Haploinsufficiency of Target of Rapamycin Attenuates Cardiomyopathies in Adult Zebrafish

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Rationale: Although a cardioprotective function of target of rapamycin (TOR) signaling inhibition has been suggested by pharmacological studies using rapamycin, genetic evidences are still lacking. We explored adult zebrafish as a novel vertebrate model for dissecting signaling pathways in cardiomyopathy.

Objective: We generated the second adult zebrafish cardiomyopathy model induced by doxorubicin. By genetically analyzing both the doxorubicin and our previous established anemia-induced cardiomyopathy models, we decipher the functions of TOR signaling in cardiomyopathies of different etiology.

Methods and Results: Along the progression of both cardiomyopathy models, we detected dynamic TOR activity at different stages of pathogenesis as well as distinct effects of TOR signaling inhibition. Nevertheless, cardiac enlargement in both models can be effectively attenuated by inhibition of TOR signaling through short-term rapamycin treatment. To assess the long-term effects of TOR reduction, we used a zebrafish target of rapamycin (ztor) mutant identified from an insertional mutagenesis screen. We show that TOR haploinsufficiency in the ztor heterozygous fish improved cardiac function, prevented pathological remodeling events, and ultimately reduced mortality in both adult fish models of cardiomyopathy. Mechanistically, these cardioprotective effects are conveyed by the antihypertrophy, antiapoptosis, and proautophagy function of TOR signaling inhibition.

Conclusions: Our results prove adult zebrafish as a conserved novel vertebrate model for human cardiomyopathies. Moreover, we provide the first genetic evidence to demonstrate a long-term cardioprotective effect of TOR signaling inhibition on at least 2 cardiomyopathies of distinct etiology, despite dynamic TOR activities during their pathogenesis. (Circ Res. 2011;109:658-669.)

Key Words: doxorubicin | cardiotoxicity | cardiomyopathy | target of rapamycin | zebrafish | anemia

Because of its advantageous embryology and unique genetic tools, zebrafish embryo has become a recognized vertebrate model for studying genetic aspects of human diseases.1 However, many human diseases, such as cardiomyopathy and heart failure, are adult diseases with a progressive pathogenesis, which are difficult to be recapitulated in zebrafish embryos. To explore the potential of adult zebrafish for cardiomyopathy studies, we developed the first adult zebrafish model for cardiomyopathy induced by anemia.2 We characterized pathogenesis in the heart of tr265, an anemic mutant incurred by a Band 3 mutation in erythrocytes that was identified from an ENU mutagenesis screen.3 The chronic anemic stress to the heart induces profound cardiac enlargement, followed by cardiomyopathy-like phenotypes.2 Because of the unique regeneration capacity of a zebrafish heart, both cardiomyocyte (CM) hypertrophy and hyperplasia contribute to the pathogenesis in the tr265 fish. To validate adult zebrafish as a conserved vertebrate model for cardiac diseases, its cardiac responses to other cardiomyopathy-inducing biomechanical stresses must be assessed.

In the present study, we report the second adult zebrafish model of cardiomyopathy induced by doxorubicin (DOX, adriamycin). In contrast to the anemia model, we found that CM hyperplasia does not contribute to this type of cardiomyopathy. DOX is an anthracyclin drug that has been used in the treatment of a broad spectrum of cancers. However, overdose of DOX imposes toxicities on the heart,4 which can be

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categorized into 2 different types.5,6 Acute cardiotoxicity occurs immediately after the administration of DOX, presenting as hypotension, transient cardiac rhythm disturbance, or reduced heart size. By contrast, chronic cardiotoxicity appears years or decades after patient exposure to DOX, typically presenting as an irreversible, cumulative, and dose-dependent cardiomyopathy that finally leads to congestive heart failure.5,6 Mechanistically, it has been hypothesized that DOX-induced cardiotoxicity is mediated by activation of free radical–induced mitochondrial damage, which in turn imposes oxidative stress on the heart and triggers apoptotic CM death.7,8

One of the major goals of cardiomyopathy studies is to identify a common pathological signaling pathway that can be intervened to achieve therapeutic benefit. Target of rapamycin (TOR) pathway is one of the candidates that interprets a variety of inputs, including nutritional status, energy level, growth factors, as well as cellular stresses, and then coordinates the regulation of cell growth, cell proliferation, and cell death.9 A cardioprotective function of TOR signaling inhibition has been suggested by pharmacological studies using rapamycin, a specific inhibitor of TOR. In mice, rapamycin prevents cardiac hypertrophy induced by aortic constriction,10,11 by thyroid hormone,12 or in the LEOPARD syndrome.13 In humans, the treatment of patients after cardiac transplantation with rapamycin significantly attenuates left ventricular hypertrophy.14 However, this notion, based on pharmacological studies, has not been supported by direct genetic analysis of components in TOR signaling.15

In this study, we first set up a DOX-induced cardiomyopathy model in adult zebrafish. We then sought to utilize the zebrafish target of rapamycin mutant (ztor), which we have isolated from an insertional mutagenesis screen, to elucidate functions of TOR signaling in both DOX and our previously established anemia-induced cardiomyopathy models. Our data uncovered dynamic activity of TOR signaling in pathogenesis of cardiomyopathies and provides the first genetic evidence to support that TOR signaling is a common pathological pathway that can be leveraged for therapeutic benefits for cardiomyopathies of different etiology.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

CM Dissociation and Primary CM Culture
CMs from dissected ventricles of Tg(cmlc2:nuDsRed) fish or tr265 fish were dissociated as described previously.2 The newly dissociated CMs usually attached to the cell culture chamber within 1 hour, which allows us to capture images for CMs area measurement by outlining each individual CM using ImageJ software (NIH).2 The CM identity was determined by either the nuDsRed reporter in the Tg(cmlc2:nuDsRed) transgenic fish, Mef2, or α-actin antibody staining. Both rod- and round-shaped CM populations were noted during the primary CM culture. Twenty to 40 rod-shaped CMs were identified for size quantification.

Cardiac Functional Analysis in Adult Fish
For fraction shortening (FS) measurement, casper:Tg(cmlc2:nuDsRed) fish were anesthetized in 0.16 mg/mL tricaine for 2.5 minutes and placed ventral side up on a slotted sponge. Movies of beating hearts were recorded using a digital camera attached to a fluorescent Leica MZ FLI III microscope. Images from movies were then used to measure the axis length between the myocardial borders of the ventricles at diastole and systole, respectively (see Online Figure II). The percent fraction shortening (FS%) was calculated using the formula FS% = (length at diastole−length at systole)/length at diastole×100. The red blood cell flow rate was measured as described previously.2 More than 6 fish were used for each experimental group.

Transposon-Mediated Insertional Mutagenesis Screen
The gene-breaking transposon P9 was generated by subcloning the SB-P6 GBT16 into a nearly full-length Tol2 vector.17 In-crosses were conducted for each founder fish family to identify families exhibiting embryonic lethal phenotypes; xu015 is 1 of the 41 embryonic-lethal mutants identified from this screen.

Statistics
All values are shown as mean±standard error (SEM, n≥3 otherwise noted). Student t test was used for comparisons between 2 special groups, and ANOVA was used for assessment differences among 3 or more groups. Probability values for the analysis of survival rates were determined by log-rank test. A value of P<0.05 was considered significant.

Results
DOX Induces Progressive and Pathological Cardiomyopathy in Adult Zebrafish
To assess DOX-induced cardiac responses in adult zebrafish, we injected DOX intraperitoneally into casper fish, ages 2 months to 1 year. The casper fish was chosen because of its transparent body at adult stage that facilitates both DOX injection and cardiac function measurement.18 On the basis of mice studies,19,20 we empirically determined the proper dose. Injection of 50 μg/g body mass (gbm) DOX led to severe mortality, as well as significant loss of body and heart mass at 4 weeks after injection, although the heart surface area/body weight index remained unchanged (Figure 1A and 1B and Online Figure I). In contrast, injection of 20 μg/gbm DOX bypasses the severe mortality. Most fish survived this
dose without a detectable change in body shape or size. However, a significantly enlarged heart was detected at both 4 weeks and thereafter, such as 12 weeks after injection (Figure 1A and 1B and Online Figure I). To assess whether the enlarged heart is due to myocyte hypertrophy, we dissociated individual hearts and isolated single CMs, whose identity was indicated by either α-actinin or MeF2 antibody staining (Figure 1C). Average size of individual CMs was significantly enlarged (Figure 1C and 1D). The width but not the length of individual CMs was increased (Figure 1E and 1F), confirming a hypertrophic growth.

Because of its small size, a beating zebrafish heart has been difficult to be documented by echocardiography-based imaging technology. We developed an alternative approach by crossing the semitransparent casper with the Tg(cmlc2:nuDsRed) line, which fluorescently labels all CMs nuclei red. The resulted casper: Tg(cmlc2:nuDsRed) fish line allows the documentation of a beating heart using a fluorescent microscope directly. The recorded movies were then used to calculate fraction shortening and heart rate (Online Figure II, A). We detected reduced fraction shortening starting at 4 weeks after injection (Figure 1G) but unchanged heart rate (Online Figure II, C). To validate the cardiac function study, we further quantified red blood cell (RBC) flow rate, an assay developed to reflect cardiac function indirectly. We detected a reduction of RBC flow rate in the DOX-treated fish beginning at 4 weeks after injection (Online Figure II, B).

We then examined hallmarks of pathological cardiomyopathy in the DOX-injected fish. Expression of the atrial natriuretic factor (anf), a fetal gene and molecular marker of CM hypertrophy, was significantly upregulated after DOX injection (Figure 1H). Muscular disarray and myofibril loss were detected at 12 weeks after DOX injection through either α-actinin antibody staining or transmission electron microscopy (TEM) verified muscular disarray and myofibril loss in fish hearts at 6 months after 20 μg/gbm DOX injection. Significantly decreased FS was detected at 4 weeks and thereafter, after DOX injection (Figure 1I). Together, these data suggest that DOX induces progressive and pathological cardiomyopathy in adult zebrafish. The progression from adaptation phase to decompensation phase occurs at around week 4 after injection, which continues to progress into more severe cardiomyopathy at later stages.

Dynamics of TOR Signaling During Pathogenesis of 2 Adult Zebrafish Models of Cardiomyopathy

To assess the role of TOR signaling in DOX-induced cardiac responses, we measured the level of pS6K, an essential downstream signaling axe of TOR. We did not detect significant changes of pS6K level at 3 days after DOX injection, whereas the total protein level of S6K remained unchanged (Figure 2A).

We also measured pS6K level in our previously established tr265 anemia model. Our previous studies of this model revealed CM hypertrophy at the 3- to 10-week-old tr265 fish. However, a small portion of the tr265 fish that survived to weeks 12 to 16 are characterized by heart enlargement consisting of increased number of CMs with smaller size. Consistent to the notion that tr265 fish at week-6 and week-12 ages represent 2 types of cardiac enlargement with different cellular nature, we detected upregulated pS6K at week 6 but downregulated pS6K level at week 12.
at week 12 (Figure 2B). Together, our data suggested dynamic TOR activity along the pathogenesis of 2 cardiomyopathy models.

Temporal TOR Reduction Through Rapamycin Treatment Attenuates Cardiac Enlargement Induced by Either DOX or Anemia

Next, we assess the effect of rapamycin treatment on different models of cardiomyopathy. First, we found that rapamycin treatment can effectively reduce pS6K level in both the DOX and anemia models (Figure 2A and 2B). Second, we detected that 1-week rapamycin treatment effectively attenuated DOX-induced cardiac enlargement at both week 4 and week 12 after injection (Figure 2C and 2D). Similarity, we also found that the heart enlargement was significantly attenuated by rapamycin treatment at both week 4 and week 7 but not week 12 fish compared with sibling with or without rapamycin (0.4 μmol/L) treatment. (*P<0.05. ns indicates not significant; V, ventricle; A, atrium; and OFT, outflow tract.

Sustained TOR Reduction Through ztor+/− Attenuates Cardiomyopathy Induced by Either Anemia or DOX

We have conducted an insertional mutagenic screen using P9, a Tol2-based transposon vector containing a gene-breaking cassette, and identified xu015, a recessive embryonic lethal zebrafish mutant (Online Figure III, A). Through linker-mediated PCR (LM-PCR), a P9 insertion was detected in the intron between exon 5 and exon 6 of the zebrafish target of rapamycin (ztor) gene (Figure 3A). The linkage between this P9 insertion and the xu015 mutant phenotypes was established by genotyping 20 xu015/xu015 embryos (LOD score >6, Online Figure III, B). It was predicted that the insertion of P9 into an intron would hijack the splicing machinery of the target gene and lead to generation of 2 end transcripts, an N-terminal cDNA consisting of the first 5 exons and a C-terminal cDNA that fused with the GFP in the Tol2 construct. Indeed, both end products can be detected by reverse transcription polymerase chain reaction (Online Figure IIIIC). The mRNA level of ztor was reduced in xu015 mutant to around 11% of that in normal sibling (Figure 3C). The TOR protein level was also reduced, as well as the body length (Figure 2F and data not shown). This observation validated the notion that 11- to 12-week-old tr265 fish represents a unique pathological stage that is characterized by reduced CM size and reduced TOR signaling. Together, our data demonstrated a conserved function of rapamycin in attenuating cardiac enlargement in zebrafish models of cardiomyopathies, as has been previously shown in mammalian models and humans.
and its downstream branches of TORC1 and TORC2 in 6-month old
staining with antibodies against PCNA and
days). p4E-BP1 level is indicated by the lower band. *p<0.05.

The ztor<sup>xu015</sup> embryos appeared normal until 6 days after
(post) fertilization (dpf), when the larvae became less active and exhibited characteristic dark layers at 7 dpf (Figure 3B). Most of the embryos died at 10 dpf. The size of ztor<sup>xu015</sup> embryonic heart appeared smaller at diastole but not systole (Online Figure III, D). Both significantly reduced CM proliferation and cell size was observed in the ztor<sup>xu015</sup> embryonic heart at 7 dpf, as revealed by immuno-
stimulating with antibodies against PCNA and β-catenin (Figure 3E through 3H). However, the ztor<sup>1/2</sup> fish exhibited no overt phenotype until at least 9 months of age, as
evidenced by their normal body length and body weight
(data not shown). The TOR protein level, however, is
reduced (Figure 3I), indicating that both TORC1 and TORC2 are affected. Similar changes of TOR downstream branches were detected in fish treated with 0.2 μmol/L rapamycin 12 hours daily for 7 consecutive days. However, fish treated with 0.4 μmol/L rapamycin 4 hours daily for 7 consecutive days exhibits reduced pS6K but activated

pAKT(ser473), which is consistent to the existence of a negative feedback regulation by TORC1 to PI3K-AKT
signaling. Consistent with the notion that 4E-BP1 is a
negative downstream effector of TOR signaling, we de-
creased upregulated p4E-BP1 level in adult ztor<sup>xu015</sup> heterozygous fish (ztor<sup>1/2</sup>) compared with that in wild-type siblings treated with or without rapamycin (0.4 μmol/L 4 hours daily or 0.2 μmol/L 12 hours daily for consecutive 7
days). p4E-BP1 level is indicated by the lower band. *p<0.05.

Figure 3. Isolation of a zebrafish target of
rapamycin hypomorphic mutant: ztor<sub>xu015</sub>. A, A P9 insertion was inserted within the 5th
intron of the zebrafish target of rapamycin (ztor) gene at chromosome 8 in the ztor<sub>xu015</sub> mutant. Locations of the primers used in
LM-PCR and genotyping are indicated by
arrows. B, The homozygous ztor<sub>xu015</sub> mutant (ztor<sup>xu015</sup> ) embryos appeared to be smaller compared with their normal siblings and displayed dark liver phenotypes at 7
days after fertilization (dpf). Arrowheads indicate location of the liver. C, Quantitative reverse transcription polymerase chain rea-
tion revealed that the mRNA level of ztor was reduced by about 90% in the ztor<sup>xu015</sup> com-
pared with that in normal siblings at 7 dpf.
D, Western blot analysis confirmed a dra-
matic reduction of Ztor protein in the ztor<sup>xu015</sup>
mutant at 7 dpf. In addition, phosphor-
ribosomal S6K was also dramatically reduced, whereas the total ribosomal S6K level remained unchanged in the ztor<sup>xu015</sup> mutant. E, Representative images of dis-
sected fish hearts at 7 dpf after coimmuno-
sted with Mef2 (green) to label CMs and
PCNA (red) to label proliferative cells. Scale bar=10 μm. Arrowhead indicates a prolifera-
ting CM. Arrow indicates a proliferating
non-CM. F, Quantification of CM proliferation index represented in E. G, Representative images of dissected fish hearts at 7 dpf after coimmunoconstaining with Mef2 (green) and
β-catenin (red) to define borders of CMs. H, Quantification of CM cell size represented in
I. Expression levels of total Ztor protein

and its downstream branches of TORC1 and TORC2 in 6-month old ztor<sub>xu015</sub> heterozygous fish (ztor<sup>1/2</sup>) compared with that in wild-type siblings treated with or without rapamycin (0.4 μmol/L 4 hours daily or 0.2 μmol/L 12 hours daily for consecutive 7
days). p4E-BP1 level is indicated by the lower band. *p<0.05.
after DOX treatment compared with that in wild-type control fish (Figure 4F).

We also assessed the long-term effect of TOR haploinsufficiency on the anemia model by generating a tr265: ztor+/− double mutant fish line. Similar to the DOX model, the enlarged heart size in tr265 was attenuated in the tr265: ztor+/− double mutant fish, as quantified by the ratio of ventricular size to body length index at 4 weeks of age (Online Figure IV, A and IV, B). In addition, an elevated RBC flow rate was observed, indicating an improved cardiac function (Online Figure IV, C). We went on to examine other hallmarks of cardiomyopathy and found that the expression level of anf was significantly reduced in the tr265: ztor+/− double mutant fish (Online Figure IV, D), as well as reduced muscular disarray and myofibril loss (Online Figure IV, F through IV, I). Importantly, the survival rate was also significantly improved in the tr265: ztor+/− double mutants than that in tr265 fish (Figure 4G). As a control, we detected no difference in the relative hemoglobin concentration at week 9 between tr265: ztor+/− and tr265 fish (Online Figure IV, E), suggesting that ztor haploinsufficiency did not restore hemoglobin level in anemic fish. Furthermore, we validated that ztor+/− fish exhibited attenuated cardiac enlargement induced by PHZ (Online Figure VI, A through VI, C). Together, the above data demonstrated that the suppression of TOR signaling exerts cardioprotective effects in both zebrafish models of cardiomyopathies.

Functions of TOR Signaling Inhibition on CM Hypertrophy

To elucidate the cellular mechanisms for the cardioprotective functions of TOR signaling inhibition, we first assessed CM hypertrophy. We found that the CM enlargement induced by DOX injection in the wild-type fish was significantly attenuated in the ztor+/− fish (Figure 5A). Similarly, the size of isolated CMs was reduced in tr265: ztor+/− double mutants compared with that in the tr265 fish at 4 weeks old (Figure 5B).

To test the hypothesis that the anti-CM hypertrophy function of TOR signaling inhibition is due to its direct action on CMs, we exploited zebrafish primary CM culture system. Immediately after dissociation and culture, we noticed rod-shaped cells whose geometry resembles typical CMs in mammals (Online Figure V, A). These CMs usually attached to the cell culture chamber within 1 hour and changed their shape after 3 days in culture, appearing to be more spread out with multiple filopodia (for a similar observation, see Reference 23), suggesting a dedifferentiation process of the CMs. To test the hypothesis, we exploited zebrafish primary CM culture system. Immediately after dissociation and culture, we noticed rod-shaped cells whose geometry resembles typical CMs in mammals (Online Figure V, A). These CMs usually attached to the cell culture chamber within 1 hour and changed their shape after 3 days in culture, appearing to be more spread out with multiple filopodia (for a similar observation, see Reference 23), suggesting a dedifferentiation process of the CMs. The sarcomere structures can be detected by either differential interference contrast imaging or α-actinin staining (Online Figure V, A and V, B). The averaged CM size remained mostly unchanged throughout the 7-day culture process, despite the change of cell shape (Online Figure V, C). Treatment with DOX for 2 hours at day 1 induced enlargement of CMs at day 5 in a dose-dependent fashion (Online Figure VII, B). This observation indicated that adult zebrafish
CMs exhibit a conserved hypertrophic response in mammals. Consistent to our in vivo studies, cotreatment with rapamycin effectively attenuated CM hypertrophy induced by DOX in vitro (Figure 5C and 5D).

Functions of TOR Inhibition on Myocyte Proliferation

In addition to myocyte hypertrophy (increased cell size), a zebrafish heart may respond to extrinsic or intrinsic stresses by changing CM cell number through cell proliferation or cell death. In the tr265 anemia model, we found that myocyte hyperplasia contributed to all stages of pathogenesis. Therefore, we assessed myocyte proliferation in the DOX model through PCNA staining of sectioned ventricles. The identity of CMs was indicated by costaining with a Mef2 antibody. We found that the CM PCNA index remained unchanged at either 3 days or 4 weeks after DOX injection (Figure 6A and 6C). There is also no significant PCNA index change detected in the tr265 fish at 6 weeks of age. Representative images of primary cultured CMs treated with 2 hours of DOX (5 μmol/L) with or without rapamycin (0.2 μmol/L) cotreatment. Scale bar = 20 μm. Quantification of CM cell size to show that DOX-induced cellular hypertrophy was attenuated by rapamycin cotreatment. *P<0.05.

The tr265 anemia model of cardiomyopathy, increased proliferation was significantly attenuated by rapamycin treatment or in tr265; ztor+/- fish (Figure 6F and 6G). The similar observation was also made in the anemic model induced by PHZ (Online Figure VI, D). To validate that the antiproliferative function of TOR signaling inhibition is due to a direct action on CMs, we measured the BrdU index of CMs in vitro. Consistently, we found that BrdU index in CMs isolated from the tr265 fish were significantly reduced on rapamycin treatment (Figure 6E and 6H). Of note, this antiproliferation function of rapamycin on CMs was also detected in wild-type siblings both in vivo and in vitro (Figure 6D, 6E, 6G, and 6H).

Functions of TOR Inhibition on CM Apoptosis and Autophagy

Previous studies indicated that CM apoptosis contributes significantly to DOX induced cardiotoxicity but not to the tr265 model of cardiomyopathy induced by anemia. Therefore, we focused on the DOX model and assessed the contribution of CM apoptosis during DOX-induced cardiomyopathy, as well as the effects of TOR signaling inhibition. We found out that apoptosis index was dramatically induced at 4 weeks but not 3 days after DOX injection (20 μg/gbm) (Figure 7A, 7C, and 7D), suggesting its contribution to DOX-induced late-onset cardiomyopathy. In our in vitro CM culture system, we also observed a dose-dependent enlargement of CM size as well as activated apoptosis and increased CM loss (Online Figure VII, A and VII, C). Reduction of TOR signaling through either ztor+/- or 1-week rapamycin treatment effectively reduced the activated CM apoptosis in vivo (Figure 7A, 7C, and 7D). Consistently, the apoptotic signal was also significantly attenuated after cotreatment with rapamycin in vitro (Figure 7B and 7E). Together, our data indicated an antiapoptotic function for TOR signaling inhibition.

Prompted by the implication of autophagy in cardiomyopathies and DOX-induced cardiotoxicity, we assessed functions of autophagy in DOX-induced cardiomyopathy model. In contrast to pS6K, which is positively regulated by TOR, autophagy is a cellular degradation process that is negatively regulated by TOR. Lc3 II (microtubule-associated protein light chain 3 form 2) and p62/SQSTM1 are 2 molecular markers that have been widely used to assess autophagic flux. The levels of Lc3 II are proportional to the number of accumulated autophagic vacuoles, whereas the level of p62 protein, which is degraded by autophagy, is inversely related to autophagy activity. We detected activated autophagy at early stages after DOX injection but suppressed autophagy at later stages, as indicated by Western blot to reveal both the Lc3 II conversion and p62 degradation (Figure 8A). This observation was validated by quantifying Lc3 aggregation dots after injecting DOX into Tg(GFP-Lc3) transgenic fish (Figure 8B and 8C). Rapamycin treatment restored autophagy to a level that is comparable to that in wild-type at 12 weeks after injection (Figure 8C). Together, our data confirmed dynamic activity of autophagy.
Distinct Functions of TOR Signaling Inhibition Between DOX-Induced Acute Cardiotoxicity and Late-Onset Cardiomyopathy

Our data in zebrafish suggest a beneficial effect of TOR inhibition/autophagy activation on DOX-induced cardiomyopathy. However, 2 recent studies in rodent models indicated either a detrimental effect of TOR inhibition or a protective effect of TOR activation on DOX-induced acute cardiotoxicity.\(^{19,29}\) Prompted by the observation that autophagy activity is opposite at early stages versus later stages after DOX injection, we hypothesized that these seemingly conflicting conclusions resulted from stage-dependent functions of TOR. To test this hypothesis, we assessed adult fish injected with a higher dose of DOX (50 \(\mu\)g/gbm) to exaggerate its acute cardiotoxicity, which is characterized by reduced heart size and severe mortality. Similar to fish injected with a low dose of DOX (20 \(\mu\)g/gbm), activated autophagy and unchanged p-S6K levels were detected in fish at 3 days after high-dose DOX injection. Cotreatment with rapamycin further enhanced the autophagy activity and abolished most of the p-S6K level in both groups but had no significant impact on the level of total S6K protein (Online Figure VIII, A).

Figure 6. Effects of DOX and TOR inhibition on myocyte proliferation. A, Merged images of sectioned fish ventricles costained with PCNA (red) and Mef2 (green) at 4 weeks after 20 \(\mu\)g/gbm DOX injection with or without rapamycin (0.2 \(\mu\)mol/L) treatment. Insets are images of higher magnification. Arrows: PCNA+/Mef2+ cells; Arrowheads: PCNA+/Mef2− cells. Scale bar=20 \(\mu\)m. B, Merged images of primary cultured CMs costained with BrdU (green) and Mef2 (red) after 2 hours of DOX treatment (5 \(\mu\)mol/L) with or without rapamycin (0.2 \(\mu\)mol/L) cotreatment. Scale bar=20 \(\mu\)m. Arrows: BrdU+/Mef2+ cells; arrowheads: BrdU+/Mef2− cells. C, Quantification of the PCNA index in fish heart represented in A. D, Quantification of PCNA index in the sectioned ventricles of \(ztor^{+/−}\) fish with or without DOX injection at both 3-day and 4-week time points. E, Quantification of the BrdU index from primary cultured CMs represented in B. F, Evaluation of hyperplasia by quantifying CM PCNA index in sectioned hearts represented in \(tr265; ztor^{+/−}\) compared with that in \(tr265\). G, Quantification of PCNA index in \(tr265\) fish with or without rapamycin (0.4 \(\mu\)mol/L) treatment at 6 weeks of age. H, Quantification of primary cultured BrdU index in \(tr265\) fish with or without rapamycin (0.4 \(\mu\)mol/L) treatment in vitro. *P<0.05. ns indicates not significant.

at different stages of pathogenesis in DOX-induced cardiomyopathy, whereas depressed autophagy reflects the later decompensational hypertrophy stage. Therefore, we propose that the proautophagy function of TOR inhibition might also be accountable for its cardioprotective effects.
function during late-onset cardiomyopathy, rapamycin co-treatment increased the CM apoptosis index in high-dose DOX-induced acute cardiotoxicity (Online Figure VIII, C and VIII, D). Second, rather than a cardioprotective effect in fish cardiomyopathy models but similar to those in acute rodent cardiotoxicity models, cotreatment of rapamycin further exerted a deleterious effect on the high-dose DOX-induced acute cardiotoxicity in zebrafish, as indicated by further increased fish mortality (Online Figure VIIIIB). Together, our data strongly suggested that TOR signaling inhibition exerts opposite effects on DOX-induced acute cardiotoxicity and late-onset cardiomyopathy.

Discussion

DOX-Induced Cardiomyopathy Exhibits Different Pathogenesis From That of the Anemia Model

In the present study, we reported the second adult zebrafish model of cardiomyopathy that is induced by DOX. Cardiac enlargement occurs at 4 weeks after DOX injection, which further progressed to decompensational remodeling at later stages, exhibiting hallmarks of cardiomyopathy including activation of fetal gene expression, muscular disarray, and myofibrillar loss. The enlarged heart and CM hypertrophy is different from other rodent models that report reduced cardiac mass, increased CM atrophy, and dilated cardiomyopathy.19,20 The discrepancy is probably caused by different doses and/or different treatment protocols of DOX stresses, partially due to different focus on either acute or chronic effects of DOX.35 At the cellular level, pathogenesis in our DOX model is different from that in the anemia model. First, CM proliferation is not activated in the DOX model, whereas activated myocyte hyperplasia occurs at all stages of pathogenesis in the anemia model. Given the high regenerative capacity of a zebrafish heart, this was a surprising observation, suggesting that activated CM proliferation is not necessarily a default stress response in a zebrafish heart. Second, CM hypertrophy contributes to the enlarged heart in the DOX model at all stages, whereas CM hypertrophy can be only detected at early stages in the anemia model.2 Third, significantly activated apoptosis was detected in CMs in the DOX model, which had also been reported in other animal models for DOX-induced cardiotoxicity.27,28 In contrast, oncrosis is a more dominant cell death pathway of CMs in the anemic model.2

It is conceivable that the distinct cellular changes in the DOX model versus the anemia model are due to different stresses imposed on the heart. Anemia mainly imposes extrinsic stresses on the heart because of the significantly increased demand from the body to compensate for the reduced red blood cell concentration. In addition, hypoxic conditions within the heart might also impose an intrinsic stress. In contrast, DOX inflicts intrinsic damage on CMs, probably as the result of its disruptive function in mitochondria and consequent activation of oxidative stress in CMs.7,8 The intrinsic nature of this stress is underscored by the observation that DOX induces cellular hypertrophy and apoptosis in cultured CMs.

TOR Signaling Inhibition Exerts Cardioprotective Effects on Cardiomyopathies

Taking advantage of the forward genetic screen in zebrafish, we identified ztor mutant. Further studies based on the ztor+/− fish provided direct genetic evidences to support a cardioprotective
function of TOR signaling reduction on cardiomyopathy. This effect has been observed in 2 types of cardiomyopathies in adult zebrafish of different etiology, prompting an exciting hypothesis that activated TOR signaling is a common pathological event during cardiomyopathy, which can be intervened for therapeutic benefits.

Our results validated the cardioprotective function of TOR signaling inhibition suggested by pharmacological studies in mice. However, cardiac-specific knockout studies of Mtor and raptor in mouse reported an accelerated heart failure progression on pressure overload, suggesting important functions of TOR signaling in normal cardiac physiology and adaptive cardiac hypertrophy. An important difference between the present study and the mouse studies is the dose of the TOR signaling inhibition. In the mouse studies, Mtor signaling was ablated to such a low level that most mice die within 8 weeks. In contrast, TOR expression is only reduced to 65% at the protein level in ztor+/− fish, which allows these fish to survive to adulthood without any noticeable phenotypes. This statement can be better appreciated in the context that extreme starvation will lead to animal death, whereas regulated caloric restriction protects heart and prolongs their lifespan. Together, our results put forward an important concept, that is, it is critical to control the dose of TOR signaling inhibition to achieve therapeutic benefits for cardiomyopathies.

TOR has been found to be involved in 2 structurally and functionally distinct complexes. Whereas TORC1 contains mTOR, mLST8, and raptor and is sensitive to rapamycin, TORC2 contains mTOR, mLST8, rictor, mSIN1, and PRR5 and is insensitive to rapamycin. The downstream signaling pathways of the TORC1 complex have been found to regulate protein synthesis, autophagy, and transcription. In contrast, the downstream signaling pathways of the TORC2 complex are implicated in regulating actin cytoskeleton structure. As shown in Figure 3I, 1-week rapamycin treatment recapitulates the attenuated TORC1 signaling in ztor+/− fish, but TORC2 signaling can be either activated or attenuated, depending on the treatment protocols. Nevertheless, both rapamycin treatment protocols effectively attenuate cardiac enlargement, suggesting that this antihypertrophy function is likely conveyed by the TORC1 rather than the TORC2 complex.

At the cellular level, our data revealed that the cardioprotective effect of TOR signaling inhibition is due to its antihypertrophy, antiapoptosis, and prosurvival functions. Although our data derived from primary CM culture support a cell-autonomous function of TOR signaling within CMs, it is important to point out that our present approach cannot exclude the possibility that TOR signaling exerts its functions through other cell types in the heart, such as endocardial cells and fibroblasts.

It is of particular interest to note that autophagy is suppressed at later cardiomyopathy stages of the DOX model. Recent studies suggested that the protective autophagy level is reduced at later stages of heart failure, whereas activated autophagy can exert cardioprotective role by eliminating damaged organelles and/or toxic proteins that contribute to the pathogenesis of cardiomyopathy. Therefore, it is tempting to propose that the cardioprotective effect of TOR signaling inhibition might be conveyed by the activated autophagy. Further investigation is warranted to test this hypothesis.

Dynamic Activities of TOR Along Pathogenesis of Cardiomyopathies

A unique feature of TOR signaling revealed is that its activity varies significantly at different stages of pathogenesis of cardiomyopathies. In the anemia model, TOR-pS6K signaling is activated concurrently with predominant CM hypertrophy at the early stages but suppressed at the later stages. In the DOX-induced models, TOR-autophagy signaling of TOR is oppositely regulated during initial acute phase and later cardiomyopathy phase. Furthermore, TOR signaling inhibition exerts opposite effects on DOX-induced acute cardiotoxicity and late onset cardiomyopathy. This stage-dependent effect of TOR signaling explains the seemingly conflicting conclusion on the beneficial effect of TOR activation on DOX-induced acute cardiotoxicity, as reported in murine models, and the cardioprotective effect of TOR signaling inhibition on cardiomyopathies. Our results underscore the concept that cardiomyopathy is a progressive disease. It is important to note that distinct signaling events and/or even opposite
activities of the same signaling pathway might occur at different stages of pathogenesis. Together, our data present adult zebrafish as a novel and conserved vertebrate model organism for eliciting molecular mechanisms of cardiomyopathies. As exemplified by this study, novel discoveries on dose- and stage-dependent functions of TOR signaling in cardiomyopathies have been made, both of which will be instructive for designing proper therapeutic strategies for DOX-induced cardiomyopathies. Complementing to the existing rodent models, further investigation utilizing this emerging animal model promises to facilitate our understanding of human cardiomyopathies.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Rapamycin is a specific inhibitor of the target of rapamycin (TOR) signaling. It exerts cardioprotective effects on cardiomyopathy; however, genetic studies of functions of TOR signaling in cardiomyopathy have yielded conflicting results.
- The first adult zebrafish model of cardiomyopathy induced by chronic anemia has been generated; however, the value of this new vertebrate model in dissecting underlying signaling pathways remains untested.

What New Information Does This Article Contribute?

- Similar to that in rodents and human, doxorubicin induces cardiomyopathy in adult zebrafish.
- Partial reduction of TOR signaling in the zebrafish tor heterozygous mutant exerts cardioprotective effects on 2 cardiomyopathies of distinct etiology, providing the first genetic evidence, indicating that TOR signaling may be a common therapeutic pathway for cardiomyopathies.
- Both dose- and stage-dependent functions of TOR must be controlled properly when developing TOR-based therapeutics for cardiomyopathies.

Zebrafish is a promising vertebrate model that has the potential to dissect molecular mechanisms of cardiomyopathies through its powerful genetics. We report the generation of the second adult zebrafish model for cardiomyopathy induced by doxorubicin, which validates zebrafish as a conserved novel vertebrate model for genetic studies of cardiomyopathy. Together with the previously reported cardiomyopathy model induced by anemia, we investigated functions of TOR signaling in these 2 types of cardiomyopathies. Despite different etiology and pathogenesis, short-term treatment of rapamycin effectively attenuated cardiac enlargement in both models. However, this antihypertrophy effect was stage-dependent because of dynamic activities of TOR along the pathogenesis of these models. We assessed the long-term effect of TOR inhibition using a zebrafish tor mutant that we identified from an insertional mutagenesis screen. We found that long-term TOR signaling inhibition improved cardiac function and fish survival rate, providing the first genetic evidence to support a cardioprotective function of TOR inhibition. Taken together, our data suggest that TOR is a common therapeutic pathway that can be leveraged to develop therapeutics for cardiomyopathies of distinct etiology. Importantly, the dose- and stage- dependent functions may be instructive to developing TOR-based therapeutic strategies.
Haploinsufficiency of Target of Rapamycin Attenuates Cardiomyopathies in Adult Zebrafish

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SUPPLEMENTAL MATERIAL

Methods

Animals

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). The Institutional Animal Care and Use Committee (IACUC) at the Mayo Clinic approved the protocol of zebrafish usage for this study (permit number: A24010). Zebrafish are handled with care. Before euthanized, they were anesthetized in 0.16 mg/mL tricaine (Western Chemical). Fish were maintained on a 14 h light/10 h dark cycle at 28.5°C. The \( tr^{265} \) homozygous embryos and their wild type siblings were manually sorted under a dissecting microscope based on the amount of red blood cells at 4 days post-fertilization (dpf). At 3 weeks post-fertilization, \( tr^{265} \) and \( tr^{265}; ztor^{+/−} \) were distinguished by genotyping PCR.

Doxorubicin injection and rapamycin treatment

*Casper* fish aged from two months to one-year old were anesthetized in 0.16 mg/mL tricaine (Western Chemical) before subjected to doxorubicin (DOX) injection. The amount of injected DOX (Sigma) was determined based on body weight. Single dose of DOX dissolved in Hank’s buffered solution was injected intraperitoneally using a 28-gauge needle. For the acute rapamycin treatment in DOX model, fish were pre-incubated with 0.2 µmol/L rapamycin 12 h before DOX injection and later on for 12 h daily for consecutive three to seven days. For rapamycin treatment at week 4 and week 12 post-DOX injection, fish were incubated with 0.2 µmol/L rapamycin 12 h daily for 7 consecutive days before sacrificed for sample collections. For rapamycin treatment in anemia model, either \( tr^{265} \) homozygous mutants or their wild type siblings were incubated with 0.4 µmol/L rapamycin 4 h daily for 7 consecutive days. Usually more than 6 fish were used in each experimental group (\( n\geq6 \)).

Measurement of ventricular area to body weight /body length index

Due to the small size of an adult zebrafish heart, we use ventricular area instead of ventricular weight to more accurately quantify heart size. Since body length changes dramatically in juvenile fish, which has been adapted as a stage marker, we use the ratio of ventricular area to body length (VA/BL) as an index to assess heart size in juvenile fish younger than three months. In contrast, body weight varies more significantly in adult fish. Therefore, we use the ratio of ventricular area to body weight (VA/BW) as an index to assess heart size in adult fish older than three months. To measure ventricular area, individual zebrafish hearts were dissected out and imaged next to a millimeter ruler with a Nikon COOLPIX 8700 digital camera attached to a Leica MZ FLI III microscope. The largest projection of a ventricle was outlined using the ImageJ (NIH) software. To measure body weight, fish were anesthetized in 0.16-mg/mL tricaine solution, semi-dried on a paper towel, and weighted on a scale. Ventricle area to body weight was then determined by the largest projection area of ventricle (in \( mm^2 \)) divided by body weight (in g). To determine ventricle area to body length, the ventricle area in \( mm^2 \) was divided by body length (in mm). Body length was manually measured with a millimeter ruler, from the tip of the mouth to the body/caudal fin juncture.
Cardiomyocyte dissociation, primary cardiomyocyte culture and drug treatment

Cardiomyocytes (CMs) from dissected ventricles of Tg(cmlc2:nuDsRed) fish or tr265 fish were dissociated as described previously. Dissociated CMs were then resuspended in L-15 media containing 10% FBS (Invitrogen), and placed in Lab-Tek eight-well chambers (Thermo Fisher Scientific, Rochester, NY) and cultured at 28.5°C. The newly dissociated CMs usually attach to the chamber within 1 h, which allowed us to capture images for CMs area measurement by outlining each individual cardiomyocyte using ImageJ software (NIH). The cardiomyocyte identity was determined by either the nuDsRed reporter in the Tg(cmlc2:nuDsRed) transgenic fish or Mef2 or α-actinin antibody staining. For DOX treatment, the newly dissociated cells were cultured for 24 h before being exposed to DOX treatment for 2 h. For rapamycin co-treatment, CMs were pre-incubated with rapamycin (0.2 µmol/L) for the preceding 2 h, incubated with rapamycin during DOX treatment, as well as thereafter during cell culture. After DOX treatment, cells were cultured in L-15 media with 10% FBS for 5 days before subjected to CM size quantification. Both rod- and round-shape CM population were noted during the primary CM culture. We usually identify 20 to 40 rod-shape CMs for size quantification.

Immunostaining

Either frozen sections (12 µm) or primary cultured CMs were subjected to immunostaining using previously described methods. The primary antibodies including PCNA, 1:3000 (Sigma); α-actinin, 1:100 (Sigma); and Mef2, 1:200, (Santa Cruz Biotechnology) were used. For in vitro 5-bromo-2-deoxyuridine (BrdU) labeling, dissociated CMs were cultured in the L-15 media containing 100 µmol/L BrdU (Sigma) for 3 days. Cells were initially stained for Mef2 (1:100, Santa Cruz Biotechnology) to identify CMs. Next, cells were re-fixed in 4% PFA for 10 min and sequentially stained with monoclonal anti-BrdU antibody (Sigma). All images were captured using a Zeiss Axioplan II microscope equipped with ApoTome and AxioVision software (Carl Zeiss).

Quantitative RT-PCR

Either fish embryos at 7 days post-fertilization (dpf) or dissected fish hearts at different stages were frozen on dry ice and homogenized with a mortar and pestle. Total RNA was extracted using the RNeasy Mini Kit (Qiagen). One µg of total RNA was then reverse-transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). To analyze the expression level of ztor or anf, quantitative PCR was performed in a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) using iQ SYBR Green supermix (Bio-Rad). 18s rRNA was used as an internal control to normalize the ztor or anf level. The primer sequences are: ztor: (forward, 5'-CTACAAGGAGCTGGACTTC-3'; reverse, 5'-ATCTCCAATTCTCATAAGTG-3'), anf: (forward: 5'- AAGCAAAGCCTGGTCTGAG-3', reverse: 5'-ACTGTATCCGATATTGCAGC-3'), 18s rRNA: (forward: 5'-CAGTGCTCCCTCAAGAAGTGCC-3', reverse: 5'-GGTGGATCCGATAACGGAACGA-3').

Western blot analysis
Fish embryos or dissected hearts from indicated stages were manually homogenized with a pestle and a 27-gauge needle. For Xu015 embryos, both wild type siblings and mutant fish were treated with 200 nmol/L bafilomycin A1 (ALEXIS Biochemicals) for 12 h at 7 days post-fertilization (dpf) to inhibit autophagosome-lysosome fusion before being subjected to protein extraction. Samples were then lysed in SDS sample buffer (1 mol/L Tris-HCl pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 3.5% SDS) with protease inhibitor included (Roche Applied Science, Indianapolis, IN). Resultant protein extracts were subjected to western blot using a standard protocol. The primary antibodies including: TOR (1:2000), p-AKT(ser473)(1:2000), p4EBP-1(1:2000), pS6K (1:6000), S6K (1:6000) and AKT (1:8000) were purchased from Cell Signaling Technology; Actin (1:4000) was purchased from Santa Cruz Biotechnology; Lc3 antibody (1:2000) from Novus Biologicals, LLC); and p62 antibody (1:2000) from Sigma were used.

Linker-mediated PCR

Genomic DNA was extracted using the DNeasy blood and tissue kit (QIAGen) from the tail fin of adult Xu015 heterozygous fish. One µg of genomic DNA was digested with MseI for 3 h at 37°C. A linker was prepared by annealing oligonucleotides 1 (5’-TAGTCCCTTCTAGGAGGT-3’) and oligonucleotides 2 (5’-GATCGTCTCTGAGGAGGT-3’) at a final concentration of 100 µmol/L each. Annealed linker (10 µmol/L) was ligated to 5 µl of digested genomic DNA. The ligation product was used as a template for the first round of PCR amplification. The following first round of PCR primers including linker primer: 5’-GGATTGGCTGCTGCTAGATGAG-3’, and 5’LTR primer: 5’LTR, 5’-CCCTAGTACTGGTACTTTCTACTTG-3’ were used. The PCR product was diluted 1:100 in 10 mmol/L Tris-HCl, pH 8.3, and then used for the second round of nested PCR. The following second round of primers including linker-nested primer: 5’-AGTACAGGGCTTTAACGAGGGA-3’, and 5’LTR nested primer: 5’-CACTTGAGTAAAATTTTTGAGTAC-3’ were used. The resultant secondary PCR products were then resolved by agarose gel electrophoresis, purified using the QIAGen gel extraction kit and sent out for sequencing and BLAST to determine the P9 insertional flanking sequences.

Genotyping PCR

Xu015 mutants and normal siblings were distinguished based on their phenotypes at 7 days post-fertilization (dpf). Genomic DNA was extracted by incubating each embryo with 50 µl lysis buffer (1 mol/L Tris PH 8.3, 0.5 mol/L EDTA, 5 mol/L NaCl, 20% SDS, with freshly supplemented proteinase K (Roche) at 55°C overnight. After centrifuged at top speed for 10 min, the supernatant was diluted in 10 mmol/L Tris buffer (1:40), heated at 98°C for 10 min to denature the proteinase K and then used as a template for PCR amplification. The following primers were used (see Online Figure 1, B through D): forward, 5’-ATAAGAAAAGAAACCACATGTCATACC-3’; reverse, 5’-CTTACCCTCAGAGGACAAAG-3’; 5’LTR, 5’-CCCTAGTACTGGTACTTTCTACTTG-3’; and 3’LTR, 5’-GTACAGTAATCAAGTAAAATTACTCA-3’.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed as described previously. Briefly, dissected fish ventricles were fixed immediately in Trump’s solution at room temperature for 1 h followed by overnight at 4°C. Fixed samples were then processed and imaged by the Mayo Clinic’s Electron Microscopy Core Facility using a Hitachi S-
4700 Field Emission Scanning Electron Microscope or Philips CM10 Transmission Electron Microscope.

**TUNEL assay**

Either cryostat-sectioned ventricles (12 µm) or primary cultured CMs were first stained with the In-Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science) according to the manufacturer’s protocol. The TUNEL stained slides were then fixed in 4% PFA and sequentially stained with Mef2 antibody (1:100, Santa Cruz Biotechnology) to identify CM.

**Quantification of GFP-Lc3 autophagic aggregates**

Frozen sections (12 µm) of ventricles from DOX treated Tg(GFP-Lc3) transgenic fish were fixed in 4% PFA for 10 min and washed three times in PBS. Samples were then mounted in vectashield mounting material (Vector Laboratories, Inc). Images were captured with the same threshold setting using a Zeiss Axioplan II microscope equipped with ApoTome (Carl Zeiss). GFP-Lc3 dots in three independent visual fields per section were quantified using the AxioVision software (Carl Zeiss).
Online Figures and Figure Legends

Online Figure I. Dosage-dependent responses in adult zebrafish after DOX injection. A, Lateral view of two-month old casper fish at 4 weeks after a single doxorubicin (DOX) injection. Compared to the control fish injected with Hanks buffer, fish injected with 50 µg/gbm of DOX were smaller, while fish injected with 20 µg/gbm of DOX had normal body shape. Scale bar=1 cm. B, Body mass of fish at 4 weeks after 20 µg/gbm DOX injection remained mostly unchanged, while 50 µg/gbm DOX caused significantly reduced body mass at 4 weeks post-injection. C, Quantification of ventricle area (VA). Low-dose DOX induced cardiac enlargement, while high-dose DOX caused fish hearts to become smaller.
Online Figure II. Cardiac function can be quantified in an adult zebrafish using casper;Tg(cmlc2:nuDsRed) fish. A, Images extracted from movies of a beating heart at systole and diastole, respectively, are shown. Dashed lines indicate the measurements used to calculate cardiac function using the formula FS\% = (length at diastole − length at systole)/(length at diastole) × 100. Scale bar=1 mm. B-C, Time courses of cardiac functions including red blood cell (RBC) flow rate (B), and heart rate (C) in casper;Tg(nuDsRed) fish injected with 20 µg/gbm DOX compared to that in control. *P<0.05.
Online Figure III. Identification of the ztor\textsuperscript{\textit{xu015}} insertional mutant. A. Schematic diagram of the P9 construct used to generate insertional mutant in zebrafish. For details of the gene-breaking cassette, see. B. The linkage between the P9 insertion and the \textit{xu015} mutant phenotypes was established by genotyping analysis. Band 1 is a predicted PCR product amplified by the primers: R+5'LTR, representing a chromosome DNA with the P9 insertion; while band 2 is a predicted PCR product amplified by the primers: F+R, representing a chromosome DNA without the P9 insertion. In 12 out of 20 normal sibling embryos examined, we detected both band 1 and band 2, suggesting their genotype as ztor\textsuperscript{\textit{xu015}} heterozygosity (ztor\textsuperscript{+/\textit{-}}). We detected only the band 2 amplification from 8 out of the 20 normal siblings examined, suggesting their genotypes as wild type. However, out of all 20 \textit{xu015} mutant embryos examined, we detected only the band 1 amplification, confirming their genotypes as ztor\textsuperscript{\textit{xu015}} homozygosity (ztor\textsuperscript{\textit{-/-}}). C. Hijacked splicing events caused by the P9 element insertion were confirmed in the \textit{xu015} mutant by RT-PCR analysis. Lower band (~120 bp) reflects a splicing fragment resulted from the splice donor of exon 5 and the splice acceptor (SA) in P9; and the upper band (~450 bp) reflects a splicing fragment resulted from the splice donor (SD) in P9 and the splice acceptor in exon 6. D. Quantification of ventricular diameter in normal siblings compared to that in the \textit{xu015} mutants at 7 days post-fertilization (dpf). Diastolic length, but not systolic length was reduced in the \textit{xu015} mutant. *P<0.05; ns, not significant.
Online Figure IV. ztor haploinsufficiency improves cardiac function, alleviates hallmarks of pathological cardiomyopathy in tr265 fish. A-E, Ventricle area to body length (VA/BL) index (A); Body length (B); Red blood cell (RBC) flow rate (C); fetal anf gene expression (D); and percent hemoglobin concentration (E) at 9-weeks old tr265; ztor<sup>−/−</sup> fish compared to that in tr265 fish. F-G, Evaluation of muscular disarray by immunostaining of the sectioned ventricle from fish at 9-weeks old stage using α-actinin antibody. Scale bar=10 µm. H-I, Evaluation of muscular disarray by transmission electron microscopy (TEM). Scale bar=2 µm.
Online Figure V. Primary cardiomyocyte culture. A, Merged images of CMs in primary culture are shown. CMs are revealed either by nuDsRed reporter in the Tg(cmlc2:nuDsRed) transgenic fish (upper panels) that labels CM nuclei with red fluorescence or by α-actinin (green, lower panels) antibody staining (B). I, CMs with rod-like normal CM cell shape; II, CMs with changed cell shape, suggesting dedifferentiation. Scale bar=20 µm. C, Quantification of CM area showed that the CM size remained mostly unchanged throughout the in vitro culture process.
Online Figure VI. ztor haploinsufficiency attenuates PHZ-induced cardiac enlargement with unchanged liver size. A, Quantification of cardiac hypertrophy evaluated by the ventricle area to body length (VA/BL) index at 9-months old fish treated with or without PHZ. PHZ-treatment induced cardiac hypertrophy in control but not in ztor+/− fish. B, Quantification of liver surface area. No significant live size change was detected by PHZ treatment. C, Quantification of CM cell size changes after dissociated from fish hearts treated with or without PHZ. D, Evaluation of CM hyperplasia by quantifying Mef2c+/PCNA+ cells in sectioned heart ventricles treated with or without PHZ. *P< 0.05; ns, not significant.
Online Figure VII. DOX induces cellular cardiomyocyte hypertrophy, apoptosis, and cell loss in a dose-dependent fashion. A, Cardiomyocytes (CMs) were dissociated from the Tg(cmlc2:nuDsRed) transgenic fish heart (in which the CM nuclei were labeled red) and subjected to different doses of DOX treatment at day 1. Obvious CM loss, as revealed by reduced CM nuclei number, was observed at day 3 after treatment with higher doses of DOX, such as 5 µmol/L or 10 µmol/L, but not with lower doses, such as 0.2 µmol/L or 1 µmol/L. Scale bar=200 µm. B, DOX induced a dose-dependent CM hypertrophy in culture. C, Significant apoptosis was observed at day 3 after treatment with higher doses of DOX, such as 5 µmol/L or 25 µmol/L, but not with lower doses, such as 1 µmol/L or 0.2 µmol/L (Data not shown). *P<0.05. ns, not significant.
Online Figure VIII. Rapamycin deteriorates high-dose DOX-induced acute cardiotoxicity. A, Western blot to assess the autophagy activity and p-S6K level in dissected fish heart at 3 days after high-dose of DOX (50 µg/gbm) injection with or without rapamycin (0.2 µmol/L) treatment. Rapamycin treatment enhanced high-dose DOX-induced autophagy activation, and abolished majority of p-S6K levels, but had no significant impact on the levels of total S6K protein. B, Kaplan-Meier survival curves for casper fish injected with 50 µg/gbm DOX with or without rapamycin (0.2 µmol/L) treatment. C, An example image of sectioned fish ventricle co-stained with TUNEL (green) and Mef2 (red) at 3 days after 50 µg/gbm DOX injection. Arrows: TUNEL+/Mef2+ cells; Arrowheads: TUNEL+/Mef2- cells. Scale bar=20 µm. D, Quantification of the apoptotic index in fish heart at 3 days after 50 µg/gbm DOX injection with or without rapamycin (0.2 µmol/L) treatment. Rapamycin treatment further activated apoptosis in the high-dose DOX-induced acute cardiotoxicity. *P<0.05. ns, not significant.
Supplemental References


