Supplemental Material to:

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Autophagy is essential for cardiac morphogenesis during vertebrate development

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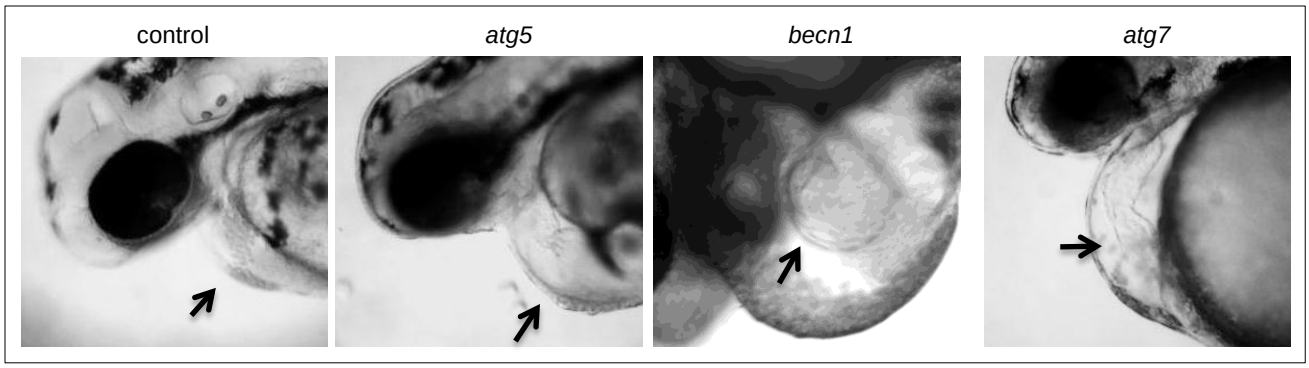

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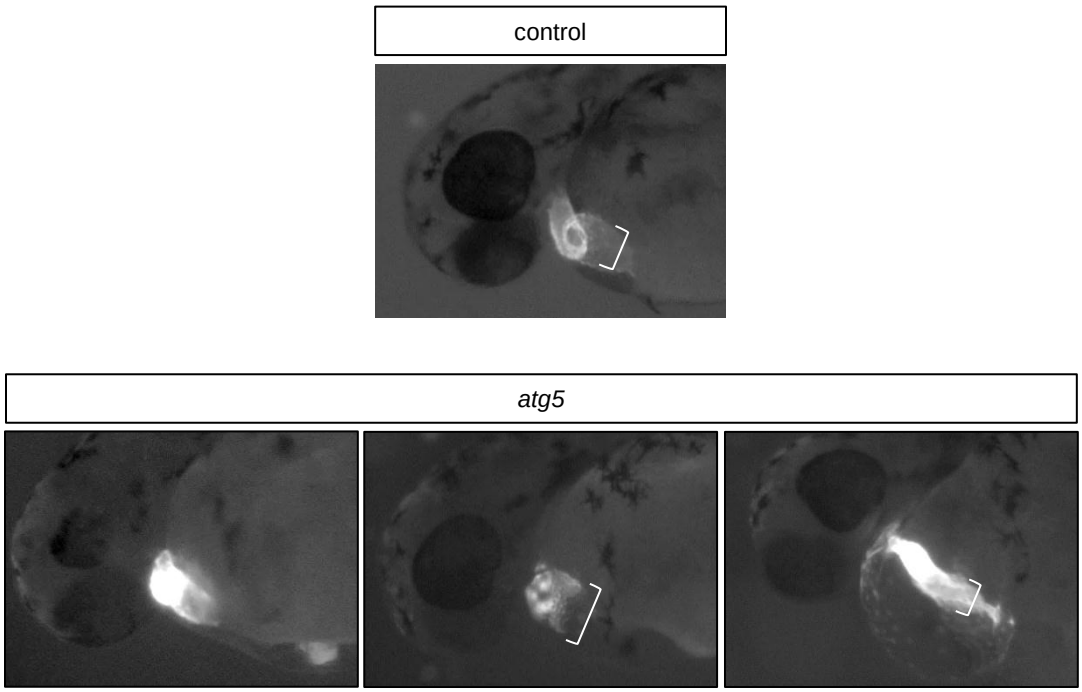

Figure S1. Autophagy gene knockdown results in cardiac defects. **(A)** Abnormal cardiac morphology in autophagy morphants including mislooping, abnormal cardiac chambers, and linearized hearts was observed at 2 dpf (arrows). **(B)** Representative images of the heart in control morphants or *atg5* morphants using *Tg(cmlc2::EGFP)* fish at 2 dpf. Compared to control, *atg5* morphant hearts show atrial enlargement (bottom; middle panel) or failure of looping with a resulting long, linear heart tube (bottom; right panel).

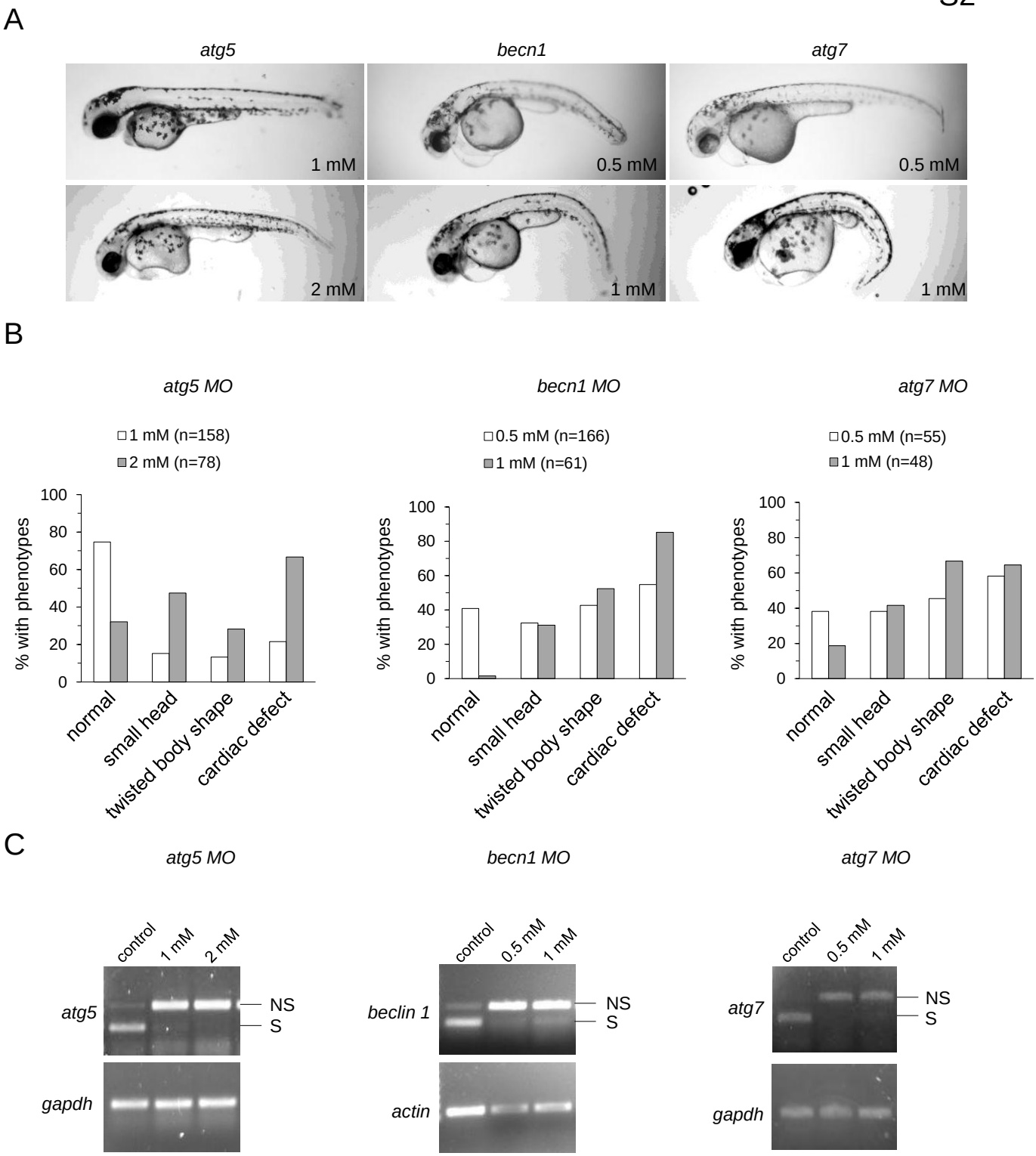

Figure S2. Autophagy is efficiently inhibited in a dose-dependent manner by slice-blocking morpholinos (MOs). **(A)** Morphology of autophagy morphants at 2 dpf. Embryos were injected with two different concentrations of each splicing MO. Phenotypes including cardiac defects were dose-dependent. **(B)** Quantification of data shown in **(A)**. **(C)** Impaired splicing by splicing MO injections was confirmed by RT-PCR. RNA was isolated from autophagy morphants at 2 dpf and PCR reactions were done using splicing site-specific primer sets. NS; non-spliced, S, spliced. In control morphants, a spliced form of RNA was mainly detected at 2 dpf by RT-PCR; however, in autophagy morphants, non-spliced RNAs were more predominant than the spliced RNA form.

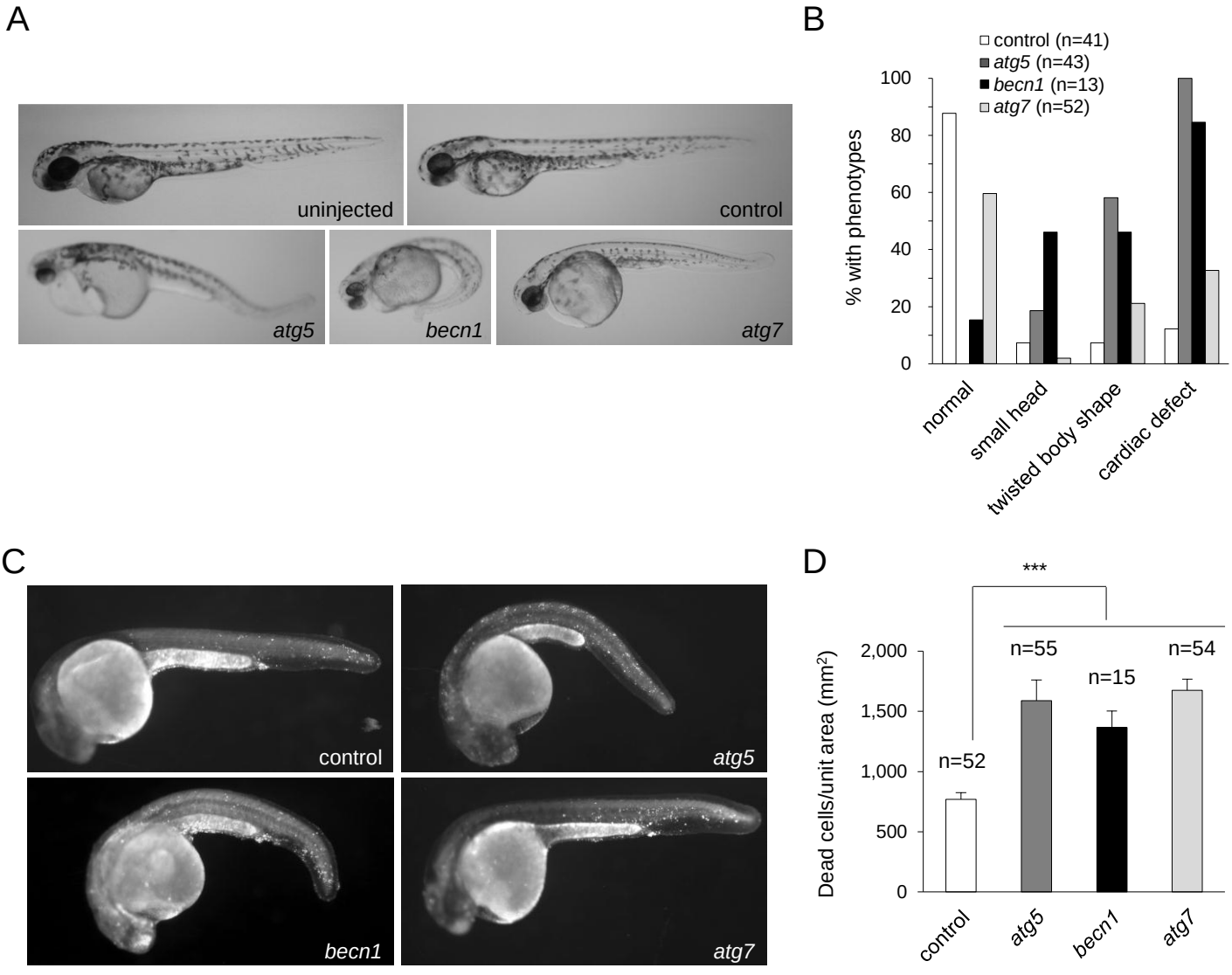

Figure S3. Autophagy gene knockdown results in developmental defects and increased numbers of dead cells in a Tp53 mutant background. **(A** and **B)** Morphology of autophagy morphants in a Tp53^{M214K} mutant background at 2 dpf. Autophagy morphants showed small heads, twisted body shapes and cardiac defects at 2 dpf. (**B**) Quantification of data shown in (**A**). More than 10 embryos in each group were analyzed. **(C** and **D)** Acridine orange (AO) staining of control and autophagy morphants in Tp53^{M214K} mutant zebrafish at 1 dpf. AO-positive cells were counted in a region spanning from the end of the yolk tube extension to the end of the tail. (**D**) Quantification of data shown in (**C**) [atg5 and atg7 should be in italics]. Results represent the mean ± SEM of more than 40 embryos in each group. ***P<0.001; one-tailed t-test.

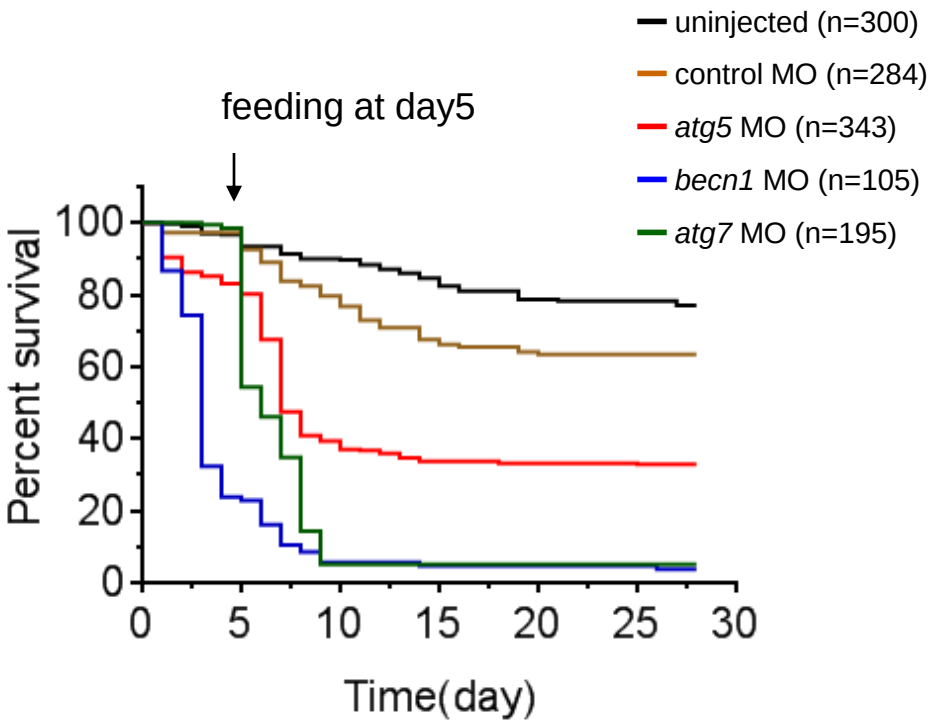

Figure S4. Effect of autophagy gene knockdown on long-term viability of zebrafish. Survival of control and autophagy gene morphants was monitored for 28 days after morpholino injection. Compared to control morphants, autophagy gene morphants exhibited impaired survival (***P<0.001 for autophagy morphants versus control morphants; Log-rank test).

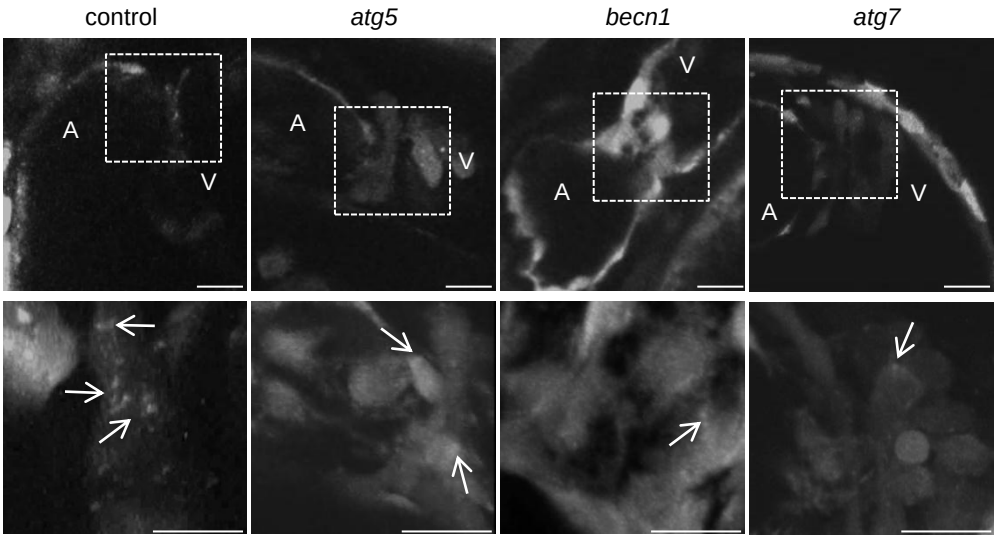

Figure S5. Autophagy is detectable in the 2 dpf heart of *GFP-LC3* transgenic embryos. Representative images showing GFP-Lc3 puncta (white arrows) at 2 dpf. *Tg(cmv:GFP-lc3)* embryos were injected with either control or autophagy MOs and imaged at 2 dpf using confocal microscopy. The areas delineated by the white dashed lines are shown as z-stack images at higher magnification in the bottom panel. Scale bar, 20 μm

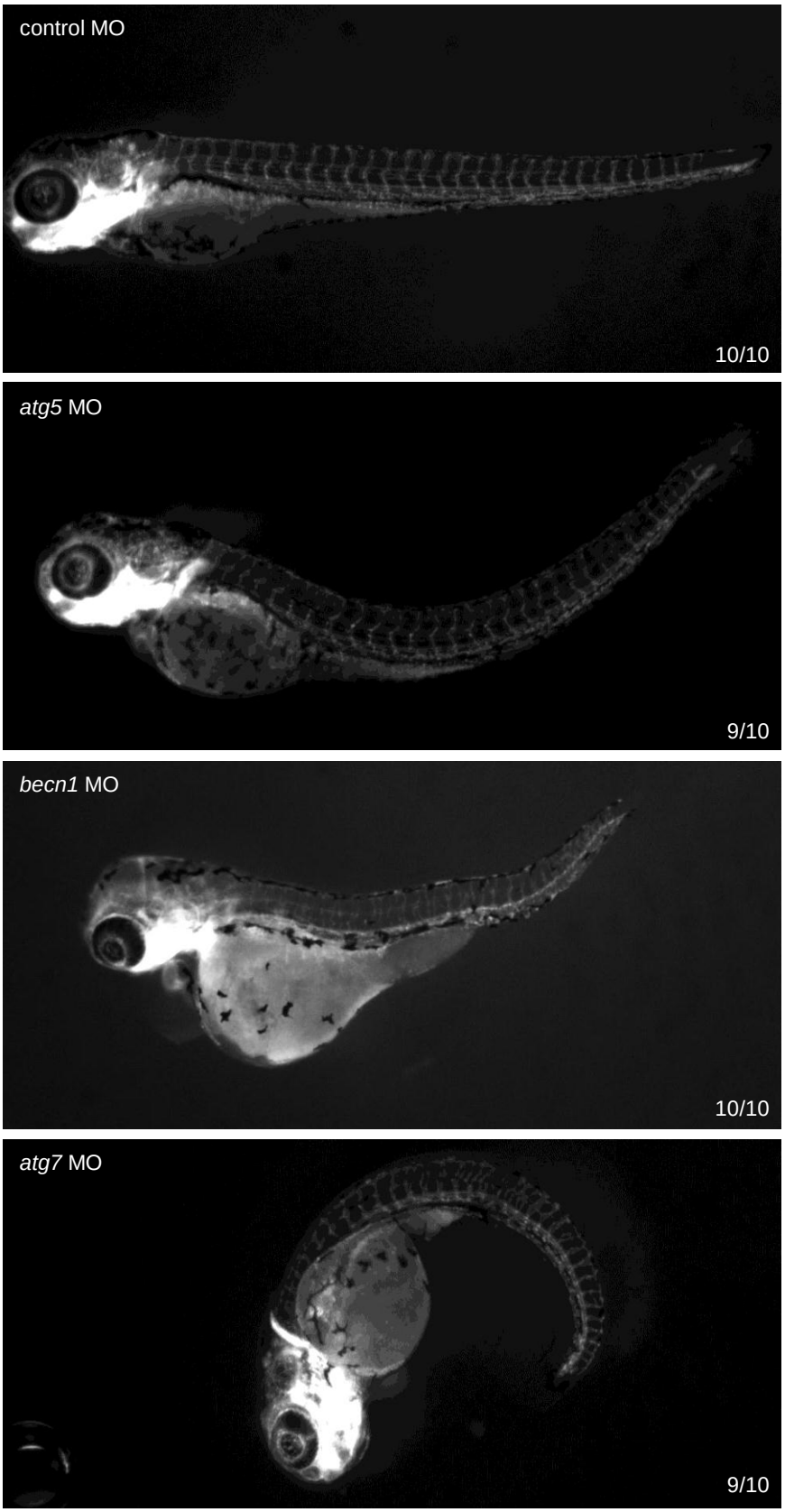

Figure S6. Autophagy morphants develop normal vasculature. Representative images of the vasculature in control or indicated autophagy morphants using *Tg(fli1::EGFP)* fish at 3 dpf. None of the control or *becn1* morphants showed abnormal vasculature. 9 out of 10 *atg5*, and 9 out of 10 *atg7* morphants showed normal vasculature.

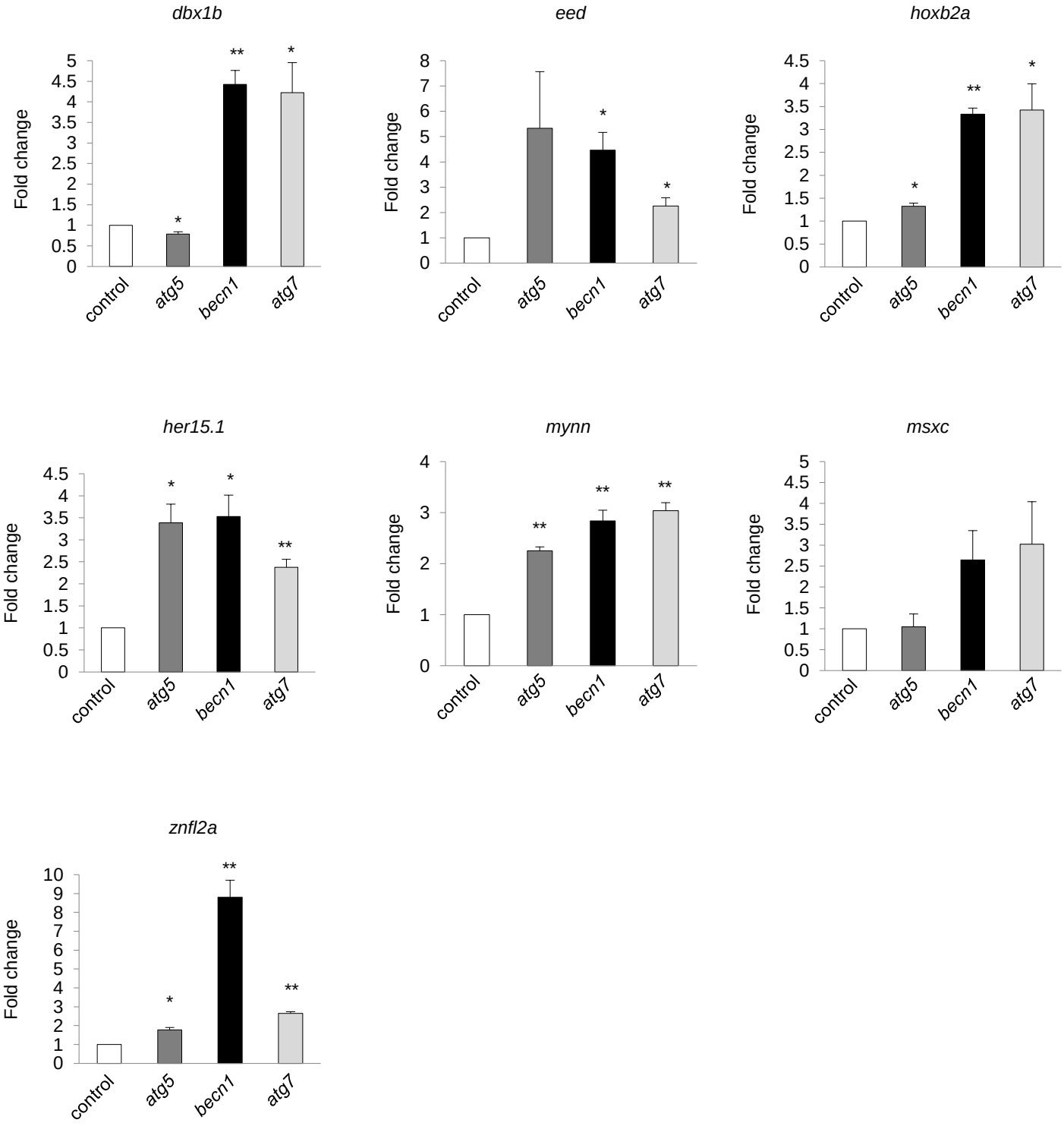

Figure S7. Autophagy gene knockdown results in upregulation of transcription factors. Real-time PCR measurement of transcripts for *dbx1b, eed, hoxb2a, her15.1, mynn, msxc,* and *znfl2a* in purified heart of autophagy morphants at 3 dpf. Results represent the mean ± SEM of triplicate samples. ***P*<0.01, **P*<0.05 for autophagy morphants versus control morphants by one-tailed t-test.

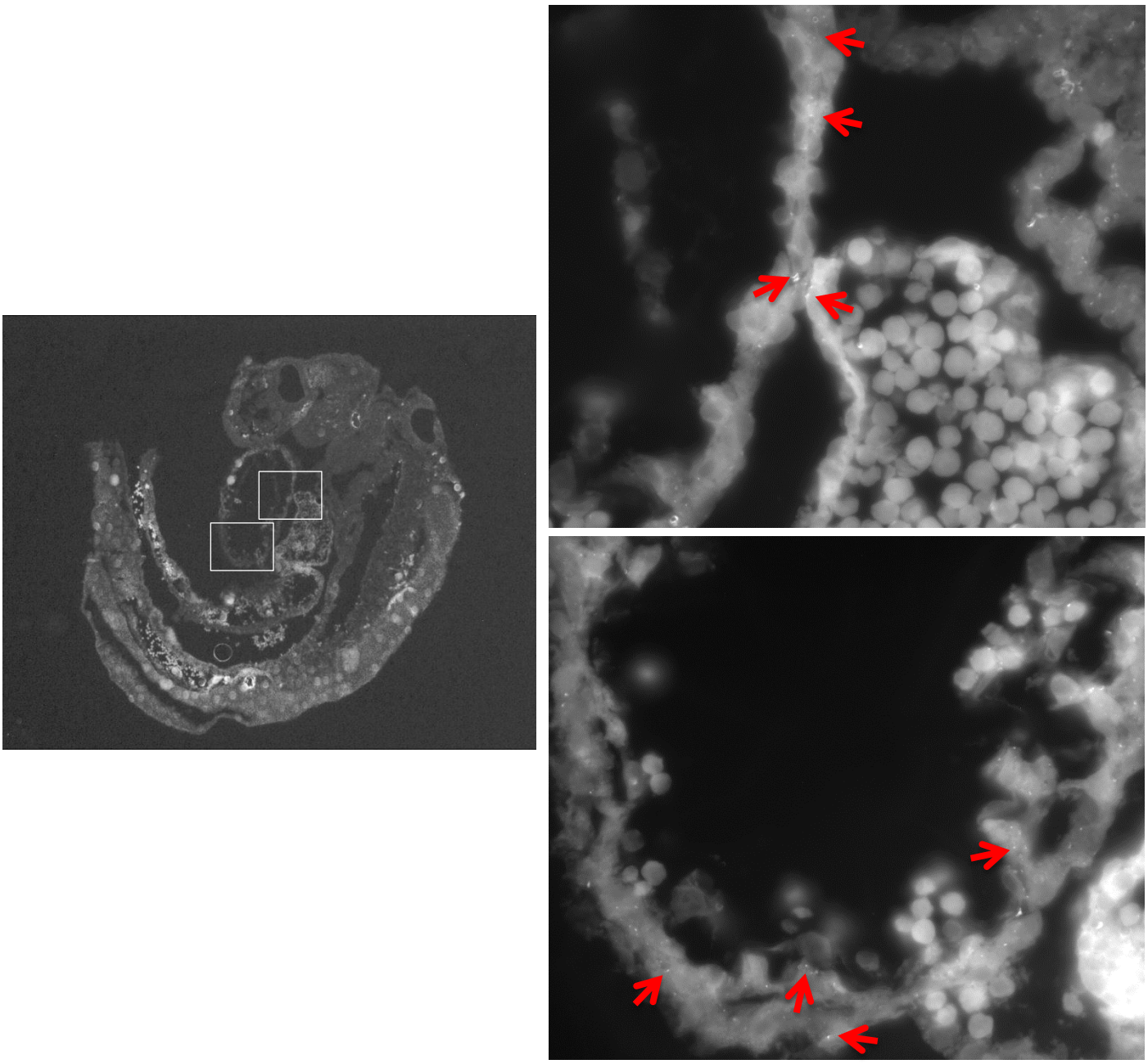

Figure S8. Autophagy is active during mouse cardiac development. GFP-LC3 puncta were visualized by fluorescence microscopy. GFP-LC3 embryos at E9.5 were dissected and sectioned in a sagittal orientation. Whole animals were visualized at 4X (left) and GFP-LC3 puncta in AVC (right, top) and cardiomyocytes (right, bottom) were imaged at 63X. Arrows denote representative GFP-LC3 puncta. Scale bar, 50 mm.

Supplementary information

Supplementary Methods

Splice-blocking morpholino injection

Splice blocking-MOs were designed to inhibit the splicing of the exon 2-intron 2 junction in *atg5*, exon 1-intron 1 junction in *atg7*, and the exon 1-intron 1 junction in *becn1* mRNA. In order to optimize MO injections, several different concentrations were tested. Splicing inhibition by MO injection was confirmed by RT-PCR.

Primers

Morpholinos	
atg5 MO	5'-CACATCCTTGTCATCTGCCATTATC-3'
becn1 MO	ACCTCAAAGTCTCCATGCTTCTTTC-3'
atg7 MO	AGCTTCAGACTGGATTCCGCCATCG-3'
Splice blocking morpholinos	
atg5 sp MO	5'-ATTCCTTTAACTTACATAGTAGGGT-3'
becn1 sp MO	5'-TGTTATTGTGTGTTACTGACTTGTA-3'
atg7 sp MO	5'-AGCTCGTTCTCCAAACTCACCGTTA-3'
Primers for RT-PCR	
atg5 exon2 F	5'-TGACAAGGATGTGCTTCGAG-3'
atg5 exon 3 R	5'-ACCACATTTCCTCCACATCC-3'
atg5 intron2 R	5'-TTTAACAACCAAATGAACACTTATGTCTATTCAACTG -3'
becn1 exon1 F	5'-ACCCACTTTGTGAGGAGTGC-3'
becn1 exon2 R	5'-GTCCCTCATCCAGCTCTTTG-3'
atg7 exon1F	5'-GATTCTGGCATCAGCTCACA-3'

atg7 exon2 R	5’-GCATCAAATGCGCTGAACT-3’
atg7 exon2 R	5’-TTTGTCGGTGGATTTGAAGG-3’
atg7 intron1 R	5’- AAGCGGGTAAGGTTAATATTGCT -3’
ulk1b F	5’-GGCAACTATGGGCAGTCTGT-3’
ulk1b R	5’-ACCTGTGGAGAGAGCTGGAA-3’
gapdh F	5’-TGGGTGTCAACCATGAGAAA-3’
gapdh R	5’-TCAACGGTCTTCTGTGTTGC-3’
actin F	5’-GCTGTTTTCCCCTCCATTGTT-3’
actin R	5’-TCCCATGCCAACCATCACT-3’
Primers for riboprobe synthesis	
cmlc2 F	5’-GTTGACCAGGCTTTTGCAGT-3’
cmlc2 R	5’ TAATACGACTCACTATAGGGAGAATGATGCTCTACTCATAGTCAAGGAA-3’
bmp4 F	5’-CTGTCCACCAGAGACATCATGATTCC-3’
bmp4 R	5’- TAATACGACTCACTATAGGGAGACTTCATGATGGAATC-3’
notch1b F	5’-GGTTTTACGGGCTCGACCTG-3’
notch1b R	5’- TAATACGACTCACTATAGGGAGAATCACACTTCCCGTTATTAAAATA-3’
tbx2b F	5’-CTGATAGCGAGCACGAAATGGAC-3’
tbx2b R	5’- TAATACGACTCACTATAGGGAGAGGTTGTCCGGTGTTTAGTGGG-3’
foxn4 F	5’-CAGACAGAAGTCTGTTGACCCTG-3’
foxn4 R	5’- TAATACGACTCACTATAGGGAGAGCCAGGCAAAGTCCATTATACTGGG-3’
tbx5 F	5’-CTACCGGCCCCTAAGCTACT-3’
tbx5 R	5’- TAATACGACTCACTATAGGGAGAGCTGGCTTCATTCCAGTCAT-3’