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 *tr265* 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, *tr265* and *tr265*; *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 *tr265* 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≥6).

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,[1] 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.[2] 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.[3] 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*, quantificative 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'-CTACAAGGAGCTGGAGTTTC-3'; reverse, 5'-ATCTCCAATTCTCCAAAGTG-3'), *anf*: (forward: 5'- AAGCAAAAGCTTGTCTGG-3', reverse: 5'-ACTGTATCCGCATATTGCAGC-3'), *18s rRNA*: (forward: 5'-CACTTGTCCCTCTAAGAAGTTGCA-3', reverse: 5'- GGTTGATTCCGATAACGAACGA-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'-TAGTCCCTCTTAAGGCCTGT-3') and oligonucleotides 2 (5'-GATCGTCCCTCTTAAGGCCT-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'-GGATTTGCTGGTGCAGTACAG-3', and 5'LTR primer: 5'LTR, 5'-CCCTAAGTACTTGTACTTTCACTTG-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'-AGTACAGGCCTTAAGAGGGA-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'-CTTACCACTCAGAGAGACCAAAG-3; 5'LTR, 5'-CCCTAAGTACTTGTACTTTCACTTG-3'; and 3'LTR, 5'-GTACAGTAATCAAGTAAAATTACTCA-3'.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed as described previously.[3] 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.

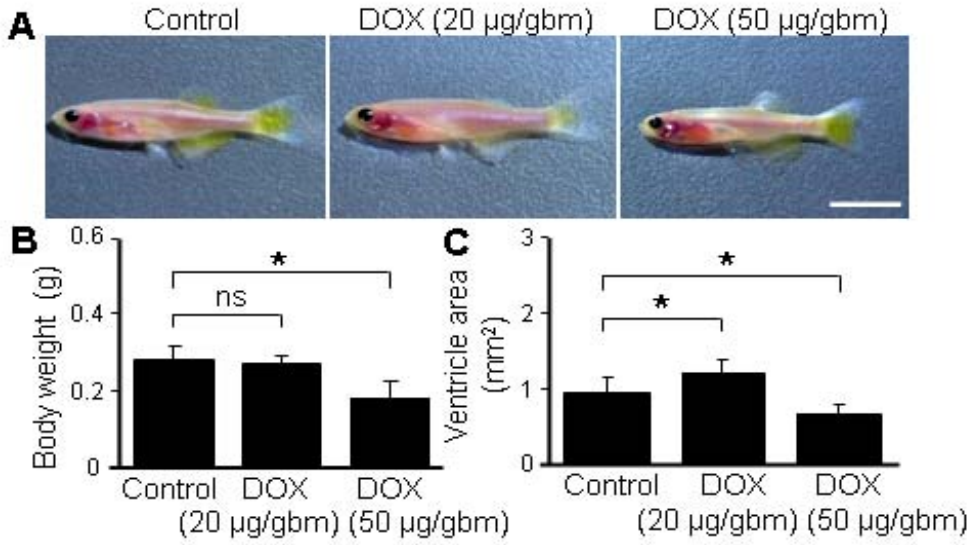
TUNEL assay

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.

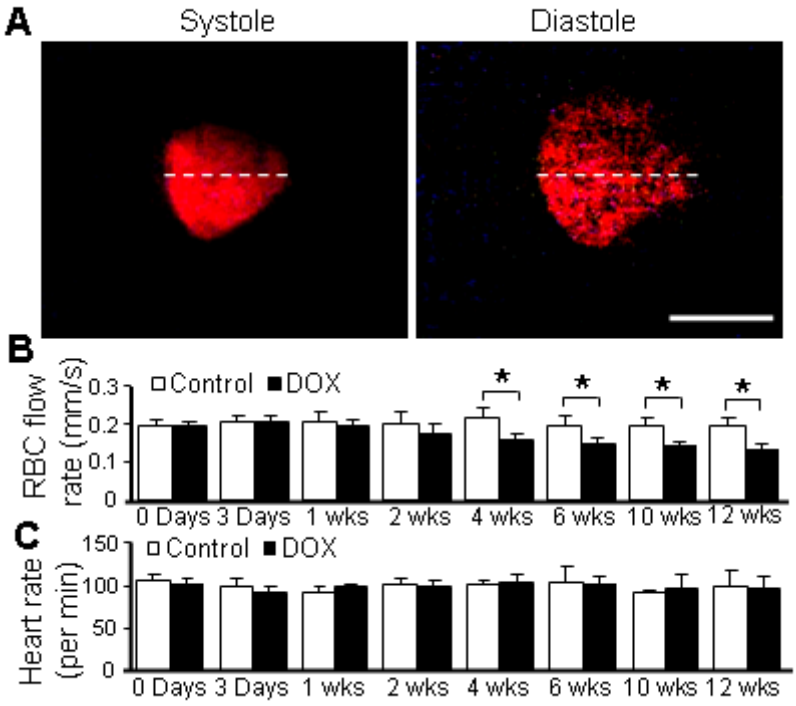
Quantification of GFP-Lc3 autophagic aggregates

Frozen sections (12 μm) of ventricles from DOX treated *Tg(GFP-Lc3)* transgenic fish[4] 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).

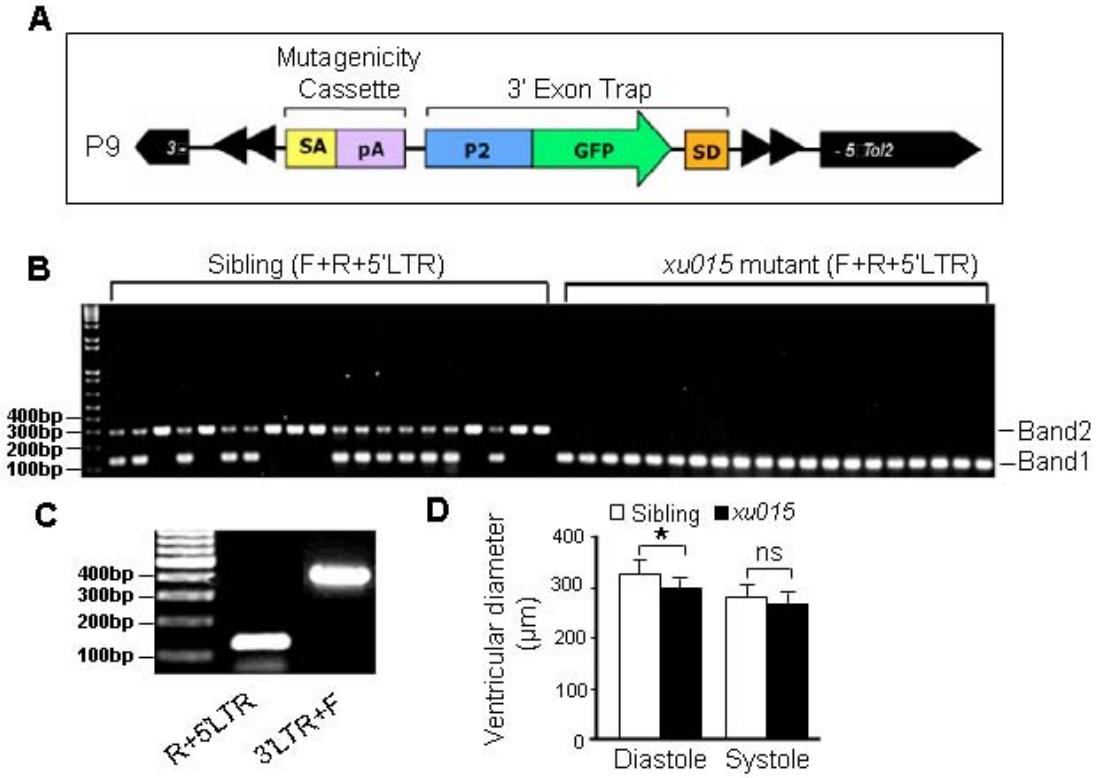
Online Figures and Figure Legends



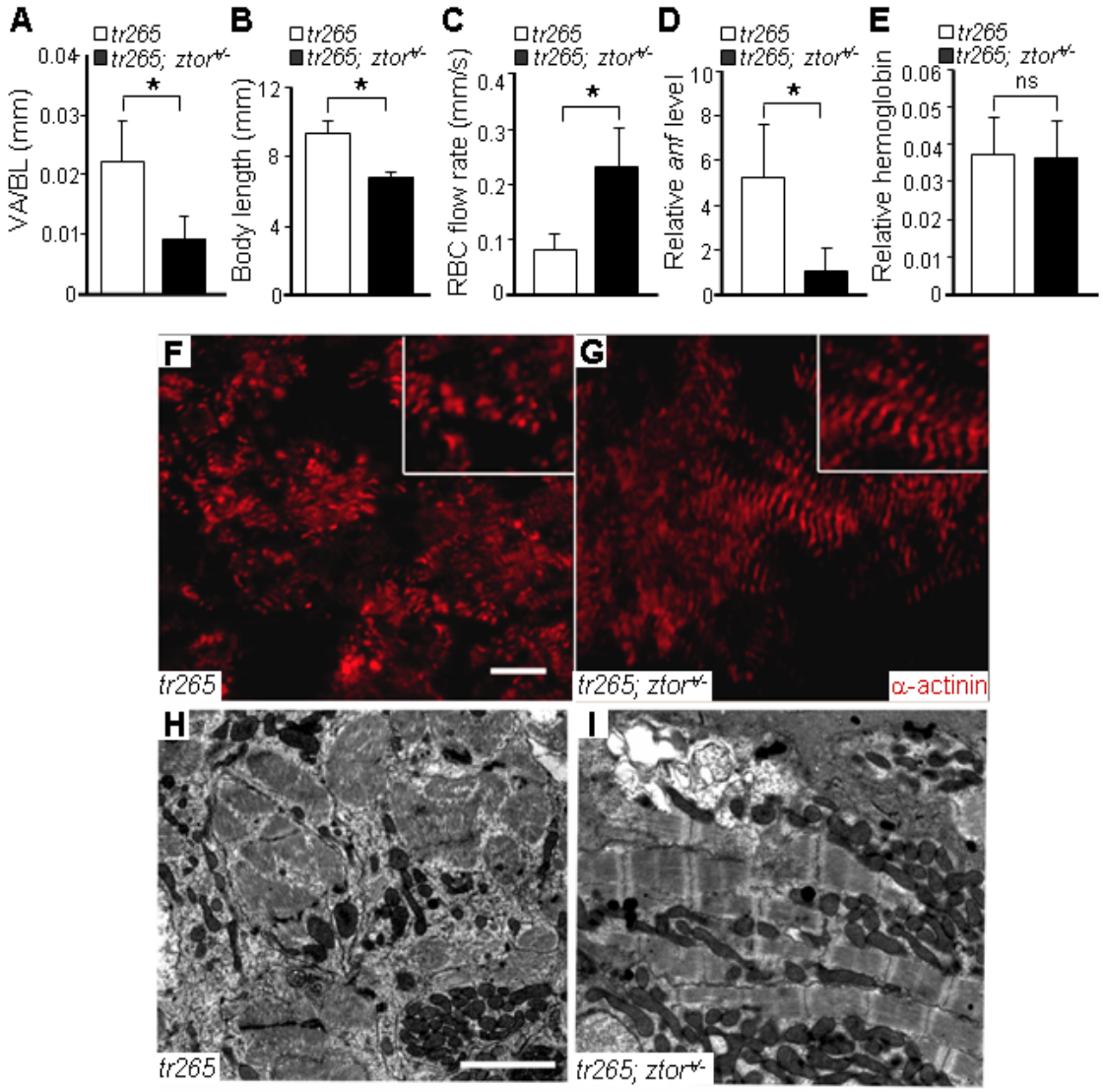
Online Figure I. Dosage-dependent responses in adult zebrafish after DOX injection. A, Lateral view of two-month old *casper* fish at 4 weeks after a single doxorubicin (DOX) injection. Compared to the control fish injected with Hanks buffer, fish injected with 50 μg/gbm of DOX were smaller, while fish 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.



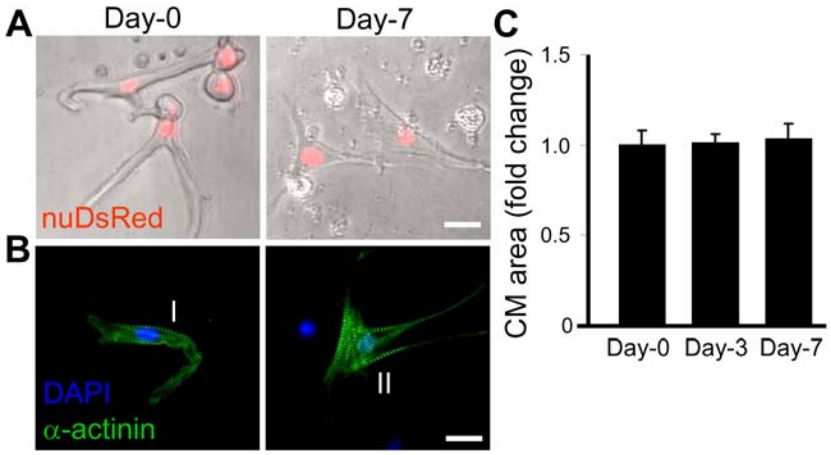
Online Figure II. Cardiac function can be quantified in an adult zebrafish using *casper;Tg(cmlc2:nuDsRed)* fish. A, Images extracted from movies of a beating heart at systole and diastole, respectively, are shown. Dashed lines indicate the measurements used to calculate cardiac function using the formula 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.



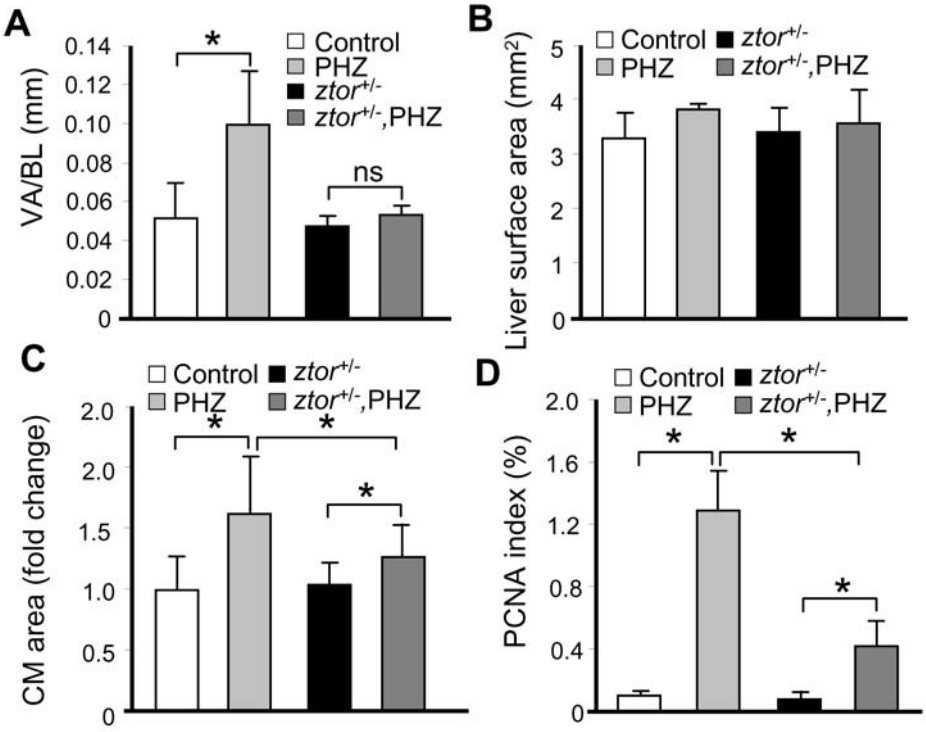
Online Figure III. Identification of the *ztor*^{xu015} insertional mutant. A, Schematic diagram of the P9 construct used to generate insertional mutant in zebrafish. For details of the gene-breaking cassette, see. [5] B, The linkage between the P9 insertion and the *xu015* mutant phenotypes was established 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*^{xu015} heterozygosity (*ztor*$^{+/-}$). 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 *xu015* mutant embryos examined, we detected only the band 1 amplification, confirming their genotypes as *ztor*^{xu015} homozygosity (*ztor*$^{-/-}$). C, Hijacked splicing events caused by the P9 element insertion were confirmed in the *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 *xu015* mutants at 7 days post-fertilization (dpf). Diastolic length, but not systolic length was reduced in the *xu015* mutant. *P<0.05; ns, not significant.



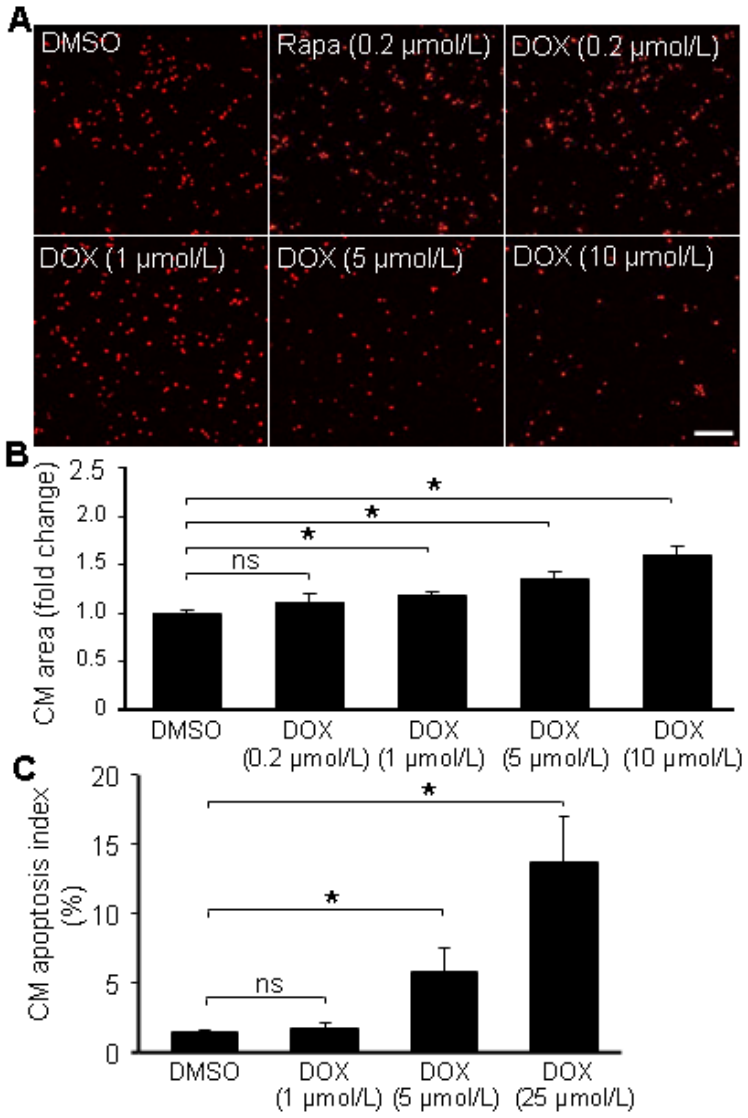
Online Figure IV. *ztor* haploinsufficiency improves cardiac function, alleviates hallmarks of pathological cardiomyopathy in *tr265* fish. A-E, Ventricle area to body length (VA/BL) index (A); Body length (B); Red blood cell (RBC) flow rate (C); fetal *anf* gene expression (D); and percent hemoglobin concentration (E) at 9-weeks old *tr265; ztor*$^{+/-}$ 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.



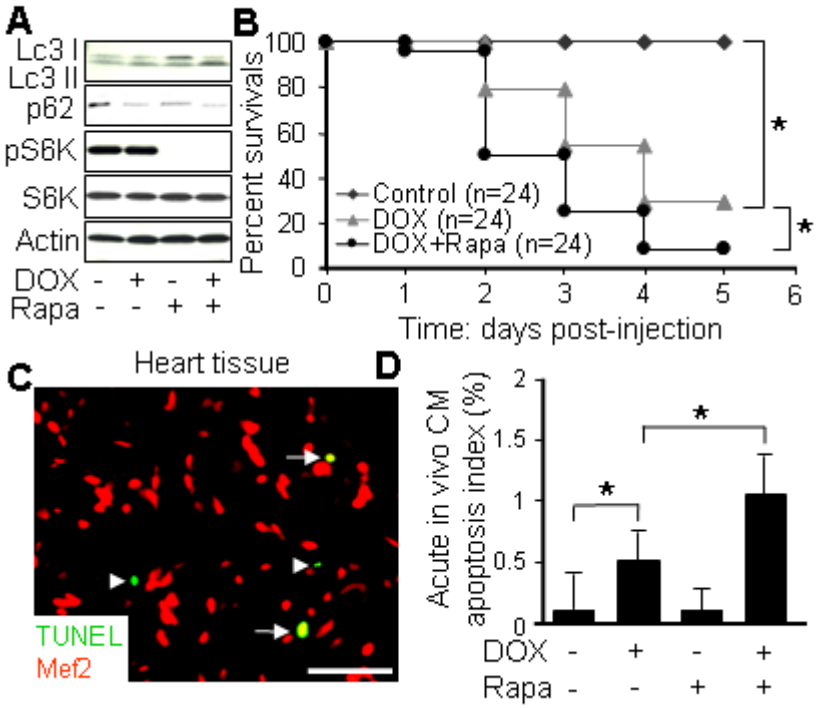
Online Figure V. Primary cardiomyocyte culture. A, Merged images of CMs in primary culture are shown. CMs are revealed either by nuDsRed reporter in the *Tg(cmlc2:nuDsRed)* transgenic fish (upper panels) that labels CM nuclei with red fluorescence or by α-actinin (green, lower panels) antibody staining (B). I, CMs with rod-like normal CM cell shape; II, CMs with changed cell shape, suggesting de-differentiation. 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

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