***mTOR* inhibition attenuates cardiac dysfunction in a zebrafish *lamp2* mutant**

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Abstract

Severe hypertrophic cardiomyopathy (HCM) with the massive autophagic vacuoles accumulation are two major characteristics of the *Lamp2*-associated Danon disease; there is no effective therapy yet to treat this most lethal cardiomyopathy in the young. Adult zebrafish is an emerging vertebrate model for studying genetic basis of cardiomyopathies, but an inherited HCM model has not been established in this species yet. Here, we enquire whether zebrafish can be used as a model of the *Lamp2*-associated Danon disease, and to develop candidate therapies in this disease. Through employing a set of emerging phenotyping tools, we showed that the *lamp2* mutants have cardiac phenotypes including decreased ventricular ejection fraction (EF), reduced exercise capacity, blunted β-adrenergic contractile response of myocardium, altered expression of the heart failure markers, and a massive subcellular accumulation of the autophagic vacuoles in the heart. We also noted the changes in the following indices suggesting cardiac hypertrophic remodeling: rounded heart shape, increased end-systolic ventricular volume, increased density of ventricular myocardium, elevated actomyosin activation kinetics together with increased maximal isometric tension at the level of cardiac myofibrils. Haploinsufficiency of *mtor* was able to attenuate certain cardiac defects of the *lamp2* knockout, including reduced ejection fraction, blunted β-adrenergic response, and the actomyosin activation kinetics, but was ineffective in repairing the expression level of LC3 and the autophagic vacuole accumulation. In summary, we demonstrate feasibility of modeling HCM in the adult zebrafish, and identify *mtor* as a candidate gene to develop potential therapeutic strategies in Danon disease.

1. Introduction

About 1% patients with hypertrophic cardiomyopathy (HCM) carry mutations in the lysosome-associated membrane protein 2 (*LAMP2)* gene [1, 2]. These patients being first described by Danon [3], usually also manifest non-cardiac symptoms such as skeletal peripheral myopathies, mental retardation, hepatic involvement, and retinopathy [4-10]. Together with the *PRKAG2*-associated glycogen-storage disease and Anderson-Fabry disease, Danon disease was recognized as one of the three metabolic causes of HCM [11]. Among HCMs of different etiology, *LAMP2*-associated HCM is the most severe type of hypertrophic response in the heart and the most lethal cardiomyopathy in the young: most patients cannot live beyond the third decade of their life [6, 12]. A mouse model of Danon disease has been established in the early 2000-s [13, 14]. About 50% *Lamp2*-deficient mice die by 20-40 days postnatal, the remaining pups survive to adult having the reduced body size and the increased heart weight / body weight ratio [14, 15]. Similar to patients with Danon disease, a massive subcellular accumulation of autophagic vacuoles has been noted in many tissues from the *Lamp2* deficient mice. No effective therapy is yet available for Danon disease.

LAMP2 encodes a type I integral membrane protein that belongs to the lysosome-associated membrane proteins (LAMP) family, which are related to autophagy. LAMP2 protein is ubiquitously expressed and localized to lysosomes and late endosomes [7]. The *LAMP2* genome locus encodes three isoforms that share common N-terminal domains but have different C-terminus due to alternative splicing of the exon 9 [7]. While LAMP2A is a major component of chaperone-mediated autophagy that regulates phagosome degradation and proteolysis of the long-lived proteins [4, 16], LAMP2B is required for the macroautophagy that regulates recycling of the damaged organelles; LAMP2C is restricted to the degradation of nucleic acids [17]. Depletion of LAMP2 impairs the autophagosome-lysosome fusion leading to the prolonged half-life of autophagosomes and accumulation of early autophagic vacuoles in the cytoplasm [7]. Induced pluripotent stem cells-derived cardiomyocytes obtained from patients with Danon disease confirmed the severe autophagy blockage [18]. Despite repairing the defective autophagy is a plausible therapeutic avenue, effective therapeutic targets remain to be discovered, partially because LAMP2 functions at the final step of macroautophagy. Known autophagy-activating therapeutic strategies such as intermitted fasting, which are effective in a model of ischemia-reperfusion, failed in the mouse model of Danon disease [19].

The zebrafish has emerged as a new vertebrate model for studying cardiomyopathies [20-22]. Acquired cardiomyopathy models induced by anemia, doxorubicin, or isoproterenol have been established, underscoring the conservation of cardiac remodeling in this species [23-28]. The majority of

the human dilated CM causative genes have their corresponding homologs in the zebrafish, supporting genetic conservation [21]. However, cardiac phenotypes in these early models are poorly defined, because of the small size of the heart, its unique sponge-like structure making difficult to determine the wall thickness, and the lack of phenotyping tools. As a consequence, it remains to be established which phenotypic traits define HCM in an adult zebrafish.

One of the major reasons for integrating the zebrafish model into cardiomyopathy studies is its powerful genetics. A mutagenesis screen-based strategy has been recently established enabling a rapid identification of new genetic factors and therapeutic targets. A zebrafish mammalian target of rapamycin (mTOR) mutant, *xu015*$^{+/-}$, was identified from this screen; we found that *mtor* haploinsufficiency can effectively attenuate cardiomyopathy induced by both anemia and doxorubicin [22, 24, 29, 30]. Our data supported known therapeutic effects of rapamycin in several types of cardiomyopathies [2, 31], and provided the first genetic evidence for mTOR as a therapeutic target. mTOR, an atypical serine/threonine kinase, is a part of two distinct complexes named mTOR complexes 1 (mTORC1) and 2 (mTORC2) [32]. mTORC1 plays an important role in regulating macroautophagy, via direct phosphorylation of the Atg1/ULK complex which is important for the autophagosome formation and/or phosphorylation of TFEB, a master transcriptional regulator of lysosome biogenesis [33]. Of note, mTOR is recruited to the surface of lysosome upon fasting where LAMP2 is also localized [34]. It has not been investigated whether mTOR can be manipulated to repair dysregulated autophagy in the *LAMP2* mutants.

Because cardiac hypertrophy is a primary manifestation of Danon disease in both humans and the mouse model, we reasoned that knockout of *lamp2* in the zebrafish might be a prototypic HCM model. Past several years witnessed a development of whole set of cardiac phenotyping tools in the zebrafish, including high frequency echocardiography [27], a Langendorff-like perfusion system [35], zebrafish electrocardiography [36, 37], cardiac imaging using light sheet microscopy [38], nuclear magnetic resonance [REF?], to name a few. We have also employed biophysical methods to study cardiac contractile function in zebrafish at the level of myofibrils [39]. Here, we deployed these new tools for detailed phenotyping of the *lamp2* knock out (KO) zebrafish model. We provide *lamp2* KO zebrafish model for studying human Danon disease with HCM, and presented *mTOR* as a candidate gene that can be a therapeutic target in Danon disease.

2. Results

2.1 lamp2$^{e2/e2}$ *zebrafish manifest compound phenotypes*

In the zebrafish, a single *lamp2* gene is found in chromosome 14. The zebrafish *lamp2* gene is predicted to consist of 9 exons, (ensemble #) with the exon 1 to 6 corresponding to the human exons 1-8. It is also predicted that alternative splicing event containing exon 8 encodes Lamp2A, while alternative splicing event containing exon 7 encodes Lamp2B, and exon 9 encodes Lamp2C. The presumed *lamp2b* encodes a 365 amino acids protein, which shares 49% identity to the human LAMP2B protein (Fig. S1).

To model Danon disease in zebrafish, we generated a $lamp2^{e2/e2}$ mutant via the TALEN technology (Fig. 1A). $lamp2^{e2/e2}$ has a 5-nt deletion in the exon 2, which results in a shift of the reading frame, a premature stop codon, and presumably truncated all three Lamp2 isoforms (Fig 1A). The transcript level of *lamp2* was reduced by 90% in the mutant, probably due to non-sense mediated RNA decay (Fig. 1F). Owing to the lack of antibodies for the zebrafish Lamp2 protein, we cannot assess expressional changes at the protein level. The $lamp2^{e2/e2}$fish appeared smaller than their wild-type siblings, as indicated by the significantly reduced body weight at the age of 9 months (Fig. 1B and C). Denser pigmentation on the skin of the back was noted (Fig. 1B). 20-80% of the $lamp2^{e2/e2}$ fish (depending on clutch) had iris defects, affecting one or both eyes (Fig. S2A, B). Histological studies discovered reduced F-actin expression in the optic nerve bundle including inner plexiform and outer plexiform layers, and layer of rods and cones (IPL, OPL, and LRC, respectively), but not in nuclear layers (Fig. S2C). Compared to their wild-type siblings, $lamp2^{e2/e2}$ fish had reduced maximum swimming capacity (U_{crit}) (Fig. 1D). $lamp2^{e2/e2}$ fish started to die at 10 months of age, and only about 60% fish could survive to 12 months of age (Fig. 1E; $P = 0.004$ to WT). Before they die, mutant fish manifest unique swirling and turning behavior, prompting neuromuscular defects.

2.2 $lamp2^{e2/e2}$ zebrafish manifest cardiac hypertrophic remodeling

Next, we conducted a detailed analysis of cardiac phenotypes. Isolated hearts from the $lamp2^{e2/e2}$ zebrafish at 10 months of age appeared less transparent (Fig. 2A). The overall heart shape became rounded in mutants (Fig. 2A), as quantified by the increased shape index (Fig. 2B). Consistent to the transparency and shape, much denser trabeculation of ventricular myocardium in the trichrome-stained heart sections were documented (Fig. 2C, D), as well as increased intensity in the red channel (RCI) of the images of the mutant hearts at diastole (Fig. 2F). In a small percentage of $lamp2^{e2/e2}$ (<5%) hearts, we noted thicker compact layer, as well as fibrosis in both trabeculated muscle layer and a compact layer (Fig. S3A, B). To enquire whether the structural remodeling occurred at the single cell level, we isolated individual cardiomyocytes by enzymatic dissociation and measured their length and width. We did not detect any differences in the size of cardiomyocytes (Fig. S3C, D). To assess cardiac pump function, we

employed our recently developed Langendorff-like ex vivo heart perfusion system [40]. We noted a significantly reduced ejection fraction (EF%), fractional area contractility (FAC%), and radial strain (RS%) at perfusion flowrate of 0.4 ml/min, indicating reduced cardiac pump function in *lamp2*$^{e2/e2}$ hearts (Fig. 2G-I). There was a mild reduction of shortening and relaxation velocities, albeit both did not reach significant differences (Fig. S4 D, E). We also provide various ex vivo measurements of the parameter of fractional shortening in Fig. S4C. Consistent to cardiac remodeling in *lamp2*$^{e2/e2}$ hearts, we detected significantly diminished expression of one of the heart failure molecular markers, namely ventricular myosin heavy-chain (*vmhc*; Fig. 2J). Other markers did not show significant differences (Fig. S5).

The absolute size of mutant hearts was smaller, as indicated by the end-systolic and end-diastolic volumes (ESV and EDV, respectively; Fig. S4A, B). However, because of the smaller body size in the mutants, the results need to be normalized with body weight [35]. We found that EDV/BW did not differ from WT at higher perfusion flowrates (0.4-0.6 ml/min) (Fig. S4A), while ESV/BW significantly increased in mutants at the low perfusion flowrate (0.05 ml/min; (Fig. 2E).

In order to assess potential electrical abnormalities in the *lamp2*$^{e2/e2}$ hearts, we conducted an electrocardiography (ECG) study using the iWorx system, employing a new ECG signal filtering method [REF?] (Fig. 3A). We did not detect any sign of cardiac arrhythmia in the *lamp2*$^{e2/e2}$ fish. Instead, we noticed significantly increased amplitudes of P-waves in *lamp2*$^{e2/e2}$ (Fig. 3A and B), suggesting either atrial enlargement or hypertrophy [11]. Because of its thin structure and the lack of fluid flow, isolated atrium in a dissected zebrafish heart easily collapses, preventing an accurate quantification of its size. To overcome this problem, we developed a method to inflate the atrium by connecting the perfusion system to a dissected heart via its outflow tract, which shall better maintain the shape of the atrium (Fig. 3C). Using this method, we noted that the atrium area in *lamp2*$^{e2/e2}$ hearts was almost doubled than that in their wild-type siblings (Fig. 3D). Compared to the thin-walled atrium in the wild type adult fish, certain areas of the atrium wall in the *lamp2*$^{e2/e2}$ hearts were thicker because of more trabeculated myocardium (Fig. 3E).

2.3 mTOR inhibition improved cardiac pump function and β-adrenergic response in lamp2$^{e2/e2}$ *hearts*

To assess whether the therapeutic effects of *mTOR* inhibition can be extended to cardiac phenotypes in *lamp2*$^{e2/e2}$, we bred *mtor*$^{xu015/+}$ into the *lamp2* mutant to generate double mutants. To simplify the labeling in the figures, we named *lamp2*$^{e2/e2}$ as LM and *lamp2*$^{e2/e2}$;*mtor*$^{xu015/+}$ as LMT. In addition to the Langendorff-like ex vivo method, we assessed cardiac pump function by using a non-invasive high

frequency echocardiography (HFE) method, because we obtained access to a high frequency echocardiography scanner at the later stage of the project. Similar to the decreased cardiac pump function revealed by the ex vivo assay, we noted significantly decreased ejection fraction (EF), fractional shortening (FS), and fraction area contractility (FAC) in *lamp2*$^{e2/e2}$ hearts (Fig. 4A to C). Importantly, we noted significantly improved EF and FAC in *lamp2*$^{e2/e2}$;*mtor*$^{xu015/+}$, prompting therapeutic effects of *mTOR* inhibition on cardiac dysfunction. The change in EF can be mainly ascribed to the change in the long axis fractional shortening (FS), but not in the short axis FS (Fig. 4C).

Because the blunted β-adrenergic response is a common feature of the failing hearts in humans [41], we went on to analyze lusi- and inotropic effects of isoproterenol using the ex vivo Langendorff-like perfusion system. We recently showed that these effects of isoproterenol can be reproduced in the zebrafish [40]. Injection of a single bolus of isoproterenol into the perfusion system induced an immediate increase in EDV and a subsequent decrease in ESV in a wild type heart (Fig. 4D, E). The first effect was blunted in *lamp2*$^{e2/e2}$, as indicated by a negative value of ΔEDV, an index reflecting the lusitropic effect, which was rescued in *lamp2*$^{e2/e2}$*;mtor*$^{xu015/+}$(Fig. 4D). By contrast, we did not notice significant changes in ΔESV, an index reflecting the inotropic effect (Fig. 4E). *mTOR* inhibition also failed to rescue the blunted ejection fraction change in *lamp2*$^{e2/e2}$, as indexed by ΔEF (Fig. 4F); neither ΔFS (Fig. S5A), nor velocities of contraction and relaxation induced by isoproterenol (Fig. S6B, C).

2.4 mTOR inhibition normalized maximal isometric tension and actomyosin activation kinetics in cardiac myofibrils

To query whether reduced cardiac pump function in *lamp2*$^{e2/e2}$ and the rescuing effects of *mTOR* inhibition occur at the level of cardiac ventricular myofibrils, we assessed kinetics of myofibril activation and relaxation. Single myofibrils were attached to glass micro-probes (Fig. 5A), and activation traces from myofibrils were obtained as deflection of the right complaint probe with known stiffness (Fig. 5B). To assess myofibril activation by calcium, we quantified the rate of calcium activation (k_{ACT}) when myofibrils start to shorten, as well as the rate of force redevelopment (k_{TR}) when a release-re-stretch maneuver is performed 5 seconds after the initial activation (shown with a star '*' in Fig. 5B). Both variables reflect the rate of cross-bridge turnover [42, 43]. To assess myofibril relaxation, we quantified the rate of exponential (fast) phase of relaxation (k_{REL}) when fast switch of a stream to the stream with zero Ca^{2+} in it triggers the relaxation process (Fig. 5B). Both k_{ACT} and k_{TR} were increased in *lamp2*$^{e2/e2}$ myofibrils, indicating faster kinetics of activation (Fig. 5C and D), and both indices were effectively rescued in *lamp2*$^{e2/e2}$;*mtor*$^{xu015/+}$. By contrast, there were no difference in kinetics of myofibril relaxation

($t_{REL, slow}$, $k_{REL, slow}$), as well as in the passive elastic properties of the skinned myocardium (Fig. 5E; Fig. S7B-D).

We detected increased maximal isometric tension in *lamp2*$^{e2/e2}$ myofibrils, supporting 'hypercontractility' that might be already suggested by the increased rates k_{ACT} and k_{TR} (Fig. 5F). mTOR inhibition effectively normalized this phenotype (Fig. 5F). By activation of myofibrils with various Ca^{2+} concentrations, one can obtain the force-pCa relationships; they were comparable between *lamp2*$^{e2/e2}$and the control group (Fig. S7A), indicating unaffected myofilament Ca^{2+} sensitivity. Intriguingly, myofilament Ca^{2+} sensitivity in the *lamp2*$^{e2/e2}$;*mtor*$^{xu015/+}$ group was reduced (Fig. 5D). We also noted a diminished Hill coefficient parameter in *lamp2*$^{e2/e2}$ mutants (Fig. 5H), suggesting reduced cooperativity in the force generation in the mutants. This parameter was not repaired by mTOR inhibition.

2.5 mTOR inhibition cannot rescue autophagic defects in lamp2$^{e2/e2}$ *hearts*

Given important functions of Lamp2 and mTOR in macroautophagy, we assessed whether the rescuing effects of *mTOR* inhibition on cardiac defects in *lamp2*$^{e2/e2}$ hearts can be ascribed to repairing of the defective autophagy. Similar to the Danon disease patients, iPSC models of Danon disease, and the *Lamp2* knockout mouse model, we detected an accumulation of LC3 in fish *lamp2*$^{e2/e2}$ mutants (Fig. 6A, B), indicating severely blocked autophagy [44]. Consistent to the effective *mtor* inhibition by *mtor*$^{xu015/+}$, we noted significantly reduced total mTOR in the *lamp2*$^{e2/e2}$;*mtor*$^{xu015/+}$ group (Fig. 6A, B). However, p-mTOR/mTOR ratio, p-S6 and LC3-II remained unchanged among the three experimental groups, suggesting that *mTOR* inhibition fails to repair defective macroautophagy *per se*.

Next, we conducted a transmission electron microscopy to assess ultrastructure of the *lamp2*$^{e2/e2}$ hearts [14]. Massive autophagic vacuole accumulation was noted in the perinuclear region of cardiomyocytes in *lamp2*$^{e2/e2}$ fish at 13 months of age (Fig 5C, quantified in the table in Fig. 5B), and this characteristic appeared as early as at the age of 6 months (J.Y.; data not shown). Despite the number of autophagic vacuoles slightly dropped in the age-matched *lamp2*$^{e2/e2}$*;mtor*$^{xu015/+}$, the drop failed to reach statistical significance when compared to *lamp2*$^{e2/e2}$ alone (Fig. 5C).

3. Discussion

3.1. lamp2$^{e2/e2}$ is a zebrafish model of Danon disease

To develop the adult zebrafish model for studying inheritable cardiomyopathies, we generate *lamp2*$^{e2/e2}$. The genotype of *lamp2*$^{e2/e2}$ was designed to recapitulate the representative genetic lesions that cause human Danon disease, since most of Danon disease-causative mutations are loss-of-function *LAMP2* mutations that resulted in truncation and/or depletion of the encoded LAMP2 protein [4]. In contrast to human *LAMP2* that sits in the X chromosome, the zebrafish *lamp2* gene is located in chromosome 14. Therefore, homozygous *lamp2*$^{e2/e2}$ can recapitulate overall LAMP2 deficiency in human, but cannot discern the different disease severity between men and women, as noted in human patients [5].

Similar to patients with Danon disease, *lamp2*$^{e2/e2}$ fish show phenotypes of hypertrophic cardiomyopathy, as will be discussed in detail below. At the same time, not all patients with Danon disease manifest HCM: DCM cases were also shown in female patients [5]. We also noted compound non-cardiac phenotypes that are reminiscent of human Danon disease, including reduced swimming capacity and the iris deformations in a portion of the mutant fish. Importantly, the massive autophagic vacuole accumulation can be detected in the heart, a characteristic feature of Danon disease. Together, our data suggest that *lamp2*$^{e2/e2}$ can be used as a zebrafish model for the human Danon disease

Despite of similarities, there are some differences among the zebrafish *lamp2*$^{e2/e2}$, mouse *Lamp2* KO models, and the human Danon disease. Cardiac phenotypes in humans are very severe - most patients die in their 30s from sudden cardiac death. By contrast, mouse model of Danon disease shows more systemic autophagic lesions: a half of *Lamp2* deficient mice die shortly after born likely due to intestinal infarctions/stenosis or pancreatic failure with massive intracellular accumulation of the autophagic vacuoles [14]. In zebrafish *lamp2*$^{e2/e2}$, cardiac phenotypes cannot be detected before 7 months of age, and we did not note cardiac arrhythmia in this model. Similar to mouse [14], some *lamp2*$^{e2/e2}$ fish have smaller body size, a phenomenon that has not been reported in human patients.

3.2. lamp2$^{e2/e2}$ zebrafish manifest cardiac phenotypes reminiscent of HCM in mammals

To determine which parts of pathophysiology of the human inheritable cardiomyopathies can be recapitulated in the zebrafish model, we conducted comprehensive studies of the *lamp2*$^{e2/e2}$ fish using the newly developed phenotyping tools [35, 39]. Using both HFE and the Langendorff-like perfusion method, we noted the diminished cardiac pump function in those fish, as indicated by reduced ejection fraction.

Together with decreased swimming capacity and changes of transcriptional levels of the heart failure molecular markers *(vmhc/vmhcl)*, these data point out to heart failure phenotypes in the *lamp2*$^{e2/e2}$fish. This conclusion was further validated by the blunted lusitropic β-adrenergic myocardium response. Blunted β-adrenergic response is a common feature of the failing human hearts [45-48], which could be due to uncoupling between myofilament Ca^{2+} sensitivity and N-terminal phosphorylation of the cardiac isoform of Troponin I by protein kinase A [45]. It remains to be investigated whether phosphorylation of thin myofilaments is affected in *lamp2*$^{e2/e2}$ fish upon β-adrenergic stimulation or treatment with protein kinase A.

In mammals, a key phenotypic trait that defines HCM is an increased thickness of the left ventricular free wall and septum [49]. However, this index cannot be measured in a highly trabeculated zebrafish heart. Instead, our studies in *lamp2*$^{e2/e2}$ suggest the following three indices that could be used to define hypertrophic remodeling in adult zebrafish. First, we noted the increased density of the trabecular muscle in the sectioned hearts, as well as significantly thickened compact layer and fibrosis in a small population of the mutant fish. Similar phenotypes have been reported in the rainbow trout hearts upon cold stress [50], suggesting that the thicker compact and the denser trabecular ventricular myocardium could be useful markers to define hypertrophic responses in the fish species. Second, using the ex vivo Langendorff-like perfusion system, we noted that ESV/BW at low perfusion flowrate was increased. It remains to be determined whether ESV/BW at low perfusion rates is a good index reflecting an increased myocardial mass. Third, at the level of cardiac ventricular myofibrils, we noted the increased maximal isometric tension and accelerated actomyosin activation, suggesting myofibrillar hypercontractility. These data are consistent to the recent 'tension index' hypothesis delineating "hypo-" and "hypercontractile" hearts [51] - whereby HCM heart has hypercontractile myofibrils, DCM heart has hypocontractile myofibrils. In contrary, in *Lamp2*-deficient mice hypocontractile phenotype has been found: intact cardiac muscle fibers developed lesser forces [14]. Possible explanation for this contradiction can be that we did not directly measure forces generated by the intact cardiac muscles obtained from the mutants; we found only increased maximal isometric tension developed by permeabilized myofibrils. The sign of contractility changes in *lamp2*$^{e2/e2}$ in vivo is still a matter for the future investigations.

One possible mechanism for the increased actomyosin kinetics could be the shift of the prevalent myosin heavy chain isoform in cardiac muscle as part of the fetal-gene re-programming process, a hallmark of the cardiac remodeling [50]. Specifically, in *lamp2*$^{e2/e2}$, we noted the reduced expression of *vmhc*. This change in the prevalent myosin heavy chain isoform may affect cross-bridge cycling, maximal isometric tension, relaxation, and energy consumption [52]. Alternatively, the increased maximal

isometric tension in *lamp2*$^{e2/e2}$ might be caused by imprecise normalization of force by cross-section area (CSA) of the myofibril due to hypertrophic remodeling. More myofibrils/CSA in *lamp2*$^{e2/e2}$ preparations could result in a bigger tension, if force is normalized by CSA. This possibility could be tested in the future by quantifying myofibril area via the electron microscopy.

In addition to ventricular hypertrophy, we also noted atrial hypertrophy in the *lamp2*$^{e2/e2}$ fish, which is unveiled by the modified ex vivo perfusion system that was originally designed to study ventricular functions. We reversed the direction of flow to inflate the atrium, enabling reliable quantification of the atrial area. The bigger and more trabeculated atrium in *lamp2*$^{e2/e2}$ are consistent to the increased magnitudes of P-waves on ECG. This result prompted future studies to determine whether enlarged atrium is part of pathogenesis in patients with Danon disease, or a fish-specific response. Nevertheless, the *lamp2*$^{e2/e2}$ fish may provide a useful in vivo model for deciphering how atrium remodeling compensates the insufficient pump function of the ventricle.

3.3 mTOR inhibition partially attenuates cardiac defects in lamp2 KO fish

Based on two acquired cardiomyopathy models, we previously demonstrated the feasibility of the adult zebrafish to assess therapeutic effects of *mTOR* haploinsufficiency [22, 24, 29]. Here, we further demonstrate that the therapeutic effects of *mTOR* inhibition can be extended to *lamp2*$^{e2/e2}$ cardiomyopathy model. The comprehensive phenotyping pointed out the perspectives of cardiac phenotypes that can be rescued by *mTOR* inhibition. We show that *mTOR* inhibition is able to rescue blunted lusitropic β-adrenergic response in *lamp2*$^{e2/e2}$fish, but not the blunted ejection fraction change, nor the differences of the velocities of contraction and relaxation induced by isoproterenol. At the myofibrillar level, *mTOR* inhibition is able to rescue maximal isometric tension and actomyosin activation kinetics, but not diminished Hill coefficient parameter. Interestingly, we note the reduced Ca^{2+} sensitivity in the double mutants. One plausible hypothesis for that is that mTORC2 modulates reciprocal interactions between protein kinase A and C [53, 54], which may in turn affect phosphorylation of myofilaments and affect myofilament Ca^{2+} sensitivity.

Cardiomyopathies are progressive diseases with a longitudinal pathogenesis that consists of initial compensational responses followed by decompensational changes. As for Danon disease, the severely blocked autophagy is believed to be a primary defect that results in the accumulation of toxic proteins inside the autophagic vacuoles, which triggers a cascade of responses in the heart that ultimately affects cardiac contractility. Because the therapeutic effects of *mTOR* inhibition are not accompanied by repaired

macroautophagy, we concluded that *mTOR* inhibition does not target the root of the disease, i.e. repairing the severely blocked autophagy. Instead, *mTOR* inhibition may exert its therapeutic effects via activating an autophagy-independent pathways, or attenuating key sequential cascade events such as those driving the switch of the pathogenesis from compensational to the decompensational phase. Our observation of the incompletely rescued cardiac defects in the double mutants favors the latter hypothesis. Future studies are warranted to discern these two potential mechanisms, which shall facilitate the development of mTOR-based therapies for Danon disease.

3.4 Zebrafish is an accessible vertebrate model for deciphering inheritable cardiomyopathies

Through the comprehensive phenotyping of *lamp2*$^{e2/e2}$, we established the first adult inheritable HCM model in zebrafish, and demonstrated its use in identifying a candidate therapeutic target. An immediate future direction is to decipher precise mechanisms of the rescuing effects of *mTOR* inhibition on cardiac contractility, including examination of the cAMP/protein kinase A pathway and testing the myosin heavy chain isoform shift hypothesis. Capitalized on the efficient zebrafish genetics, a unique future research direction is to extend the modifier screening strategy to the Danon disease model for systematically discovering gene modifiers. We are particularly interested in finding more genes with similar therapeutic capacity. Upon successful completion of the screening, this gene/therapy strategy can be extended to another inheritable cardiomyopathy models which can be easily generated in zebrafish. In summary, the present work launches zebrafish as an efficient vertebrate model for developing individualized medicine for different types of inheritable cardiomyopathies.

4. Methods

4.1 Fish husbandry

Zebrafish were handled under the guidelines of the Mayo Clinic Institutional Animal Care and Use Committee (IACUC protocol # is A00003513-18). WIK was used as a wild type strain. The fish were maintained at 28°C under 14 hours light - 10 hours dark cycle.

4.2 Generation of lamp2^{e2} via TALEN

To generate *lamp2*^{e2} mutants, we injected WIK embryos with TALEN RNA at one-cell stage. TALEN pairs were designed using Zifit (http://zifit.partners.org/ZiFiT/ChoiceMenu.aspx) and assembled using a Golden Gate Kit (Addgene) (ref). The target sequences and TALEN constructs are listed in Fig.1A. Capped mRNAs were synthetized using mMESSAGE mMachine kit (Ambion). Primers and enzyme for genotyping are listed in Supplemental Table 1.

4.3 Swimming tunnel assay

A swimming tunnel respirometer was used (Mini Swim 170, Loligo Systems, Denmark) and a protocol was modified from [55, 56]. Adult fish (N = 10) were placed into the swimming tunnel with an initial water flow at 9 cm/s for a 20-minute acclimation. Water flow was then gradually increased by 8.66 cm/s (Ti) every 150 s (Tii) until all fish were exhausted. The values of speed at the last stage (Uii) and the previous stage (Ui) were recorded for each individual fish. The critical swimming capacity (Ucrit) was calculated with the following formula: Ucrit=Ui+[Uii*(Ti/Tii)]. Ucrit was then normalized to an average body length in the group (BL). Measurements were done in duplicate.

4.4. Quantitative PCR

Zebrafish hearts were excised from the anaesthetized fish by 0.16 mg/ml tricaine. Ventricles were dissected in cold phosphate buffered saline, frozen in liquid nitrogen, and kept at -80°C. Total RNA was extracted with TRIzol Reagents (Thermo Fisher) using the Advanced Blender System with RNase-free 0.5 mm steel beads (Advanced Blending Solution, USA). Extracted RNA was used as a template for reverse transcription using the SuperScript® III Reverse Transcription kit (Invitrogen, USA). Real-time PCR was performed using the Applied Biosystem VAii 7 System (Thermo Fisher). Primers are listed in

Supplemental Table 1. Expression of genes was normalized to GAPDH and by –ΔΔCt (cycle threshold) values. N=7(5) for WT group, N=7 for LM group and N=4 for LMT group.

4.5 Ex vivo cardiac pump function assay

Our method was described in details previously [35]. Briefly, hearts were excised from anaesthetized by tricaine (0.16 mg/ml in ice-cold water) 10-months-old fish of both sexes and tied to the tip of 34G blunt catheter for perfusion. Perfusion of the Ca^{2+} containing fish Tyrode was done with the help of peristaltic pump EP-1 Econo Pump (Bio-Rad). Setup is built on a Leica M165C stereo microscope equiped with a 14-megapixel Amscope MU1403 camera (Amscope). Perpendicular plane of heart image was obtained by using a 45-degree mirror (Thorlabs). Hearts were electrically stimulated using a MyoPacer stimulator (Ionoptix). The fish Tyrode solution was modified from [57], which contains (in mM) 132 NaCl, 2.5 KCl, 4 $NaHCO_3$, 0.33 NaH_2PO_4, 1 $CaCl_2$, 1.6 $MgCl_2$, 10 HEPES, 5 glucose, and 5 sodium pyruvate. pH was adjusted to 7.5 with NaOH. All experiments were performed at room temperature.

End-diastolic and end-systolic volumes have been calculated using bi-plane area-length (half-ellipsoid volume) formula:

(1) $V = 2/3A*L$,

where A is ventricle area in the transverse plane (short axis), and L is ventricle length in longitudinal plane (long axis).

(2) $EF\% = (EDV – ESV) / EDV$;

where EDV and ESV are ventricle volumes at diastole and systole, respectively.

(3) $FAC\% = (EDA – ESA) / EDA$,

where EDA and ESA are ventricle areas in the transverse plane at diastole and systole, respectively.

(4) $FS\% = (L_d – L_s) / L_d$,

where L_d and L_s are ventricle lengths at diastole and systole, respectively. Length of ventricle can be measured at various ways: short or long axis in longitudinal or transverse plane. Radial strain (RS%) is a modification of FS% - instead of ventricle length we used average radius of ventricle in the transverse plain.

Image series were analyzed using ImageJ software (NIH) and custom-written Matlab code (MathWorks). The code was used for the edge detection and strain/velocity calculations on a frame-to-

frame basis [35]. Maximal contraction and relaxation velocities were obtained by differentiation of the ventricle wall deformation function (at transverse plane) and by finding the extremums of that function. To normalize ventricular dimensions from the fish of different size, we normalized them using body weight (BW, g) as denominator.

We analyzed 15 hearts from WT, 16 hearts from LM group, and 11 hearts from LMT group. These numbers already excluded hearts with the visible damages of the ventricle during surgery. Then, during analysis, we excluded 3 hearts in WT and 3 hearts in LM groups for ex vivo functional parameter assessments (final N=12, 13 and 11 for WT, LM, and LMT groups, respectively). Exclusion criteria were the following: 1) a shallow increase in EDV with the linear increase in a flowrate (from 0.05 to 0.8 ml/min) suggesting leakage, 2) quick drop of ejection fraction values <30% at the higher flowrates due to invisible damages.

Isoproterenol injection

A bolus of isoproterenol (Isoprenaline hydrochloride; Sigma-Aldrich, 10^{-7} moles in 0.2 ml of fish Tyrode) was injected by syringe directly into the perfusion line right before the heart via injection port at flow rate of 0.2 ml/min. Immediately after injection we recorded a series of video files within a minute; the effect usually develops within 10 seconds, lasts for 40-60 seconds, and then vanishes. We averaged results from 3 videos within a minute after injection.

4.6. Shape index and red channel intensity

To calculate ventricular shape index, we employed the following formula:

$$SI = A / P^2 \quad (5)$$

where A is area, and P is perimeter of ventricle averaged from the two perpendicular planes at diastole. If ventricle has bigger amount of muscle fibers per volume, denser muscle tissue will affect the shape of the heart - more tissues will result in more concave shapes. Biggest area in smaller perimeter is a well-known characteristic of circle: in circle, SI equals ¼π or 0.0796; in equilateral triangle, this index is close to 0.048.

Red channel intensity (RCI) was assessed in the region of interest (400 x 400 pixels) in images of the ex vivo Langendorff-like perfused hearts at diastole using ImageJ software (National Institutes of Health).

4.7. Quantification of atrial volume

We employed the same setup as we used for Langendorff-like heart perfusion. Instead of inserting a catheter via atrioventricular orifice, we inserted the cannula via outflow tract to introduce retrograde flow.

For the atrial area quantification, we took photos of none-beating inflated atria from two perpendicular planes using the 45° mirrors. Images were analyzed using ImageJ software (National Institutes of Health). We averaged areas obtained from these two perpendicular planes and normalized them by BW. N=3 atria were analyzed for each group (*lamp2*$^{e2/e2}$ and their wild-type siblings; no exclusion).

4.8. In vivo echocardiography

We employed Vevo 3100 high-frequency imaging system equipped with a 50 MHz linear array transducer (FUJIFILM VisualSonics Inc.). Acoustic gel (Aquasonic®) was applied over the surface of the transducer to provide adequate coupling with the tissue interface. Adult zebrafish were anesthetized in tricaine (0.16 mg/ml) for 5 minutes, placed ventral side up into the sponge. The 50 MHz (MX700) transducer was placed above the zebrafish to provide a sagittal imaging plane of the heart. B-mode images were acquired with an imaging field of view of 9.00 mm in the axial direction and 5.73 mm in the lateral direction, a frame rate of 123 Hz, with medium persistence and a transmit focus at the center of the heart.

Image quantification was performed using the VevoLAB workstation similar to [27]. Ventricular chamber dimensions were measured from B-mode images in the sagittal plane: area of ventricle and long and short axis's lengths of ventricle at end-diastole and end-systole (L_{long} and L_{short}, respectively). Volume was calculated using a single-plane formula:

(6) $V = 8A^2 / 3\pi L_{long}$

whereby, A is area, and L_{long} is long axis dimension. For every fish/heart, an average of 3-5 consequent cardiac cycles were taken. All indices (EF, FAC, FS) were calculated similar to ex vivo (see Methods section 4.5).

4.8. Trichrome staining of paraffin sections

Whole hearts of adult zebrafish were fixed in 4% formaldehyde. Samples were embedded in paraffin by the Pathology Research Core in Mayo Clinic and sectioned at 5 μm thickness. The sections were stained with the Accustain Trichrome Stain (Masson) Kit (Sigma- Aldrich, HT 15) and mounted with Permount mounting medium (Fisher Chemical). Images were obtained using a Nikon Eclipse microscope equipped with a SPOT RT3 Camera.

Density of myofibers (myocardium area in % to total area) was calculated in 3 standard areas of images (400 x 400 pixels) obtained from the trichrome stained sections using a 20x objective. To label myocardium tissue we applied standard thresholding function in ImageJ (NIH). The threshold value was set the same through the analysis. Accuracy of thresholding was controlled by placing side-by-side with unprocessed images. We checked three clutches of fish (WT, LM; LMT – one clutch) for consistency; for the analysis we took the last clutch with N=6 for wild-type and N=5 for *lamp2*$^{e2/e2}$.

4.9. Single cardiomyocyte isolation and morphometry

Single ventricular cardiomyocytes were isolated by enzymatic digestion as previously described [39, 58]. Briefly, excised hearts were perfused with low calcium fish Tyrode solution containing collagenase type II (0.2 mg/ml; Worthington Biochemical Corporation) and trypsin type IX-S (0.12 mg/ml; Sigma) for 15-20 min at 0.5 ml/min flowrate. Cardiomyocytes were photographed in fish Tyrode solution in tissue chamber on inverted microscope Nikon Axiovert 135 TV with the help of standard camera. Length and width of the cells were measured using ImageJ software (NIH).

4.10. Electrocardiography and signal filtration

Zebrafish were anaesthetized 5 min in 0.16 mg/ml buffered tricaine (MS-222) solution, and then placed in a shielded chamber with ECG electrodes (iWorx Inc.). An open-chest surgery was carried out prior to data acquisition in order to enhance the signal quality [59]. A standard ECG gel was applied between electrodes and the skin. ECG signals were recorded for 2 minutes at the sampling rate of 1000 Hz (iWorx). Signals were filtered using the Wavelet and thresholding techniques in Matlab (MathWorks) [37, 59].

Here, a novel signal processing scheme was applied to clear the unwanted components and to provide fast automated ECG analysis [60]. First, the ECG signal was processed to remove glitches, mostly caused by spontaneous or accidental extreme movements of the fish or probe station. Second, the Wavelet filtering and thresholding technique was applied to further filter out the non-ECG components [36, 37, 60]. Specifically, glitches of the coming ECG signals were detected by finding the maximum and minimum values and thresholds were defined. The ECG data would be then reconstructed with the non-ECG components being removed. The ECG data were then further processed to remove noise components such as baseline wander (breathing/gill motion interferences) caused by low-frequency and high-frequency noise. Data analysis was achieved by an automated manner as previously reported in [37, 60].

4.11. Single myofibril technique

Single myofibrils were prepared as previously described [39, 61, 62]. Briefly, extracted hearts were washed in phosphate buffered saline, frozen in liquid nitrogen and kept at -80 ºC. On a day before experiment, hearts were placed to 1% (v/v) Triton in standard relaxing buffer supplemented with protease inhibitors at 4ºC overnight. Then, permeabilized zebrafish heart ventricles were homogenized in ice-cold relaxing buffer at 20,000 rpm for 10 s (homogenizer MDT500, 5 mm probe, MicroDisTec, Switzerland). The pellet of myofibrils can be stored at 4ºC for up to 3 days. A droplet of myofibril suspension was placed into the tissue bath for subsequent mounting to fire-polished glass micro-tools in relaxing solution containing 10 mM EGTA. The left probe was stiff serving for fiber stretching; the right probe was complaint serving as a force transducer. The tip of the right probe was ~1 μm and its stiffness was calibrated to be 10-20 nN/μm. The attached myofibril was then superfused with alternating solution streams of relaxing and activating solutions (1 mM EGTA) emanating from a double-barreled pipette (~180 μl/min) that was mounted on a translation stage capable of rapid (<5ms) solution switches. Bath solution contained 10 mM EGTA.

Activation of myofibril led to its shortening, therefore displacement of the right probe. Position of the left and right probes and a single myofibril sarcomere length were detected optically with the use of fast camera Teledyne Dalsa Genie HM640 and HVSL software (Aurora Scientific, Canada). Release-restretch manneur (release is ~20% of the fiber length) was performed by using piezo controller Thorlabs (USA) driving the left probe. All motors, perfusion system (Warner Instruments) and recordings were controlled by custom written Labview software (by courtesy of Dr. Pieter de Tombe). Sarcomere length 2.0 μm was used for force-pCa experiments. In order to generate force-pCa curve, myofibrils were

subjected to various pCa activating buffers (in a range of 5.5 - 6). Passive stiffness test was performed by releasing of fiber at gradually increasing sarcomere/fiber length (from slack position to SL=2.4 μm).

Rates of activation (k_{ACT}), rapid release-restretch force redevelopment (k_{TR}), and bi-phasic relaxation (k_{lin} and k_{exp}), together with the duration of the slow linear relaxation phase (T_{lin}) were analyzed by linear- and exponential- curve fitting using offline custom in-house written software (Labview, MathWorks). The compositions of bath, relaxing, and activating solutions were as previously described [39]. Exclusion criterion for fibers was rundown of developed tension more than 20% per set of contractions (6-10). All zebrafish single myofibril mechanics experiments were done at 10°C.

4.12. Western Blotting

Zebrafish hearts were excised from anaesthetized 11 months-old fish after 24-hrs fasting. Ventricles were dissected in cold phosphate buffered saline and frozen in liquid nitrogen. Ventricles were mechanically homogenized in RIPA buffer (Sigma) supplemented with the Protease Inhibitor Cocktail (Sigma). Western blotting was performed according to standard protocols. The following antibodies were used: anti-mTOR(1:1000, Cell Signaling Technology), anti-phospho-mTOR (1:1000, Cell Signaling Technology), anti-S6 ribosomal protein (1:1000, Cell Signaling Technology), anti-phospho-S6 ribosomal protein (1:1000, Santa Cruz Biotech) and anti-LC3 (1:1000, Novus Biologicals). Anti-GAPDH (1:2000, Santa Cruz Biotech) was used as an internal control.

We assessed three clutches of fish. For statistical analysis we took the last clutch with N=4 fish for each group after 24-hrs fasting (no exclusion).

4.13. Transmission Electron Microscopy

Excised hearts were rinsed in a phosphate buffered saline and then fixed in the Trump's solution (4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2) at room temperature. Fixed tissues were then processed and imaged at the Mayo Clinic Electron Microscopy Core Facility using a Philips CM10 transmission electron microscope. We assessed one clutch of fish. For statistical analysis we employed N=3 fish for each group.

4.14. Statistical analysis

Unpaired two-tailed Student's *t* test was used to compare 2 groups. One-way analysis of variance (ANOVA) was used to analyze differences among multiple groups. The log-rank test was used to determine the difference in animal survival. In graphs, each value represents the Mean ± SD if not otherwise mentioned. *P* values less than 0.05 were considered to be significant.

Author contribution

A.V.D., J.Y., X.X. designed experiments, A.V.D., J.Y., M.W. performed experiments and analyzed data, A.V.D. wrote the manuscript, X.X. edited the manuscript

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Figure Captures

Figure 1. Generation of *lamp2*$^{e2/e2}$. A. 5-nt deletion in the 2nd exon of *lamp2* gene results in premature stop codon. Sequences targeted by TALEN are underlined. SacI recognition site is boxed, which was used for genotyping. B. Representative images of wild-type (WT) and *lamp2*$^{e2/e2}$ (LM) fish at 9 months of age. Scale bar is 1 cm. C. Body weight is reduced in *lamp2*$^{e2/e2}$ fish (N=12). D. Swimming capacity (U_{cri}) is decreased in *lamp2*$^{e2/e2}$ fish (N=7). E. Survival plot (N=21). F. *lamp2* transcript is effectively depleted, as shown by qRT-PCR (N=3).

Figure 2. Ventricular remodeling in *lamp2*$^{e2/e2}$ hearts. A. Images of isolated ex vivo perfused hearts. Ventricles in *lamp2*$^{e2/e2}$ appear rounder. Scale bar is 1 mm. B. Shape index (area over perimeter squared) is increased in *lamp2*$^{e2/e2}$ fish (N=15 for wild-type and 16 for *lamp2*$^{e2/e2}$). C. Trichrome-stained heart slices show denser trabeculae myocardium. Scale bar is 50 μm. D. Quantification of C (N=6 hearts for wild-type and N=5 for *lamp2*$^{e2/e2}$). E. Increased end-systolic volume at low flow (0.05 ml/min; ESV_{05}) in *lamp2*$^{e2/e2}$ hearts. F. Red channel intensity is bigger in *lamp2*$^{e2/e2}$ hearts, supporting denser tissue. G. Reduced ejection fraction (EF%), H. Reduced fractional area contractility (FAC%), and I. reduced radial strain both suggest compromised cardiac pump function in *lamp2*$^{e2/e2}$ (LM). Shown in G-I are ex vivo studies of Langendorff-like perfused hearts (N=12). J. qRT-PCR to quantify transcripts of fetal gene program (N=6 and 7 for WT and LM, respectively).

Figure 3. Atrial hypertrophy in *lamp2*$^{e2/e2}$. A. Representative electrocardiograms. Note increased magnitudes of P-waves in *lamp2*$^{e2/e2}$ (LM, right panel). B. Quantification of ECG parameters: P/R ratio is increased (*: $p<0.05$). C. Representative images of the inflated atrium. Atria in *lamp2*$^{e2/e2}$ appear bigger and less transparent. Scale bar is 0.8 mm. D. Quantification of the area of atria averaged from two perpendicular planes and normalized by BW (N=3). E. Trichrome staining of the atrial slices. Additional trabeculae were noted in atria of *lamp2*$^{e2/e2}$. Scale bar is 100 um.

Figure 4. *mTOR* inhibition alleviates cardiac dysfunction and blunted β-adrenergic response in *lamp2*$^{e2/e2}$. A-C. In vivo parameters of cardiac pump function via HFE. Both ejection fraction (EF%) and fractional area contractility (FAC%) were reduced in *lamp2*$^{e2/e2}$ (LM), and were partially rescued in *lamp2*$^{e2/e2}$; *xu015*$^{+/-}$ (LMT). Fractional shortening (FS%) at the long axis (LAX), but not short axis (SAX), was rescued. D-J. Ex vivo indices of the response to isoproterenol. The ISO-induced increase of EDV (ΔEDV) and EF (ΔEF) were blunted in LM, but rescued in LMT group. By contrast, the ISO-induced increase in ESV (ΔESV) was not affected. (Mean ± SEM in the ex vivo ISO response experiment). * - $p <$ 0.05 to WT # - $p<0.05$ to LM group (rescue)..

Figure 5. *mTOR* inhibition normalizes maximal isometric tension and kinetics of activation in single myofibrils. A. A representative image of a single myofibril preparation attached to the glass micro-tools. B. Schematics of the calcium activation and force re-development traces in the single myofibril during release-re-stretch maneuver (*) in WT and LM groups; C and D. Rates of calcium activation (k_{ACT}) and force re-development (k_{TR}) are both higher in *lamp2*$^{e2/e2}$ (LM) and normalized in double mutant (LMT). E. Parameters of the rate of fast exponential relaxation (k_{EXP}) have no difference among three groups. F. Maximal tension is increased in *lamp2*$^{e2/e2}$ (LM), but rescued in *lamp2*$^{e2/e2}$*; xu015*$^{+/-}$ (LMT). G. Calcium sensitivity (pCa_{50}) is reduced only in double mutant (LMT). H. Hill coefficient is decreased in both LM and LMT groups. * - $p < 0.05$ to WT # - $p<0.05$ to LM group (rescue).

Figure 6. Autophagy defects in *lamp2*$^{e2/e2}$. A. Shown are western blots of the cardiac protein extracts to analyze mTOR-autophagy signaling. B. Quantification of Western Blotting (A) and numbers of the autophagic vacuoles (C). C. TEM photographs of cardiac ultra-thin slices. Arrowheads indicate autophagic vacuoles. Scale bar (upper panel): 5 μm. Scale bar (lower panel): 1 μm. *: $p<0.05$ with wild-type. **: $p<0.01$ with wild-type.

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