SUPPLEMENTAL MATERIALS

Activation of Autophagic Flux Blunts Cardiac Ischemia/Reperfusion Injury

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Running Title:
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Expanded Materials & Methods

Animal studies

C57BL/6N mice were obtained from Charles River.

The ATG7$^{F/F}$ mouse was provided by Dr. Massaki Komatsu[31], and the αMHC-merCremer mouse was obtained from Jackson lab[32]. All animal studies were conducted according to ethics guidelines provided by the Institutional Animal Care and Use Committee at UT Southwestern. To induce MerCreMer (MCM) activity, tamoxifen (Sigma, T5648) dissolved in corn oil was administered intraperitoneally (IP, 20mg/kg per day x 5 days). Tissue- and induction-specific recombination was confirmed in αMHC-MCM lines by genomic DNA isolation (Zymo gDNA kit, D4068) and PCR reaction around the target exon detailed in Dr. Masaki Komatsu's paper[31].

Primers that were used:
First Pair: HIND-FW:GGCTGCTACTTCTGCAATGATGT;
PST-RV: CAGGACAGAGACCATCAGCTCCAC; (WT 1500bp, Floxed 500bp).
Thermocycler conditions: cycle 1 (1X), 95.0ºC for 10 min; cycle 2 (35X), step 1 at 95.0ºC for 30 sec, step 2 at 62.0ºC for 30 sec; cycle 3 (1X), 72.0ºC for 10 min; then 4 ºC.

Second Pair: ATG7EX14F: TCTCCCAAGACAAGACAGGGTGAA;
ATG7EX14R: AAGCCAAAGGAAACCAAGGGAGTG. (WT 300bp, Floxed non band)
Thermocycler conditions: cycle 1 (1X), 95.0ºC for 10 min; cycle 2 (35X), step 1 at 95.0ºC for 30 sec, step 2 at 60.0ºC for 30 sec; cycle 3 (1X), 72.0ºC for 10 min; then 4 ºC.

WT animals used in the αMHC-MerCreMer study were mixed cohorts of ATG7$^{F/F}$ Cre- and WT αMHC-MCM+ littermates, all of which were treated with the same tamoxifen regimen as the experimental group. Animals were maintained in 12hr light/dark cycles with a standard chow diet ad libitum.

RNA purification and RT-qPCR

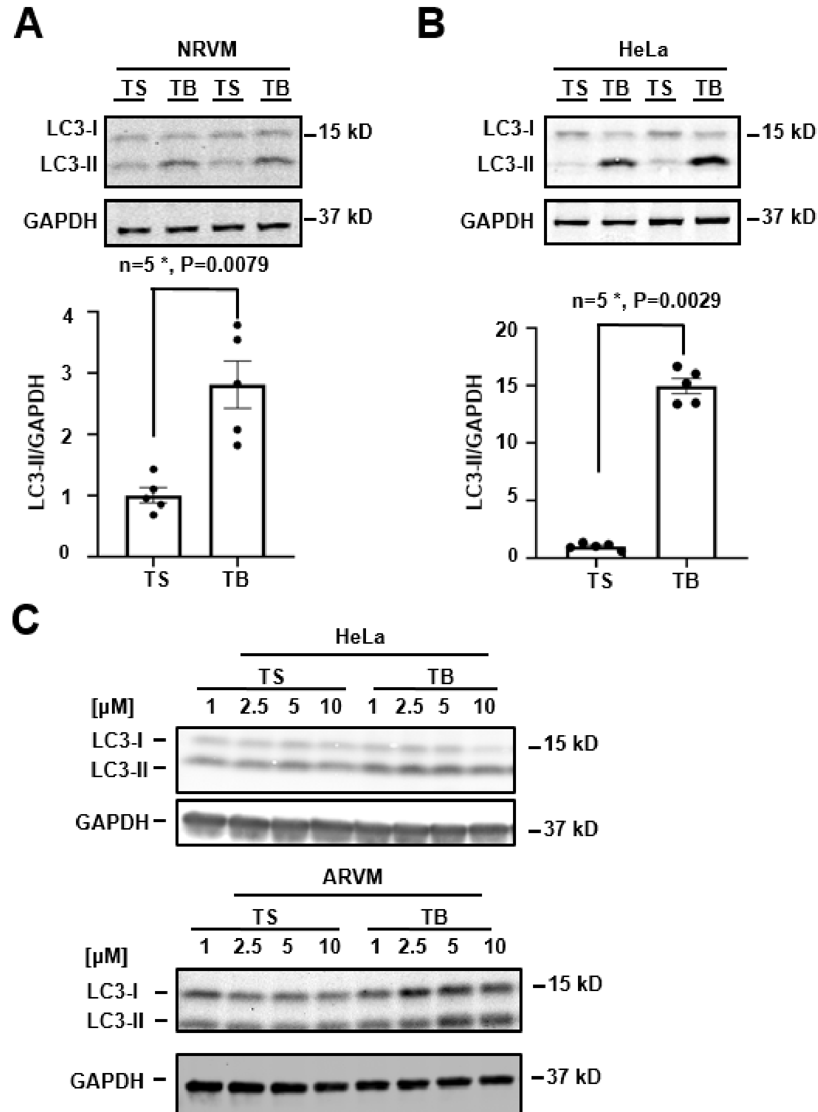
Snap-frozen tissues were disrupted in TRIzol (Fisher, 15596026) by bead beater (MP, FastPrep-24) using disposable ceramic beads (Fisher, 6913-100) to extract total RNA using the Fatty and Fibrous Tissue RNA kit (Biorad, 7326870). For isolated cells, a Quick RNA microprep kit (Zymo, R1051) was used to extract total RNA; cell pellets were resuspended in RNA lysis buffer and disrupted by homogenizer (Polytrone, PT-MR2100) before extraction. A total of 500ng of purified RNA was used for reverse-transcription reactions (Biorad, 170-8841). Quantitative PCR reactions were run with cDNA libraries in duplicate with SYBR Green master mix (Biorad, 1725125) on a Roche Lightcycler 480. All of the primer sets were validated for doubling efficiency using cDNA standard curves. Quantification was performed using the ΔΔCt method to obtain relative fold change to WT or untreated sample in each sample set, normalized against a housekeeping gene (tubulin for mouse). ATG7 primers that are used for mouse ATG7 expression are: M-ATG7 311-330: TGGAGTTCAGTGCTTTTGAC, M-ATG7 387-370: GGTGTTGTGCAGGGTTCC. Internal control used is mouse beta-actin. The primers are: mBAF: TCACCCACACTGTGCCCATCTACGA, mBAR: CATCGGAACCGCTCGTTGCCAATAG. Thermocycles: cycle 1 (1X), 95.0ºC for 1.5 min; cycle 2 (30X), step 1 at 95.0ºC for 20 sec, step 2 at 61.0ºC for 30 sec; cycle 3 (1X), 95.0ºC for 1 min; cycle 4 (1X), 55.0ºC for 1 min, cycle 5 (40X) 55.0ºC for 10 sec with an increase of 1.0ºC after each repeat for collecting melt curve data.

Western blotting

Snap-frozen tissue was resuspended in ice-cold modified RIPA buffer (50mM Tris-HCl pH=7.4, 150mM NaCl, 0.1% SDS, 1% TritonX-100, 0.5% sodium deoxycholate, 5mM EDTA, 2mM EGTA) with protease/phosphatase inhibitor cocktail (Thermo, A32961). Tissues were processed using a Dounce homogenizer on ice. For tissue cultures, 1XSDS buffer was used to collect cells. Primary antibodies used: LC3-II (rabbit anti-LC3 prepared in Hill Laboratory at UT Southwestern), ATG7 (Anaspec 54231 and Cell Signaling 2631), GAPDH (Fitzgerald 10R-G109a), Oxyblot Protein Oxidation Detection Kit S7150 - Millipore, pS6(Ser235/236) Cell Signaling 4858, pmTOR (Ser2448) Cell Signaling 2971, mTOR Cell Signaling 2972, p4EBP1 (Thr37/46) Cell Signaling 2855 , 4EBP1 Cell Signaling 9452.

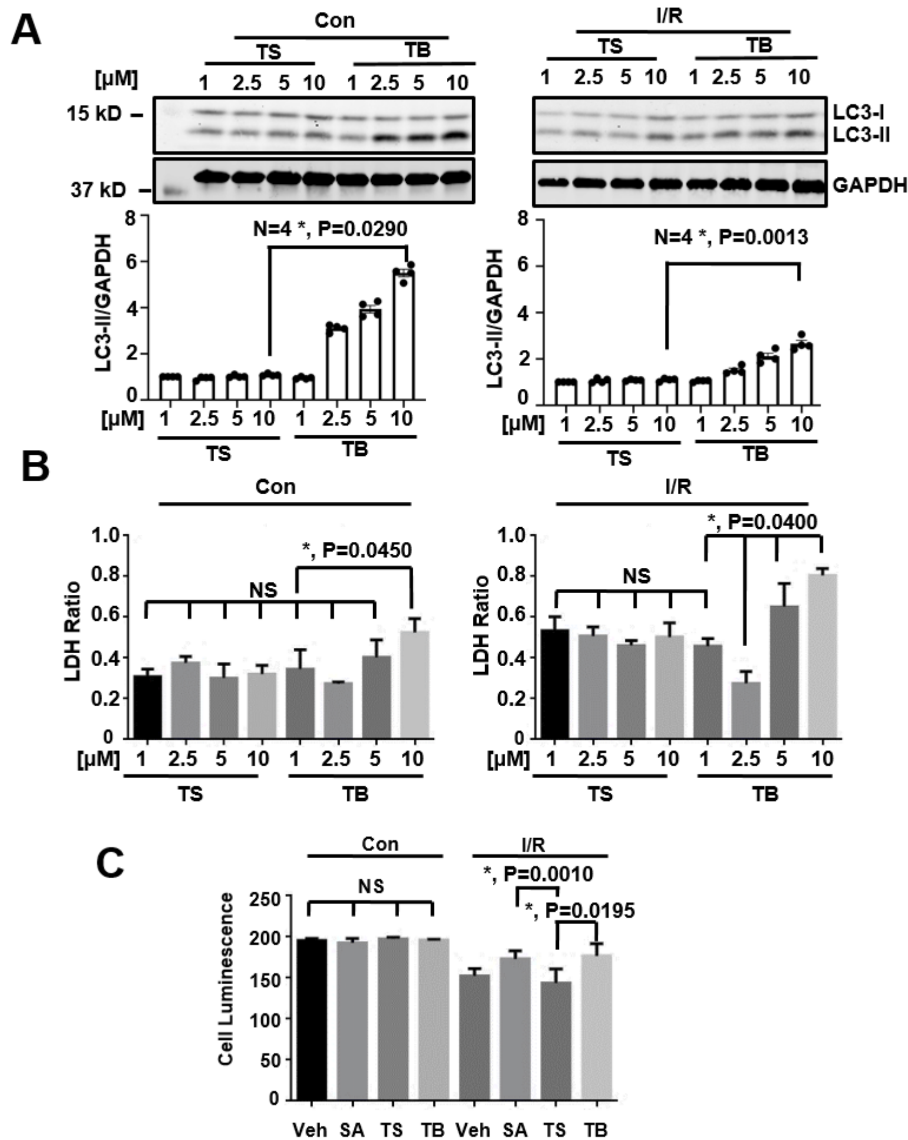
After incubation with near infrared fluorophore-conjugated secondary antibodies (LI-COR, 926-68020 and 926-32211, two colors), membranes were imaged with an Odyssey scanner (LI-COR) and quantified using ImageStudio software.

Online Figures



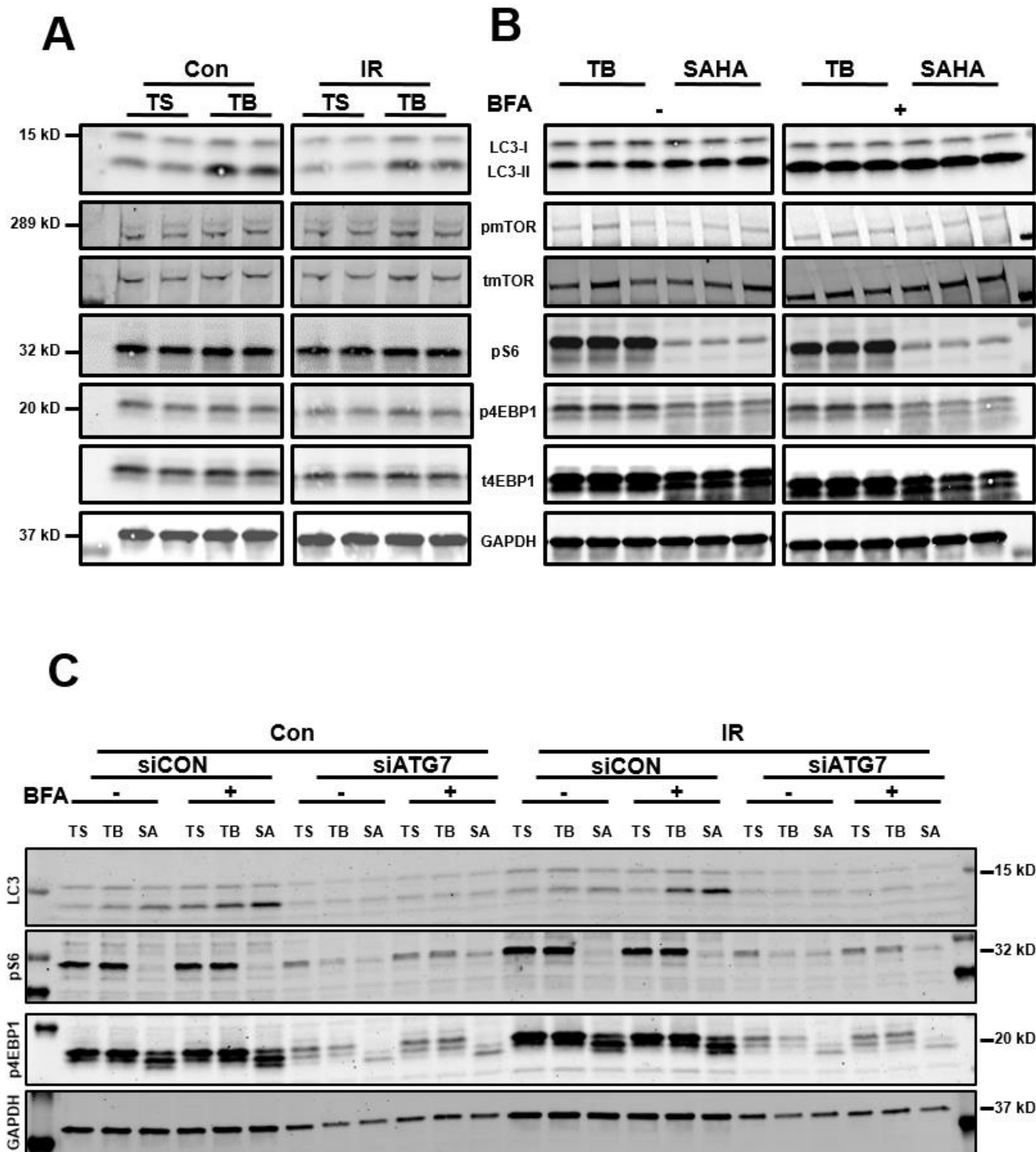
Online Figure I. Tat-Beclin peptide stimulates autophagy in cardiomyocytes and other cell lines.

(A) Immunoblot and quantification of LC3 normalized to GAPDH revealing increased autophagy in NRVMs treated with TB. Mann-Whitney test was used, NRVM, N = 5, P = 0.0079. Hela Cells, N = 5, P = 0.0029. (B) Immunoblot and quantification in HELA cells of LC3 normalized to GAPDH showing increased autophagy with TB treatment. (C) Immunoblot of LC3 normalized to GAPDH demonstrating dose curves of TS and TB administration in HELA cells.



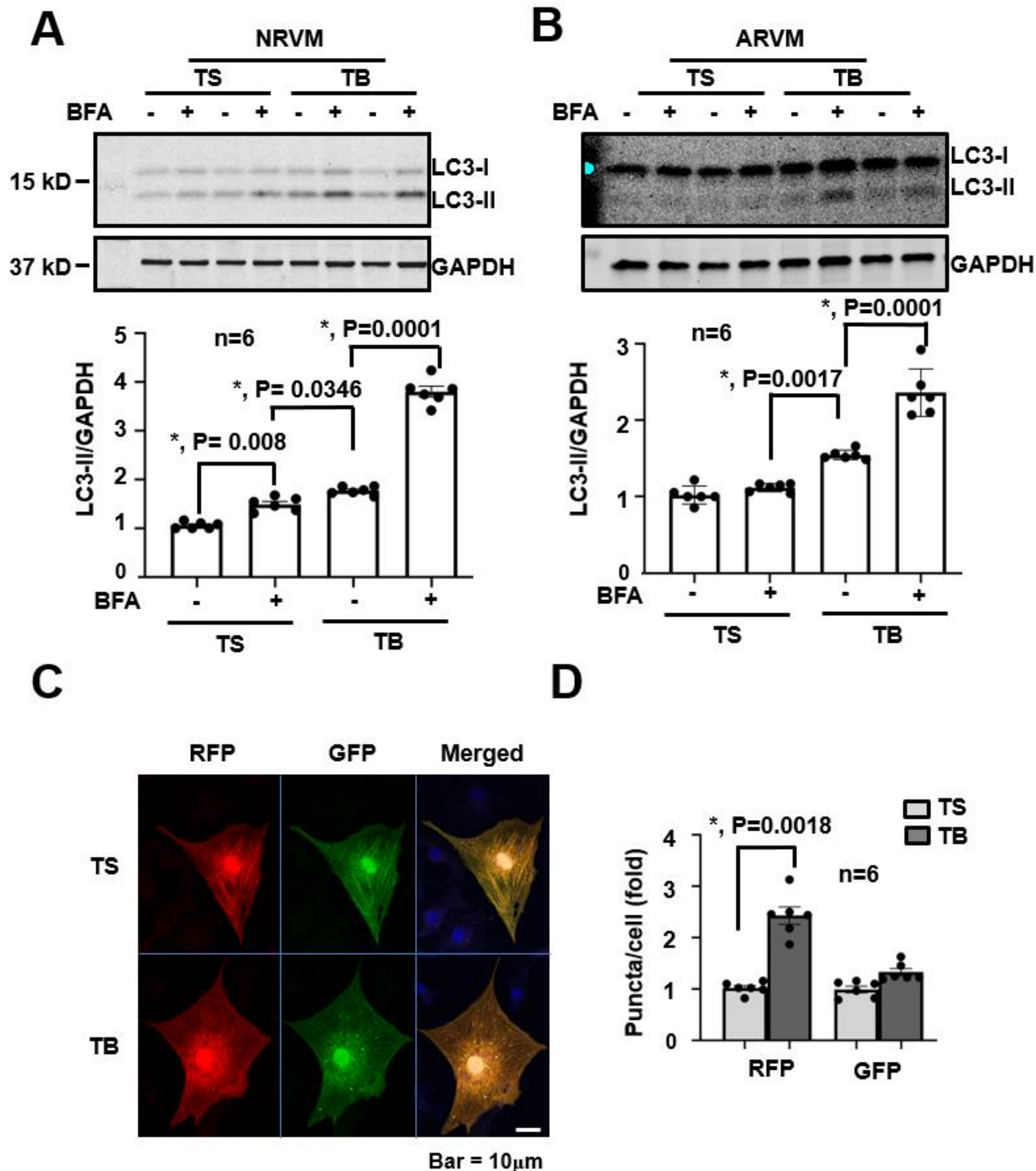
Online Figure II. Tat-Beclin's autophagy inducing effects are dose dependent.

(A) Immunoblot and quantification of LC3 normalized to GAPDH demonstrating dose curves of TS and TB administration in control versus simulated I/R conditions in NRVMs, with only TB stimulating autophagy in both states. Kruskal-Wallis test with Dunn's multiple comparisons test, TS 10 vs TB 10, Control, N = 4, P = 0.029, I/R, P = 0.013. (B) LDH cell death assay evaluating NRVM treatment with TS [1-10 μM] or TB [1-10 μM] in control and simulated I/R conditions, showing TB [2.5 μM] is the optimal dose to reduce I/R induced cell death. (C) Cell luminescence assay showing improved NRVM survival with SAHA and TB administration after simulated I/R treatment in NRVMs. N = 3-5 independent experiments in replicates for each group.*P<0.05.



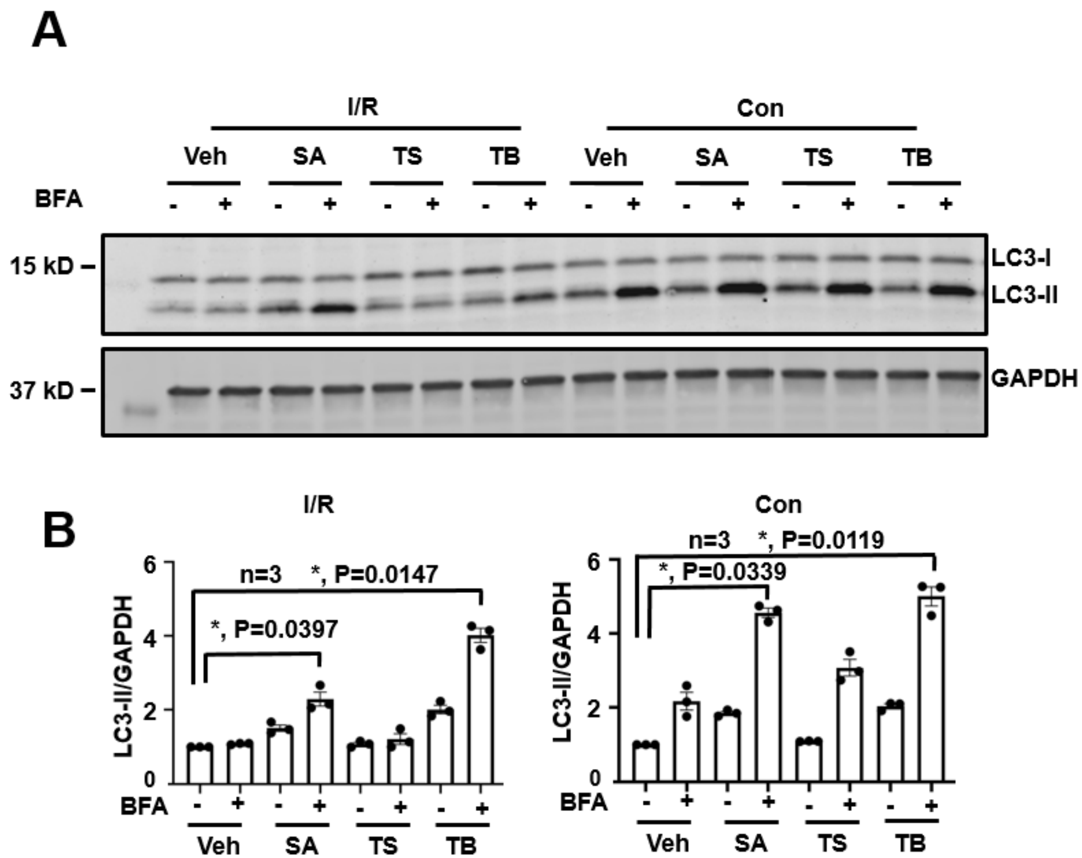
Online Figure III. Tat-Beclin induced autophagy is independent of mTOR.

(A, B, C) Immunoblot detection the mTOR signaling cascade proteins extracted from isolated NRVMs normalized to GAPDH revealing increased autophagy and autophagic flux with both TB and SAHA (SA) measured by LC3-II conversion, but that SAHA inhibits mTOR signaling (downregulation of pS6 and p4EBP1) whereas TB acts independently of the mTOR pathway (no changes in pS6 and p4EBP1 compared to control).



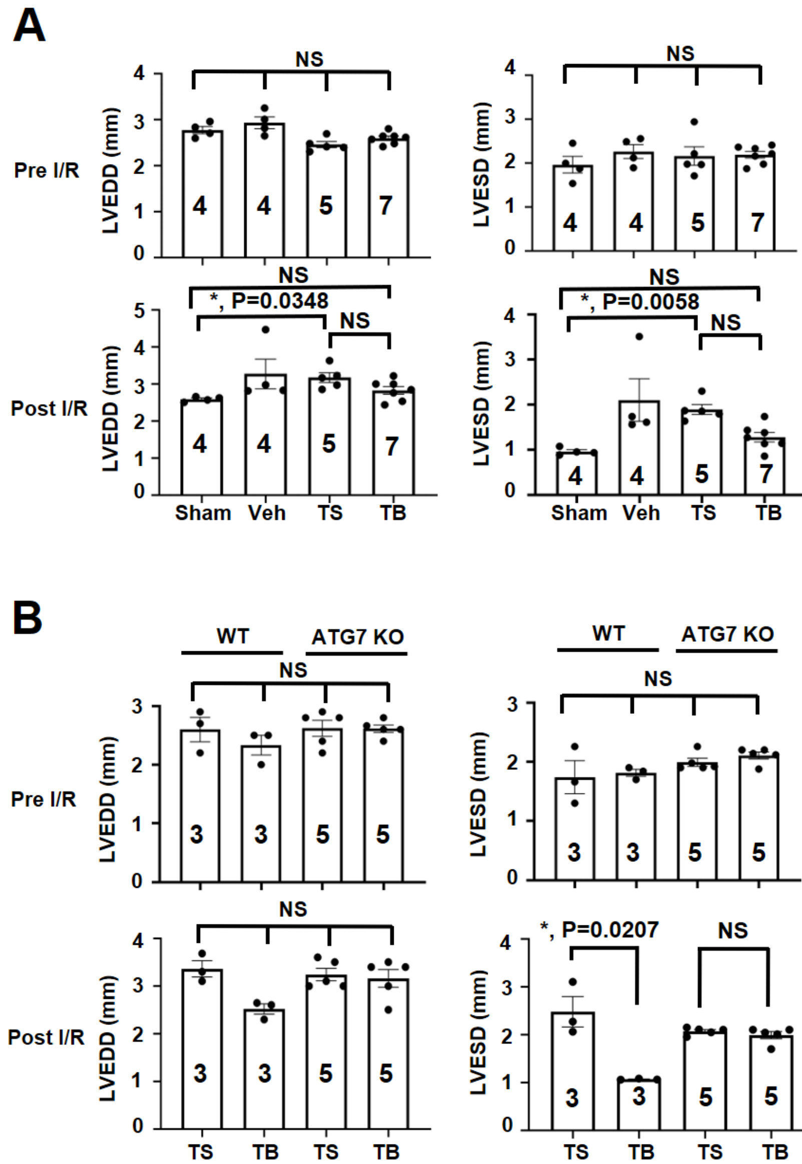
Online Figure IV. Tat-Beclin peptide induces autophagy in both adult and neonatal cardiomyocytes.

(A, B) Immunoblot and quantification of LC3 normalized to GAPDH in NRVMs and ARVMs with or without bafilomycin A (BFA) revealing increased autophagic flux only in the TB-treated groups. (C, D) RFP-GFP-LC3 adenovirus-treated NRVMs showing increased RFP-LC3 conversion only in cells receiving TB peptide. (A, B). N = 6 in all panels and passed normality test. One-way ANOVA followed by Tukey's post-hoc multicomparison test was used. P values are indicated on the figure.



Online Figure V. Tat-Beclin and SAHA both stimulate autophagic flux during I/R injury.

(A, B) Immunoblot and quantification of LC3 normalized to GAPDH in NRVMs with or without bafilomycin A (BFA) revealing increased autophagy in cells treated with TB or SAHA compared with those receiving control vehicle or TS. Kruskal-Wallis test with Dunn's multiple comparisons test was used. N number and P values are indicated on the figure.



Online Figure VI. Tat-Beclin improves post I/R dimensions of the left ventricle, and the loss of ATG7 abolished this protective effect.

(A) Left ventricular end-diastolic and systolic dimensions (LVEDD and LVESD) of wild-type mice treated Tat-Scrambled (TS) and Tat-Beclin (TB). (B) LVEDD and LVESD of wild-type and ATG7 cardiac knockout mice treated with TS and TB. Kruskal-Wallis test with Dunn's multiple comparisons test was used. N number and P values are indicated in the figure.