

# Experimental models of Barth syndrome

William T. Pu<sup>1,2</sup> 

<sup>1</sup>Department of Cardiology, Boston Children's Hospital, Boston, Massachusetts, USA

<sup>2</sup>Harvard Stem Cell Institute, Harvard University, Cambridge, Massachusetts, USA

## Correspondence

William T. Pu, Department of Cardiology, Boston Children's Hospital, 300 Longwood Ave., Boston, MA 02115, USA.  
Email: william.pu@cardio.chboston.org

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## Abstract

Mutation of the gene *Tafazzin* (*TAZ*) causes Barth syndrome, an X-linked disorder characterized by cardiomyopathy, skeletal muscle weakness, and neutropenia. *TAZ* is an acyltransferase that catalyzes the remodeling of cardiolipin, the signature phospholipid of the inner mitochondrial membrane. Here, we review the major model systems that have been established to study the role of cardiolipin remodeling in mitochondrial function and the pathogenesis of Barth syndrome. We summarize key features of each model and provide examples of how each has contributed to advance our understanding of *TAZ* function and Barth syndrome pathophysiology.

## KEYWORDS

Barth syndrome, cardiolipin, disease model, tafazzin

## 1 | INTRODUCTION

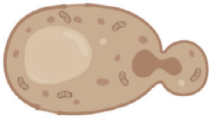




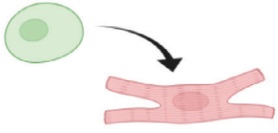
Mutation of the gene *Tafazzin* (*TAZ*) causes Barth syndrome.<sup>1</sup> *TAZ* is an acyltransferase required for the remodeling of cardiolipin (CL), a hallmark phospholipid of the mitochondrial inner membrane. In normal tissues with high metabolic demand, the four acyl chains of CL have a narrow, characteristic composition (eg, tetralinoleoyl-CL in striated muscles). Because enzymes in its synthetic pathway lack acyl chain specificity, de novo synthesized CL has greater acyl chain diversity and tends to be more saturated. Remodeling CL into its mature form involves exchange of acyl chains, through phospholipase-catalyzed removal of one acyl chain to form monolysocardiolipin (MLCL) followed by reacylation, or through transacylation, both catalyzed by *TAZ*.<sup>2,3</sup> Patients lacking functional *TAZ* have decreased total CL, increased CL saturation and diversity of acyl chains, and increased MLCL to CL ratio.<sup>2,4</sup> These abnormalities of CL composition impair the function of proteins of the inner mitochondrial membrane, including F1F0 ATPase, components of the electron transport chain, which collectively lead to the

manifestations of Barth syndrome. While *TAZ* is ubiquitously expressed and CL abnormalities in Barth syndrome are widespread, the cardinal disease manifestations—cardiomyopathy, skeletal myopathy, and neutropenia<sup>5,6</sup>—are tissue restricted. The precise mechanisms by which mutation of *TAZ* and impaired CL biosynthesis lead to these tissue restricted phenotypes remain imprecisely defined.

Although CL composition varies between species, the steps of CL biogenesis and its impairment by *TAZ* mutation are well conserved throughout eukaryotes, from yeast through humans. To improve biological understanding of *TAZ* function and Barth Syndrome pathogenesis, and to expedite development of effective therapies, diverse model systems have been established to study the consequences of *TAZ* mutation. Here we review these major model systems and highlight important findings (Figure 1).

## 2 | YEAST

*Saccharomyces cerevisiae*, baker's yeast, are eukaryotic cells that are readily cultured and genetically

Model	Advantages/Disadvantages	Barth Phenotypes
 Yeast	<ul style="list-style-type: none"> <li>• inexpensive, fast</li> <li>• well characterized CL biogenesis and metabolic pathways</li> <li>• powerful genetic tools</li> <li>• rich literature</li> </ul> <hr/> <ul style="list-style-type: none"> <li>• evolutionary distance</li> <li>• unicellular organism</li> </ul>	<ul style="list-style-type: none"> <li>• abnormal CL composition</li> <li>• growth on non-fermentable substrates</li> </ul>
 Fly	<ul style="list-style-type: none"> <li>• inexpensive, relatively fast</li> <li>• powerful genetic tools</li> </ul> <hr/> <ul style="list-style-type: none"> <li>• evolutionary distance</li> <li>• lack of heart tube phenotype</li> <li>• relatively understudied</li> </ul>	<ul style="list-style-type: none"> <li>• abnormal CL composition</li> <li>• male sterility</li> <li>• decreased climbing and flight endurance</li> </ul>
 Zebrafish	<ul style="list-style-type: none"> <li>• potential for genetic or small molecule screens</li> </ul> <hr/> <ul style="list-style-type: none"> <li>• understudied</li> <li>• no reported genetic mutant</li> </ul>	<ul style="list-style-type: none"> <li>• abnormal CL composition</li> <li>• embryonic lethality; reduced heart rate and contractility; abnormal cardiac looping</li> </ul>
 Mouse - Taz-KD	<ul style="list-style-type: none"> <li>• mammal</li> <li>• cardiolipin defects similar to patients</li> </ul> <hr/> <ul style="list-style-type: none"> <li>• residual <i>Taz</i> mRNA and TAZ protein</li> <li>• greater phenotypic variability</li> <li>• relatively mild and late onset phenotypes</li> <li>• synthetic phenotypes not observed in genetic mutant</li> </ul>	<ul style="list-style-type: none"> <li>• abnormal CL composition</li> <li>• mild cardiac dysfunction</li> <li>• reduced endurance</li> <li>• depending on Dox dose, embryonic lethality with LV non-compaction</li> </ul>
 Mouse - Taz-KO	<ul style="list-style-type: none"> <li>• mammal</li> <li>• cardiolipin defects similar to patients</li> <li>• no residual TAZ protein</li> <li>• recapitulate most phenotypes of Barth patients</li> <li>• potential for tissue specific gene inactivation</li> </ul> <hr/> <ul style="list-style-type: none"> <li>• need to account for perinatal lethality and strain dependence in experimental design</li> </ul>	<ul style="list-style-type: none"> <li>• abnormal CL composition</li> <li>• progressive, severe cardiac dysfunction</li> <li>• reduced endurance</li> <li>• growth retardation</li> <li>• perinatal lethality (of <i>Taz</i><sup>null/Y</sup> in C57BL6/J)</li> <li>• reduced neutrophil count</li> <li>• phenotypes depend on strain</li> </ul>
 Human iPSC, iPSC-CM	<ul style="list-style-type: none"> <li>• human</li> <li>• potential for patient-specific models</li> <li>• cell culture model -- accessible for interventions and measurements</li> <li>• model cardiac-specific effects in iPSC-CMs</li> </ul> <hr/> <ul style="list-style-type: none"> <li>• iPSC-CMs are immature</li> <li>• culture is relatively expensive, slow, and labor intensive</li> <li>• skeletal muscle model has not been reported.</li> </ul>	<ul style="list-style-type: none"> <li>• abnormal CL composition</li> <li>• abnormal mitochondrial function</li> <li>• excessive mitochondrial ROS</li> <li>• impaired sarcomerogenesis</li> <li>• reduced force generation</li> <li>• abnormal Ca<sup>2+</sup> transients</li> </ul>

**FIGURE 1** Experimental models of Barth syndrome. The figure lists major model systems, some advantages or disadvantages of each model system, and key phenotypes reported. Images from biorender.com

manipulated. Powerful genetic and biochemical tools have been developed to study yeast. These features, combined with their relatively low cost and short experimental timelines, make yeast a superb model system to study gene functions that are highly conserved and manifest in unicellular organisms. At the same time, yeast cannot model aspects of gene function that are less conserved or selectively expressed in multicellular organisms, and it has limited utility as a preclinical model to test candidate therapies.

Yeast lacking *taz1*, the yeast homolog of *TAZ*, were unable to grow on nonfermentable carbon sources at elevated temperature.<sup>7,8</sup> Like human *TAZ* mutant cells, yeast *taz1* mutants had reduced overall CL, reduced unsaturated acyl chains, and elevated MLCL. Human *TAZ* complemented the yeast *taz1* mutation, indicating functional conservation.<sup>8</sup> These features make yeast a highly tractable model system to study the function of *TAZ* and CL.

Several key findings have been made in the yeast system. In yeast, the phospholipase that catalyzes CL deacylation to MLCL is CLD1<sup>9</sup>; the corresponding enzyme(s) in mammals have not been identified. *Cld1* deletion blocks CL remodeling such that de novo synthesized, saturated CL predominates. Remarkably, *Cld1* deletion rescued the growth defect of  $\Delta$ *taz1* yeast.<sup>10,11</sup> Changes in MLCL/CL and total CL level, but not CL saturation, mirrored the severity of the growth defect: compared to wild-type,  $\Delta$ *taz1* had low overall CL level, reduced unsaturation, high MLCL/CL, and impaired growth, whereas  $\Delta$ *cld1* $\Delta$ *taz1* had normal overall CL level, reduced unsaturation, low MLCL/CL, and normal growth. These data suggest that saturated CL can fulfill many functions of unsaturated CL, and that elevated MLCL/CL or reduced total CL cause the deleterious effects of *TAZ* mutation. Studies of yeast with mutations in CL biosynthesis genes also implicated CL in the regulation of mitochondrial iron homeostasis and the iron-sulfur cluster biogenesis,<sup>12,13</sup> intermediary metabolism including the TCA cycle,<sup>14,15</sup> and to increased oxidative stress,<sup>16</sup> which may contribute to Barth syndrome pathophysiology in humans and mice (see below). Genetic screens in yeast have identified genetic modifiers of *taz1* mutation. A synthetic screen for genes whose inactivation results in growth deficiency in combination with *taz1* mutation identified Yme1, a mitochondrial quality control protease<sup>17</sup> On the other hand, screens for *taz1* suppressors identified Odc1p, a conserved carrier of  $\alpha$ -ketoglutarate and other Krebs cycle intermediates located in the mitochondrial inner membrane,<sup>18</sup> and cycloheximide, a protein synthesis inhibitor.<sup>19</sup> It will be interesting to determine if these genes and pathways also modify *TAZ* deficiency in mammalian models.

The yeast system has also been useful to functionally assess *TAZ* splice isoforms and human *TAZ* mutations. Interestingly, of several human *TAZ* splice isoforms tested, only one, lacking the primate-specific exon 5, complemented, indicating that only this isoform possesses conserved acyltransferase activity in yeast.<sup>8</sup> Yeast have also been used to dissect the pathogenic mechanism of human mutations. Based on genetic complementation and biochemistry, 21 conserved human missense mutants were classified into those that affect *TAZ* catalytic activity, localization, and stability.<sup>20-22</sup>

### 3 | FRUIT FLY

*Drosophila melanogaster* is a powerful invertebrate system that has been employed to investigate the pathobiology of *Taz* mutation. Like yeast, advanced genetic approaches and reagents have been developed to dissect pathogenic mechanisms that are conserved between humans and flies. Flies also have specialized cell types, including muscle cells and a rudimentary heart tube, that are relevant to Barth syndrome, and have matured as a model to study diseases of these organ systems.<sup>23,24</sup> However, because of the evolutionary distance between flies and humans, flies are an imperfect model of human pathophysiology.

*Taz* null flies had reduced total CL and reduced unsaturated acyl chains.<sup>25</sup> Mutant indirect wing muscles contained clusters of mitochondria with abnormal cristae.<sup>25</sup> Although not grossly apparent, in quantitative assays mutant adult flies had muscle weakness, as demonstrated by reduced ability to fly and to climb against gravity.<sup>25,26</sup> Moreover, *Taz* mutant flies had reduced endurance that failed to improve with exercise training,<sup>26</sup> a phenotype highly relevant to patients, who commonly manifest muscle weakness as profound fatigue following minimal exertion.<sup>27</sup> However, the beat rate, contractility, and compliance of *Taz* mutant fly heart tubes was normal<sup>25,28</sup> and did not show increased susceptibility to pacing-induced heart failure.<sup>26</sup> Flies were also used to test human and *Drosophila Taz* splice isoforms. Unlike yeast, in which only the isoform lacking primate-specific exon 5 was active,<sup>8</sup> in flies isoforms both with and without exon 5 had transacylase activity.<sup>29</sup>

Male *Taz* null flies were also sterile as a result of defective spermatid individualization.<sup>30</sup> Although male sterility is not a feature of patients with Barth syndrome, this phenotype nevertheless was useful to establish the importance of CL metabolism in mediating the pathogenic effects of *Taz* mutation in animals. Analogous to the finding that *cld1* mutation mitigated the phenotype of *taz1* mutant yeast, mutation of *Pla2-VIA* in flies

suppressed the male sterile phenotype of *Taz* mutant flies. *Taz* mutation decreased CL levels, increased MLCL/CL ratio, and increased the diversity of CL species. *Pla2-VIA*; *Taz* double mutants restored CL levels and MLCL/CL ratio but did not alter the diversity of CL species. However, in wild-type flies *Pla2-VIA* was not required for CL remodeling. The effect of *Pla2-VIA* mutation on the muscle phenotypes of *Taz* mutant flies was not reported. These findings suggest that suppression of the phospholipase(s) that mediate CL deacylation in humans may be a therapeutic strategy to treat Barth syndrome. However, the identity of the responsible phospholipase(s) and their function in other aspects of cellular homeostasis are currently unknown.

The reproducible exercise and sterility phenotypes of *Drosophila* could be readily adapted to take advantage of the power of fly genetics to identify genes that genetically interact with *Taz* and modulate the phenotypes of heart, skeletal muscle, and sperm.

#### 4 | ZEBRAFISH

*Danio rerio* has matured into a potent vertebrate model system amenable to both genetic and small molecule screens. As inexpensive vertebrates with well-developed forward and reverse genetics and well established systems to study cardiac and skeletal muscle physiology, zebrafish occupy a unique position among the major model organisms.<sup>31,32</sup> Morpholino knockdown of *taz* in zebrafish embryos yielded among the first animal models of Barth syndrome.<sup>33</sup> Morpholinos reduced heart rate, contraction, and looping, and caused embryonic lethality. Specificity of these effects was supported by phenotypic rescue by co-injected morpholino-resistant *taz* mRNA.

Despite this promising beginning, zebrafish have not subsequently been used to model Barth syndrome. CRISPR/Cas9 has now enabled generation of targeted mutations in zebrafish,<sup>34</sup> and comparison between morpholino and targeted mutant phenotypes has raised questions about morphant specificity.<sup>35</sup> Surprisingly, genetic *taz* mutant fish have not been reported. Such fish could be used to study mature heart phenotypes in *taz* mutant fish, to perform genetic interaction studies, and to conduct small molecule screens for suppressors of the *taz* mutant phenotype.<sup>36,37</sup>

#### 5 | MICE

*Mus musculus* is the most frequently used mammalian model of disease. Some key advantages of mice are that they are amenable genetic manipulation, their husbandry

is less expensive compared to other mammals, and an enormous set of investigative tools, reagents, and reference literature on mice has arisen as a result of their intensive study.

Initial efforts to create genetically modified *Taz* mutant mice were unsuccessful; in retrospect, this was likely due to male sterility of *Taz* mutant mice (discussed below), which precluded germline transmission of genetic modifications made in male embryonic stem cells. To circumvent this difficulty, a genetic model was created by developing transgenic mice in which administration of doxycycline (Dox) induced expression of a short hairpin RNA that depleted *Taz* mRNA by over 90%. This *Taz* knockdown (*Taz*-KD) model yielded the first mammalian model of *Taz* depletion.<sup>38,39</sup> Induction of *Taz* knockdown by administration of Dox in chow at 200 to 625 mg/kg throughout most of gestation and postnatal life resulted in 90% to 97% reduction of *Taz* mRNA in heart. Tetralinoleoyl-CL, the most abundant CL species in mammalian striated muscle, was markedly decreased, MLCL accumulated, total CL was reduced, and unsaturated CL decreased. In younger mice (2 months), cardiac mitochondrial genome copy number was increased; however, significantly more frequent alterations in mitochondrial ultrastructure, most commonly involving cristae morphology, were not evident until 8 months. At 2 months of age, cardiac function was normal. Mild cardiomyopathy was evident at 7 to 10 months. Skeletal muscle force-frequency was only mildly impaired in *Taz*-KD. However, by 5 months of age *Taz*-KD mice exhibited a dramatic reduction in maximal exercise capacity. Indirect calorimetry revealed that *Taz*-KD mice did not differ significantly from controls at baseline. During exercise, *Taz*-KD mice had markedly impaired oxygen utilization, earlier switch to carbohydrate substrates, and increased lactic acid production,<sup>40</sup> which parallel observations made in Barth patients.<sup>41</sup> Reduced neutrophil counts were alluded to but not explicitly described in the initial characterization of the *Taz*-KD model.<sup>39</sup> These investigators also noted that Dox itself may affect neutrophil counts, potentially confounding studies of neutropenia using this model.

Another group studied the same *Taz*-KD mice but used a different Dox dosing regimen—2 mg/mL Dox in 10% sucrose drinking water—which resulted in an estimated 3 to 10× higher Dox intake.<sup>42</sup> *Taz*-KD had reduced fetal survival and those that survived to term had high perinatal lethality. Cardiac histological sections demonstrated myocardial noncompaction and diminished cardiomyocyte proliferation, and electron microscopy revealed that fetal cardiomyocytes had abnormal mitochondrial ultrastructure and sarcomeric organization. Fetal heart systolic function was not significantly



reduced, although reduced peak velocity in the dorsal aorta may have been consistent with subtle reduction of systolic function. The doppler blood flow pattern in the dorsal aorta also suggested possible diastolic dysfunction. These data were interpreted to indicate that *Taz* deficiency in Taz-KD caused abnormal fetal heart development, cardiac noncompaction, and fetal and perinatal death. Notably, fetal loss in this model was more severe than observed in genetic *Taz* null embryos (see below), suggesting that factors in addition to *Taz* deficiency likely contribute to this embryonic demise. For example, Dox affects mitochondria<sup>43</sup> and metalloproteases,<sup>44</sup> suggesting the possibility that high Dox interacts with *Taz* deficiency to impair fetal survival. Although germline *Taz* null mutation in C57BL6/J mice causes high perinatal loss,<sup>45</sup> this was not due to inactivation of *Taz* in fetal cardiomyocytes, since conditional ablation of *Taz* early after cardiogenesis by *Tnnt2-Cre* did not impair fetal or perinatal survival (Wang and Pu, unpublished).

Studies using the Taz-KD model need to be interpreted with several caveats in mind. First, TAZ is incompletely ablated. Unlike most patients with TAZ loss of function mutations, Taz-KD had 3% to 10% residual *Taz* mRNA,<sup>38,39</sup> and protein levels as high as 45% of controls.<sup>46</sup> Second, the genetic background of the mice in initial reports was “C57BL6/129S6,” and studies of the germline null *Taz* mouse have now revealed that strain background is a critical variable (see below). Third, the dose of Dox is an important variable<sup>47</sup> and can account for differences between studies. Moreover, nursing pups likely receive a low dose of Dox. Although murine data are not available, human data suggest that Dox excretion into milk is low, so that infant serum levels are approximately 6% those of the mother (LactMed: <https://www.ncbi.nlm.nih.gov/books/NBK500561/>), which may contribute to late onset and mild phenotypes observed in Taz-KD mice. Fourth, there are inconsistencies between the knockdown and knockout models that suggest that some aspects of the Taz-KD phenotype cannot be explained solely by depletion of *Taz*. High embryonic attrition reported for Taz-KD mice under the high Dox dosing regimen described above is one example. Another is the development of increased left ventricular wall thickness with preserved systolic function reported as the cardiac phenotype in some studies of aged Taz-KD mice.<sup>48</sup> To test the hypothesis that increased mitochondrial production of reactive oxygen species (ROS) contributes to the pathogenesis of heart disease in Barth syndrome, Taz-KD mice were crossed to mice that express mitochondrially targeted catalase (MCAT).<sup>48</sup> This study found that Taz-KD mice did have elevated levels of ROS, but the cardiac phenotype was not altered by the MCAT transgene, leading the authors to conclude that

elevated ROS is not required for cardiac pathology in this model. However, the cardiac pathology reported, left ventricular wall thickening, was not observed in the initial description of Taz-KD mice on the same Dox dose, and furthermore increased wall thickness was not observed in *Taz* knockout mice. Although a hypertrophic cardiomyopathy pattern has been described in some Barth syndrome patients, the inconsistent cardiac phenotype in the Taz-KD model across studies and the possibility that the hypertrophic phenotype is synthetic and not solely attributable to *Taz* deficiency preclude reaching a clear conclusion about the contribution of ROS to Barth syndrome cardiomyopathy. Notably, another study of the Taz-KD model did not find elevated production of mitochondrial oxidants and questioned its contribution to Barth syndrome pathogenesis.<sup>49</sup>

Mice with genetically targeted *Taz* have now been developed. A conditional *Taz* allele was created by flanking exons 5 to 10 with loxP sites. Chimeric mice transmitted this allele in the germline, yielding the *Taz*<sup>lox</sup> allele. Germline Cre-mediated recombination deleted exons 5 to 10, resulting in the constitutive *Taz*<sup>null</sup> allele.<sup>45,50</sup> *Taz*<sup>null</sup> and *Taz*<sup>lox</sup> alleles were extensively backcrossed into the C57BL6/J background, and initial cardiovascular characterization of these mice was performed in this background. Western blotting confirmed that this allele is protein null.<sup>45</sup> As expected, *TAZ*<sup>null/Y</sup> had elevated MLCL/CL and increased CL diversity. Mutant cardiac mitochondria were smaller but more numerous and had simplified cristae. *TAZ*<sup>null/Y</sup> mice had slightly reduced survival to term, but most of these neonates die in the first several days after birth. *TAZ*<sup>null/Y</sup> neonates had lower body weight than control littermates, and those most likely to perish had the most severely reduced birth weight. Cardiac selective inactivation of *Taz* did not impair fetal or neonatal survival, suggesting that perinatal death was noncardiac (see below). These neonates had reduced motor activity and righting behavior, and less frequent milk spots, suggesting that skeletal muscle weakness, maternal culling behavior, and inability to compete with more vigorous littermates caused postnatal demise.

*Taz*<sup>null/Y</sup> mice had lower body weight and length than their littermates, similar to prepubertal growth retardation observed in Barth patients. However, in humans this growth delay is often abrogated by late catch up growth,<sup>6</sup> whereas *Taz*<sup>null/Y</sup> mice were smaller than littermates throughout life.<sup>6,51</sup> *Taz*<sup>null/Y</sup> mice had progressive dilated cardiomyopathy, with cardiac systolic function becoming measurably depressed by 3 months of age.<sup>45</sup> Hearts also exhibited myocardial fibrosis and increased cardiomyocyte apoptosis. These mutant mice also had skeletal muscle disease, as demonstrated by markedly reduced

endurance on exercise treadmill testing. Neutrophil counts were lower in  $Taz^{null/Y}$  mice than littermate controls but remained within the normal range for mice.  $Taz^{null/Y}$  male mice are sterile due to defective spermatogenesis.<sup>50,52</sup> Mechanistic investigation revealed that *Taz* and mitochondria make a novel contribution to acrosomes, specialized structures within the head of sperm.

The perinatal lethality of C57BL6/J  $Taz^{null/Y}$  mice can make this model cumbersome for studies of postnatal phenotypes. Conditional mutagenesis can be used to circumvent this difficulty. *Myh6-Cre* selectively inactivates floxed alleles in cardiomyocytes, usually by embryonic day 12.5. *Myh6-Cre; Taz<sup>fl/Y</sup>* mice lack *Taz* expression in postnatal cardiomyocytes.<sup>45</sup> These mice survive normally and develop progressive dilated cardiomyopathy that is slightly more aggressive than observed in  $Taz^{null/Y}$  mice, perhaps because the most severely affected  $Taz^{null/Y}$  mice die perinatally, resulting in survivor bias.

A critical factor to consider in the design and interpretation of experiments using *Taz* mutant mice is strain background. By crossing  $Taz^{null/WT}$  female mice in the C57BL6/J strain background with mice in other inbred strains, we obtained  $Taz^{null/Y}$  F1 males with different strain backgrounds. Survival, cardiomyopathy, and skeletal muscle involvement varied tremendously by strain background, suggestive of strong genetic modifiers (Wang and Pu, unpublished). Identification of these genetic modifiers may lead to novel therapeutic strategies to treat Barth syndrome and may explain the highly variable expression of *TAZ* mutation in Barth patients. From an experimental standpoint, one can manipulate strain background to adjust survival and the severity of cardiomyopathy or skeletal muscle phenotypes. At the same time, the experimental design must strictly control for effects of genetic background, and greater variability can be expected in mixed genetic backgrounds.

The *Taz*-KD,  $Taz^{null/Y}$ , and *Myh6-Cre; Taz<sup>lox/Y</sup>* mouse models have all been used to test the efficacy of AAV-*Taz* gene therapy.<sup>45,46</sup> *Taz*-KD mice were treated with self-complementary AAV9 that expressed full length human *TAZ* from the *Desmin* promoter.<sup>46</sup> At a dose of 1E13 vg/kg, the vector increased *TAZ* protein levels from 45% of control to approximately 115%. The fraction of transduced cardiomyocytes was not measured. This treatment significantly improved cardiac systolic function above untreated levels, but function remained well below control levels. Skeletal muscle function, measured by spontaneous activity measurements after light exercise, were improved to normal levels.  $Taz^{null/Y}$  mice were treated with standard or self-complementary AAV9 that expressed full length human *TAZ* from the synthetic CAG promoter.<sup>45</sup> AAV, administered on the second postnatal day at a dose that transduced ~65% of

cardiomyocytes and ~60% of skeletal muscle cells, rescued perinatal lethality, and returned cardiac function to normal. However, at 4 months of age, treated mice exhibited declining heart function, likely reflecting loss or dysfunction of nontransduced cardiomyocytes. The cardiac specific *Myh6-Cre; Taz<sup>lox/Y</sup>* model was used to further study dose-response. A dose of 2E13 vg/kg, which transduced ~70% of adult cardiomyocytes, prevented cardiac dysfunction for over 4 months, whereas a dose that transduced ~30% of cardiomyocytes had a more variable and less durable effect. Moreover, the higher dose also was able to reverse mild established cardiac dysfunction and improve exercise capacity. Overall, both gene therapy studies provide proof-of-concept that AAV-*TAZ* gene therapy could be effective therapy for Barth syndrome, although transduction of a large majority of muscle cells is likely required for durable efficacy. One question not yet addressed is the optimal *TAZ* splice isoform to use for gene therapy. Both full length and exon 5-deleted splice isoforms have transacylase activity, but their relative activity in rescuing BTHS phenotype needs to be established.

In summary,  $Taz^{null/Y}$  and *Cre; Taz<sup>lox/Y</sup>* mice are excellent models of Barth syndrome to study disease mechanisms and to test the efficacy and safety of proposed therapies.

## 6 | HUMAN INDUCED PLURIPOTENT STEM CELLS

A number of mammalian cell models have been developed to study the effects of *TAZ* deficiency, including immortalized patient-derived lymphocytes<sup>53</sup> or skin fibroblasts,<sup>54</sup> C2C12 murine skeletal myoblasts with engineered *TAZ* mutation,<sup>55</sup> patient-derived induced pluripotent stem cells (iPSCs) and iPSC-derived cardiomyocytes (iPSC-CMs),<sup>56,57</sup> and human iPSCs and iPSC-CMs with engineered *TAZ* mutation.<sup>56</sup> Here we focus on human iPSC and iPSC-CM models as these are unique human models of relevant affected cell types.

iPSCs harboring disease-causing mutations can be reprogrammed from patients' somatic cells or generated from reference wild-type iPSC lines by introduction of patient-derived mutations through genome editing. For studies of biological mechanisms, genome-edited wild-type and mutant lines are often preferable, because the control cells should be isogenic except for the introduced mutation. Patient-derived lines afford the additional opportunity to investigate effects of the patient's genetic background on disease manifestations and treatment responses. Wild-type reference iPSC lines can be used as nonisogenic controls, or the patient mutations can be

corrected through genome editing to yield an isogenic control for each patient-derived mutant line. iPSCs are differentiated into iPSC-CMs by well-established, highly efficient protocols, yielding cardiomyocyte-like cells.<sup>58,59</sup> These cells spontaneously beat and develop cardiomyocyte-like action potentials and calcium transients. However, their structural and functional properties are comparable to fetal or neonatal cardiomyocytes, and in most cases these cells lack properties of adult cardiomyocytes, such as rectangular shape, T-tubules, highly ordered sarcomeres, and reliance on oxidative phosphorylation.<sup>60</sup> The immaturity of iPSC-CMs is an important caveat that should be kept in mind while interpreting the results of experiments using iPSC-CMs.

Patient-derived and genetically engineered iPSCs<sup>57</sup> and iPSC-CMs<sup>56,61</sup> that lack TAZ have expected derangements in CL biogenesis. Mitochondrial function assays reproducibly demonstrated marked reduction in maximal electron transport chain activity, which has been linked to disassembly of respiratory chain supercomplexes and cardiac-specific succinate dehydrogenase deficiency.<sup>61</sup> TAZ mutant iPSC-CMs had high levels of mitochondrial ROS,<sup>56</sup> likely reflecting escape of electrons from partially disassembled respiratory chain complexes. Sarcomere assembly was markedly impaired in TAZ mutant iPSC-CMs, and correspondingly engineered heart tissues built from TAZ mutant iPSC-CMs had severely compromised ability to generate contractile force.<sup>56</sup> These phenotypes were rescued by a mitochondrially targeted ROS scavenger, but not by culture conditions that normalized ATP levels,<sup>56</sup> suggesting that elevated ROS generation participates in the pathogenesis of contractile dysfunction, at least in this cell culture model.

One mechanism by which mitochondrial ROS might affect sarcomere assembly and contractile function is through oxidation and activation of protein kinases. A key ROS-sensitive kinase in the heart is Ca<sup>2+</sup>-calmodulin-dependent protein kinase II (CaMKII).<sup>62</sup> Excessive activation of this kinase is pro-arrhythmic and deleterious to heart function.<sup>63</sup> In TAZ mutant iPSC-CMs, ROS activated CaMKII, which increased phosphorylation of RYR2, the main cardiomyocyte intracellular Ca<sup>2+</sup> release channel, on serine 2814, resulting in elevated diastolic Ca<sup>2+</sup>, reduced Ca<sup>2+</sup> transient amplitude, and increased frequency of spontaneous Ca<sup>2+</sup> release events.<sup>64</sup> These findings were suppressed by ROS scavenger, CaMKII inhibitor, and genetic ablation of the RYR2-S2814 phosphorylation site. CaMKII inhibition likewise improved the function of *Taz* mutant cardiomyocytes isolated from *Myh6-Cre; Taz<sup>fl/y</sup>* mice. These data implicate ROS activation of CaMKII in the pathogenesis of cardiac dysfunction and arrhythmia, at least in the iPSC-CM Barth syndrome model.

These studies show that iPSCs and iPSC-CMs are useful models to dissect disease mechanisms, despite their immaturity. These models are amenable to genetic and small molecule screening approaches, which could be used to further develop mechanistic insights or novel therapies in Barth syndrome. iPSCs can now also be differentiated into human skeletal muscle-like cells.<sup>65</sup> Given the importance of muscle fatigue in Barth syndrome manifestations,<sup>65</sup> an iPSC-derived skeletal muscle model would appear to be a promising direction for future studies. Similarly, a human iPSC-based model could provide insights into the mechanisms by which TAZ mutation causes neutropenia. Patient-derived iPSCs may be useful to build patient-specific disease models, which it is hoped will enable precision medicine approaches by predicting therapeutic responses of individual patients.<sup>66</sup> However, accuracy with which such patient-specific models predict individual responses has not yet been established.

## 7 | CONCLUSIONS

In summary, a wealth of model systems have been developed to study the pathological effects of TAZ mutation. These model systems have put researchers in an excellent position to dissect the pathophysiology of Barth syndrome and to discover new therapeutic approaches. While much progress has been made, key questions remain regarding the mechanistic links between TAZ mutation and abnormal CL composition and the manifestations of Barth syndrome. For instance, the contribution of elevated ROS to Barth syndrome pathogenesis remains controversial. Is there a mammalian counterpart of yeast *Cld1* and *Drosophila Pla2-VIA*, and if so could its inhibition ameliorate Barth syndrome manifestations? What are the mechanisms by which TAZ mutation causes neutropenia and growth delay? What is the mechanism that accounts for the wide variation in disease severity observed between Barth patients, and between inbred mouse strains, and could this mechanism be therapeutically exploited? The TAZ-KO model will also be invaluable for preclinical testing of emerging candidate therapies. An important caveat is that mice and humans have considerable physiological differences, and it currently is not known the degree to which therapeutic efficacy in the TAZ-KO model predicts the responses of patients. One important practical hurdle is optimizing this model to study skeletal muscle disease and therapies targeting this indication: in the pure C57BL6/J background, high neonatal loss is likely due to skeletal muscle disease and therefore cannot be circumvented by tissue-specific gene knockout. Potential solutions are temporally

controlled gene inactivation or selection of a different inbred strain background that circumvents lethality yet exhibits significant skeletal muscle disease.

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## CONFLICT OF INTEREST

William T. Pu previously received sponsored research funding from Stealth BioTherapeutics to study elamipretide in Barth syndrome. WTP holds intellectual property on gene therapy for Barth syndrome. WTP is on the Scientific and Medical Advisory Board of the Barth Syndrome Foundation.

## ORCID

William T. Pu  <https://orcid.org/0000-0002-4551-8079>

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