 Morpholino oligonucleotide stocks (3 mmol/L) were diluted to 0.3 to 1.2 mmol/L in Danieau solution (58 mmol/L NaCl, 0.7 mmol/L KCl, 0.4 mmol/L MgSO4, 0.6 mmol/L Ca(NO3)2, 5 mmol/L HEPES, pH 7.6) before injection into the yolks of freshly fertilized fish eggs. Approximately 1 nL of morpholino oligonucleotides was injected into 40 to 180 embryos at the 1- to 4-cell stage, and the embryos were allowed to develop at 29°C. Phenotypes were scored at various hours post-fertilization (hpf) using a stereomicroscope and the following criteria: unaffected embryos were defined as being indistinguishable from noninjected embryos and affected embryos had obvious cardiac (generalized edema, dysmorphic heart) abnormalities, decreased growth (i.e., were hypomorphic), delayed development, and ultimately died.

Figure 1. Deduced protein sequences and genomic structures of tafazzins. a, Sequence comparison among predicted open-reading frames of human, mouse, and zebrafish tafazzins. Sequence identities are shown with a black background, and conserved substitutions are highlighted in gray. Human and zebrafish Taz proteins share 60.3% amino acid identity and 70.2% similarity. Corresponding exons for human Taz are depicted. The sequence corresponding to exon 5 in human Taz is absent in mouse and zebrafish homologs. The conserved G197 often mutated in Barth syndrome is boxed. Solid, black diamonds depict missense mutations in Barth patients (http://www.barthsyndrome.org).3 29 The arrowhead indicates the alternative exon 3 translational start methionine. Solid black bars depict domains conserved among acyltransferases. b, Genomic structures of human and zebrafish tafazzins. asMO-binding sequences at the bottom have coding sequences capitalized. “*Altered” indicates the 7/25 nucleotide altered Taz mRNA MO binding site used in Taz mRNA rescue experiments. c, cDNA sequences corresponding to zebrafish Taz exons 1 to 4 encoding N-terminal full-length and ∆ex 2 to 3 variant Taz with predicted amino acid sequences (coding nucleotides capitalized). Translation start codons for both full-length and ∆ex 2 to 3 tafazzins are underlined. The predicted mitochondrial targeting sequence of full-length Taz is boxed (mitochondrial targeting sequence and cleavage site were predicted using MitoProt server at http://mips.gsf.de/cgi-bin/proj/medgen/mitofilter).
or/and shortened tails. The number of dead embryos was determined by subtracting the number of unaffected and affected embryos from the total number of injected embryos.

Whole-Mount In Situ Hybridization
Taz cDNA was subcloned into the pCRII dual promoter vector for both the production of in vitro synthesized transcripts for “rescue” experiments and generation of RNA probes for in situ hybridization. Cardiac myosin light chain (cmlc) cDNA clone was from Dr T. Zhong (Vanderbilt University). Antisense mRNA probes for Taz and cmlc were transcribed from linearized cDNAs using T7 RNA polymerase (Ambion) and digoxigenin-11-UTP (Roche). Sense control probes were transcribed in the opposite direction with SP6 RNA polymerase. For in situ hybridization, embryos were fixed overnight in 4% paraformaldehyde at 4°C. In situ hybridization was performed at 65°C overnight. Anti-digoxigenin alkaline phosphatase–conjugated antibodies were used for detection. Washes, antibody incubations, and color development were done as described.21

Phenotypic Rescue With Zebrafish Taz mRNAs
cDNA clones in the pCRII vector containing the full-length coding sequences of normal (wild-type) and G197R mutant zebrafish Taz mRNAs were used as templates for RNA synthesis. Mutations were created using the QuickChange site-directed mutagenesis kit (Stratagene). mRNAs were synthesized using T7 RNA polymerase and the Message Machine transcription kit (Ambion). For rescue experiments, 2.5 ng of mRNA encoding normal or G197R mutant Taz mRNAs was injected into 4- to 16-cell-stage embryos initially (10 to 15 minutes earlier) injected with Taz mRNA specific asMO.

Results
To assess Taz function and its role in cardiac development and contractility, we explored the feasibility of creating animal models of human Barth syndrome. Using genomic databases, we, and others,3 determined that Taz amino acid sequences are highly conserved across species (Figure 1a). The Danio rerio Taz gene (Figure 1b) contains only 10 exons because the zebrafish genome lacks the exon corresponding to human Taz exon 5. We assigned exon numbers (exons 1 to 4, 6 to 11) corresponding to human Taz exons (Figure 1b). 5’-RACE of total RNA from 24-hour zebrafish embryos revealed 2 Taz transcripts with open-reading frames (not shown). The first, full-length mRNA contains sequences from all 10 exons with translation initiated from the most 5’ AUG codon in exon 1. The second, alternatively spliced, truncated form (Δex 2 to 3 or “short” Taz mRNA), ~10% of the total, lacks sequences corresponding to exons 2 and 3 (Figure 1a and 1c). This short Taz mRNA retains an open-reading frame encoding a 198 amino acid (aa) Taz splice variant that differs from the full-length form because the 31 N-terminal residues are derived from a different initiator methionine in a different exon 1 reading frame from the normal, longer form (Figure 1a and 1c). The biological role of Δex 2 to 3, short Taz mRNA is unclear, because alteration of the N-terminal peptide would likely disrupt mitochondrial localization and direct this form to a different subcellular localization. Like human tafazzin,22 the zebrafish Taz gene contains a putative alternative start codon in exon 3, raising the possibility that additional variant Taz mRNAs might exist. However, none of these exon 3-initiated Taz mRNA forms was detected by our 5’-RACE study, suggesting that this alternative start site is not used in developing zebrafish.

Temporal and Spatial Patterns of Tafazzin mRNA Expression in Developing Zebrafish Embryos
To elucidate the temporal and spatial patterns of Taz expression in a developmental-, cell-, and tissue-specific manner, we performed quantitative RT-PCR and in situ hybridization in zebrafish at different developmental stages (Figure 2a). Before gastrulation, at approximately the 2000-cell stage (3.3 hpf), Taz expression is barely detectable. At the 75% epiboly stage of the gastrula (7 hpf), Taz is sharply upregulated, and high-level expression continues throughout the segmentation (12 hpf) and pharyngula (24 to 30 hpf) stages. Developmental stages between 75% epiboly (7 hpf) and the pharyngula period (24 to 30 hpf) are characterized by migration of cardiac progenitor cells from the ventral and lateral regions of blastula toward the future hindbrain, formation of primitive myocardial tubes, and subsequent fusion into the definitive heart tube with cardiac pumping.23,24 At the hatching period (51 hpf), Taz mRNA expression declines dramatically. Drastic changes in Taz gene expression in these critical stages of zebrafish development are consistent with an essential role of Taz for overall development, and high-level expression coincides with cardiac organogenesis and development.

To study the spatial and tissue-specific patterns of Taz expression, we performed whole-mount in situ hybridization of zebrafish embryos at different stages of development with a Taz-specific 755 nucleotide–long antisense (as) RNA probe. Sense, control hybridizations showed no detectable labeling (not shown). At 10 hpf, Taz is expressed ubiquitously, including in cardiac progenitor regions, but the strongest expression is observed in the head area (Figure 2d and 2e). By 24 hpf, Taz is highly expressed in the head, eyes, and tail (Figure 2f). By 30 hpf, expression becomes more restricted to the head, heart, eyes, and the region next to the yolk, corresponding to endodermal tissue (Figure 2g and 2h). Only by 51 hpf does Taz mRNA expression become highly restricted to the heart (Figure 2i and 2j). This highly regulated temporal and spatial specific pattern of Taz expression suggests that Taz might be essential throughout zebrafish development.

mRNA-Specific, Antisense Morpholino Oligonucleotide–Mediated Knockdown of Taz in Zebrafish Embryos
To elucidate the role of Taz in development, we blocked Taz mRNA translation or expression in zebrafish. The zebrafish is an ideal model organism for investigation of perturbation of normal cardiac developmental programs.25 The embryonic heart of zebrafish resembles that of a human embryo at approximately 3 weeks of gestation, being divided into atrial and ventricular chambers.26 Cardiac beating commences as a peristaltic wave at 22 hpf, and the heart loops and develops coordinated contractions by 36 hpf.25 Zebrafish are not dependent on circulation for survival at these early stages of embryogenesis, and perturbations in heart development do not result in immediate death of embryos.25

We used the antisense morpholino oligonucleotide (asMO)-mediated knockdown approach to block Taz gene function.27,28 First, we used an asMO specific to the first translation initiation site and flanking sequences of Taz
mRNA (Figure 1b and 1c). This asMO is designed to transiently block Taz mRNA translation. Microinjection of this asMO produced a highly specific, dose-dependent phenotype. After 51 hours, embryos injected with 0.3 or 0.6 pmol of Taz mRNA asMO were morphologically identical to those injected with a control MO (data not shown). However, injection with 0.9 pmol of asMO resulted in severe developmental abnormalities. In these affected embryos, termed “morphants,” the most severely affected structures were the heart and tail (Figure 3b). Some eye abnormalities were observed. Morphant embryos at 51 hpf developed marked edema with large pericardial effusions; dysmorphic, slowly beating hearts; shortened curved tails (Figure 3a and 3b); and absent blood circulation (Movie in the online data supplement, available at http://circres.ahajournals.org). The cardiac abnormalities are obvious in whole-mount embryos stained with a cmlc-specific antisense mRNA probe (Figure 3c and 3d), in which the heart is linear, without looping. Wild-type embryos at 51 hpf have a fully formed two-chamber heart (Figure 3c and 3c'). In contrast, Taz morphants at 51 hpf have a highly dysmorphic, tube-shaped heart (Figure 3d and 3d').

The Taz mRNA asMO also evoked dose-dependent bradycardia (Figure 3e) (supplemental Movie). Injection of 0.9 and 1.2 ng of asMO caused markedly slower heart rates, from 142±10.2 to 38±1.4 bpm at 29°C (P<0.01). Even 0.6 ng of asMO caused a statistically significant reduction in heart rate (Figure 3e) but did not cause aberrant cardiac morphology. asMO injection also caused a dramatic, dose-dependent increase in embryonic lethality, from 21% to 22% in control MO-injected embryos to 48% (P<0.05) (0.9 pmol; supplemental Table I) or 68% (1.2 pmol; supplemental Table II) in asMO-injected embryos. Together, these results show that Taz is essential for zebrafish embryonic survival and development.

**Phenotypic Rescue**

To ensure the specificity of the effect of Taz asMO on morphogenesis, bradycardia, and mortality, phenotypic rescue experiments were performed (supplemental Table II). Freshly fertilized eggs were coinjected with 1.2 pmol of Taz mRNA antisense MO and 2.5 ng (approximately 0.01 pmole) of capped full-length (789 nt) synthetic Taz mRNA. To avoid the sequestering of the asMOs by injected exogenous Taz mRNA, the morpholino binding site in “normal” Taz mRNA was altered at 7/25 nucleotides with changes that did not alter codons (Figure 1c). As a control, injection of normal Taz mRNA alone in embryos did not produce any changes in embryonic heart rate, morphology, or survival rate (data not shown). Injection of 2.5 ng of Taz mRNA into the embryos previously injected with 1.2 pmol of Taz mRNA antisense MO significantly increased morphant survival at 24 hpf, from 68% mortality with asMO injections alone to 29% mortality with asMO and normal Taz mRNA (supplemental Table II). In addition, rescue with normal Taz mRNA maintained the heart rate at 82% of normal at 29 hpf (P<0.01) (Figure 3e).
This rescue by exogenous supplementation with Taz mRNA proves that the observed asMO phenotype was solely attributable to the Taz knockdown and was not associated with nonspecific toxicity of the asMO.

Pre-mRNA Morpholinos

It has been proposed that the human Taz gene contains 2 alternative 5\textquotesingle ends secondary to differing translational initiation sites,\textsuperscript{22} although more recent data are not consistent with this hypothesis.\textsuperscript{3} The zebrafish Taz gene alternative start codon is conserved, raising the possibility that truncated protein generation from the alternative in-frame AUG in our initial asMO morphants might partially mask the Taz knockdown phenotype. To address this, we generated MOs designed to target genomic exon/intron junction sites and block pre-mRNA splicing. Splice-blocking MOs have the advan-
tages that the efficacy of gene knockdown can be quantified by RT-PCR or Northern blot analysis and that these asMOs specifically target newly transcribed, zygotically, and not maternal transcripts. Two different such splice-site, 25-mer MOs complementary to the exon 3 or exon 4 splice–donor sites (designated E3 or E4, respectively; Figure 1b) were used.

First, we determined whether injection of the splice–targeted MOs altered splicing of the Taz gene. By RT-PCR, injection of E3 asMO into freshly fertilized embryos caused a marked decrease in overall Taz mRNA. Moreover, the altered mRNA size (from 448 bp normally to 300 bp in the E3 asMO) suggested mRNA missplicing (Figure 3h). DNA sequence analysis revealed that this shorter, misspliced mRNA variant lacks exons 2 and 3 and, therefore, is similar to the short Taz mRNA in humans. In contrast, E4 asMO injection resulted in absence of any detectable Taz mRNA in morphants, a complete knockout of Taz expression.

Microinjection of E3 MO into freshly fertilized embryos produced phenotypic characteristics similar those observed for the Taz mRNA asMO. However, the E3 asMO is more potent because injection of 0.3 pmol gave rise to embryos with severely dysmorphic hearts (Figure 3i) (supplemental Movie). E4 asMO injection led to an even more obvious phenotype, with more than 50% embryos dead after the gastrulation stage. Moreover, >35% of surviving embryos were severely dysmorphic, with absent heart and head (supplemental Table II; Figure 3j). Thus, block of Taz embryonic gene expression with exon 3 or 4 splice junction asMOs emphasizes that Taz is essential for zebrafish development.

**Partial Phenotypic Rescue With a Human Barth Syndrome Mutant mRNA**

Human Barth syndrome is caused by more than 90 different mutations in the human Taz gene that are clustered in exons 1 to 3 and 6 to 10 and some adjacent consensus splice site sequences (Figure 1a). The glycine 197 codon in human Taz exon 8 is a “hot spot” because several known mutations alter this codon. G197 (residue 165 in fish Taz) is absolutely conserved among species, suggesting functional importance (Figure 1a). To elucidate whether this Taz mutant is capable of rescuing the Danio morphant phenotype, we coinjected 1.2 pmol of Taz mRNA asMO with 2.5 ng of full-length zebrafish mRNA encoding the G197R mutant tafazzin. The G197R mutant mRNA rescued the lethal phenotype of Taz mRNA–injected morphants at 30 hpf. The number of unaffected embryos was significantly lower in G197R mRNA–injected morphants (26%) than in the wild-type mRNA–injected group (50%) (P < 0.05). One third of embryos rescued with the G197R mutant mRNA had characteristic generalized edema and an enlarged heart (supplemental Table II; Figure 3g), but the overall morphology of G197R rescued embryos was less affected than morphants injected only with asMO. Most of the G197R-injected embryos displaying an aberrant cardiac phenotype had normal tails, and the heart rate of G197R-rescued morphants was 108 ± 8.6, not significantly different from wild-type mRNA–rescued morphants (116 ± 5.7). Thus, the G197R embryos, like their human Barth syndrome counterparts, were abnormal but did survive gestation. Mutant G197R Taz mRNA was sufficiently active to prevent both bradycardia and aberrant tail development, consistent with the previous observations that these phenotypes are more sensitive to Taz knockdown (Figure 3).

This experiment also demonstrates that our zebrafish asMO Taz knockdown model can be used to assess the severity of human Taz mutants in a semiquantitative fashion. This rescue assay will be helpful in studying designed mutants altering potential Taz functional domains.

**Discussion**

Although human Barth syndrome is caused by mutations in tafazzin, and Barth patients exhibit abnormalities in cardiolipin and mitochondrial function, the precise enzymatic or biological function(s) of Taz in vertebrates and the developmental-, cell-, and tissue-specific patterns of Taz expression remain unknown. However, high degree of sequence similarity of Taz to other known phospholipid acyltransferases, including conservation of key motifs common to several categories of acyltransferases, raises the possibility that Taz has this role in mitochondrial phospholipid or cardiolipin remodeling (Figure 4).

We used a vertebrate model of Taz deficiency, in zebrafish, to begin to answer these important questions. Our studies provide several key conclusions. First, Taz is required for normal development of zebrafish, including cardiac, tail, and eye development. We believe this is the first proof of this critical role of Taz. Second, the Taz knockdown in zebrafish provides a phenotype that resembles human Barth syndrome, with abnormal cardiac function; bradycardia, edema and pericardial effusions consistent with heart failure; and aberrant tail/skeletal muscle development. The human Barth syndrome mutation, G197R, partially rescued this phenotype. Third, this latter result and the ability to rescue the zebrafish Taz knockdown with normal mRNA show that this model system can be used to study Taz functionality.

The use of whole-mount in situ hybridization with Taz-specific antisense RNA probes and the optical clarity of the zebrafish embryo allowed us to investigate the temporal and spatial patterns of Taz expression for the first time in a vertebrate. Taz is ubiquitously expressed at a high level in early stages (10 to 30 hpf) of development, a somewhat surprising result in view of the focus on Taz deficiency in skeletal and cardiac muscle in human Barth syndrome. These expression studies suggested that Taz would be essential for normal zebrafish development of many tissues and organs. Using asMO oligonucleotide-mediated inhibition of Taz protein expression through inhibition of Taz mRNA translation, we, in fact, confirmed this prediction, demonstrating an essential role of Taz gene products in normal zebrafish development. This key finding suggests that Taz may also play an important role in human development. If this were true, many human males with severe Taz mutations would be predicted to die in utero, perhaps consistent with the rarity of human Barth syndrome.

Our expression experiments revealed that Taz is present in the developing heart throughout zebrafish embryogenesis and becomes restricted to the heart late in gestation. Video recording of live embryos revealed that the heart is the most obviously affected organ in asMO-generated Taz morphants. Poor cardiac contractility is evident by 30 hpf and is followed by alteration of
cardiac anatomy and deterioration of cardiac function by day 3. No blood flow is visible in most of the affected morphants. In morphant embryos, reduced cardiac contractility produced chamber enlargement consistent with heart failure. Most affected morphants displayed poorly developed, curved tails.

Splice-site–specific, asMO-mediated knockdown, an approach that blocks Taz gene transcription and/or precursor mRNA processing, was more potent in evoking phenotypes than the Taz mRNA specific asMO. The injection of E3 asMO caused splicing out of Taz mRNA exons 2 and 3. This missplicing creates a frame shift in the 5′-H11032-coding sequence and could result in expression of a truncated form of Taz that lacks the 95 aa–long hydrophobic N-terminal domain (Figure 1). It is conceivable that this leads to disruption of mitochondrial localization and/or proper folding of truncated protein product. However, residues 96 to 262 of 5′-H9004 ex 2 to 3 Taz are identical to that segment of full-length Taz and may retain partial enzymatic activity (Figure 1a). Although E3 asMO-mediated knockdown produces an obvious phenotype, most embryos are still viable at 51 hours, a milder phenotype than asMO morphants. These embryos develop dysmorphic hearts and tails, but the heads and eyes are largely unaffected (Figure 3i). It is conceivable that truncated 5′-H9004 ex 2 to 3 Taz that lacks an N-terminal stretch of hydrophobic residues and is expected to function as a membrane anchor may partially retain its biological function. In contrast, the E4 asMO completely abolishes Taz mRNA expression and causes a very severe developmental phenotype. At 51 hpf, affected embryos lack heart, eyes, and head; and embryogenesis is arrested at the 13-somite stage (Figure 3j), consistent with Taz being absolutely crucial for normal development.

Perhaps our most helpful finding is that the knockdown phenotype can be rescued by simultaneous injection of normal zebrafish Taz mRNA into embryos. This experiment demonstrates the specificity of the asMO approach, showing that Taz mRNA can efficiently reverse all of the abnormalities produced in the asMO knockdown embryos. In contrast, a mutant mRNA, encoding the common G197R human Barth Taz mutant, was less effective in rescuing the zebrafish knockdown phenotype, as the developing embryos still displayed signs of heart failure and through 2h) that is rich in mitochondria.31 This location suggests a potential role of Taz in mediating nutrient uptake from the yolk or in energy generation using these nutrients. Because the progeny of endodermal stem cells contribute to liver, swim bladder, intestine, stomach, pancreas, esophagus, and pharynx organogenesis,34 disruption of mitochondrial energy production by abolishing Taz expression in this area may detrimentally affect overall embryonic development.

Our second major conclusion is that the zebrafish Taz knockdown provides a useful animal model partially resembling human Barth syndrome, a disorder primarily manifested by cardiomyopathy and skeletal myopathy with weakness, as well as neutropenia and growth abnormalities. The asMO knockdown phenotype includes aberrant, hypomorphic tail skeletal muscle development, consistent with a key role of Taz in skeletal muscle function. The cardiac manifestations include bradycardia, arrested heart development with a linear, nonlooped heart, and development of edema and pericardial effusions. Barth patients do have severe heart failure with left ventricular noncompaction, a known phenotype of abnormal myocardial development, and dilated cardiomyopathy, often causing death early in life, superficially similar to abnormal embryonic cardiac function of the fish.

Figure 4. Alignment of zebrafish Taz (AY576995) with murine acyl–coenzyme A (acyl-CoA):lysocardioliin acyltransferase (ALCAT1, XM_128781) and human acyl-glycerophosphate acyltransferase-1 (AGPAT1, Q99943). Numbering corresponds to the zebrafish Taz sequence. Solid diamonds indicate fish Taz amino acids corresponding to those mutated in Barth patients. Conserved amino acids are shown with a black background. Conserved motifs are noted.

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cardiac dysfunction. Glycine 197 is absolutely conserved from yeast to humans and is located within a highly conserved domain, suggesting its importance for enzymatic functionality. The effects of this mutant mRNA rescue were easily measured, as hundreds of embryos were injected, allowing statistically significant quantification of mortality and morbidity, including correction of heart rate in comparison to complete Taz knockdown fish. Thus, using this mutant rescue approach and until an adequate in vitro Taz enzyme assay is developed, we will be able to assess the severity of various Barth syndrome mutations in an in vivo assay system. More importantly, this in vivo Taz assay provides a useful tool to examine the effects of designed Taz mutations on Taz functionality. That is, we can create mutations in amino acid residues predicted by sequence similarity comparisons as essential for the postulated Taz acyltransferase activity and test the mutants for effectiveness in rescue, thus determining whether or not the proposed function is correct.

In conclusion, morpholino-mediated knockdown of Taz in zebrafish demonstrates that tafazzin is essential for normal heart development. This strategy, in conjunction with mRNA rescue, has enabled us to create a model similar to human Barth syndrome in zebrafish that will be helpful in defining and predicting effects of Barth mutations on Taz functionality.

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Disclosures
None.

References
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### Supplementary Table 1. Phenotypic responses to Taz morpholinos at 51 hpf.

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<th>Unaffected, %</th>
<th>Affected, %</th>
<th>Dead, %</th>
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<td>Control MO, 0.9 pmol/embryo</td>
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<td>71.9 ± 5.4</td>
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<td>E4 asMO, 0.3 pmol/embryo</td>
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<td>13.4 ± 7.7^A</td>
<td>34.9 ± 7.2^B</td>
<td>51.8 ± 11.0^B</td>
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n – Total number of injected embryos. Values are the mean ± standard error. Significance of differences in comparison with control asMO injected embryos (A - P<0.01, B - P<0.05).

### Supplementary Table 2. Phenotypic rescue of Taz mRNA morpholino phenotypes by coinjection of WT or G197R mutant Taz mRNA at 24 hpf.

<table>
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<th>Morpholino</th>
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<th>Affected, %</th>
<th>Dead, %</th>
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<td>Rescue with G197R mRNA</td>
<td>71</td>
<td>26.1 ± 5.1^A,D,E</td>
<td>32.5 ± 1.6^B,D</td>
<td>41.4 ± 5.5^B,D</td>
</tr>
</tbody>
</table>

n – Total number of injected embryos. Values are the mean ± standard error. Significance of differences in comparison with: Control asMO injected embryos (A - P<0.01, B - P<0.05), Taz mRNA asMO injected embryos (C - P<0.01, D - P<0.05) and Taz WT mRNA rescued embryos (E - P<0.01).