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Supplemental Material to:

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Upregulated autophagy protects cardiomyocytes from oxidative stress-induced toxicity

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SUPPLEMENTAL INFORMATION

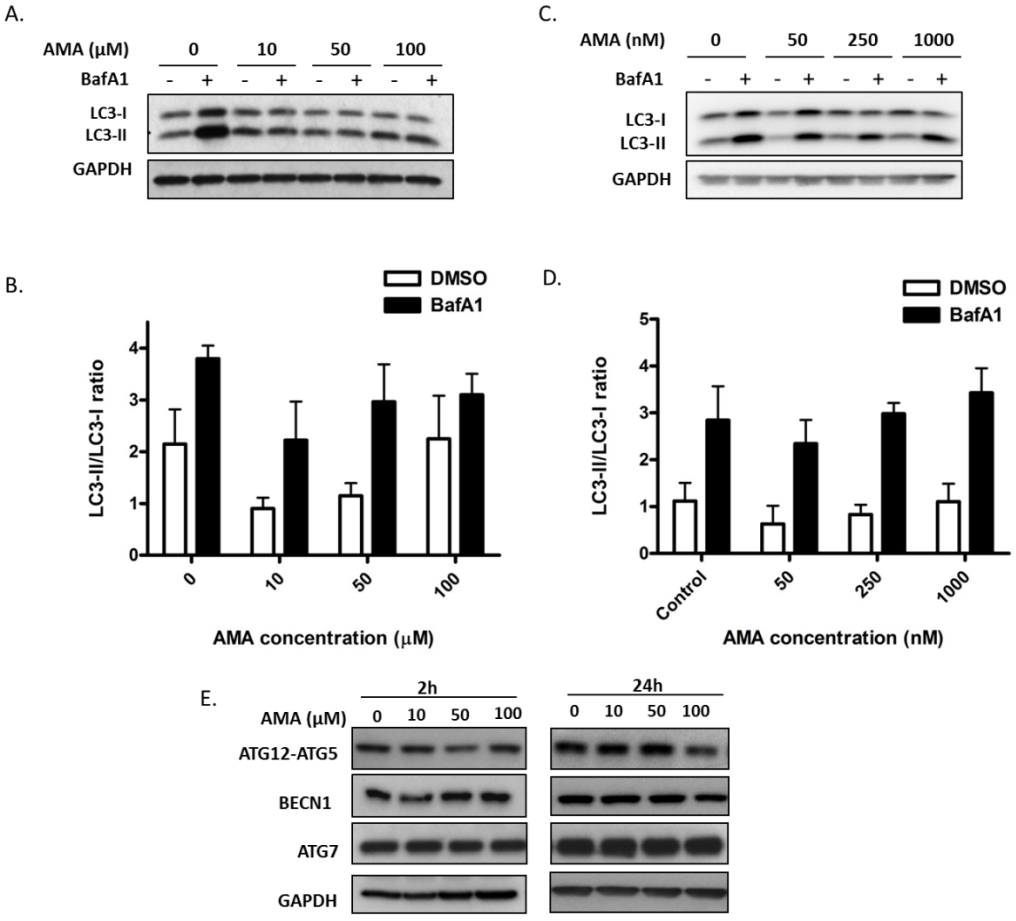

Figure S1. AMA does not induce autophagy in HL-1 cells. (A) Cells were incubated with increasing concentrations of AMA for 24 h, in the presence of DMSO (vehicle) or 75 nM BafA1, added during the final 2 h of incubation. Cells were subsequently lysed and immunoblotted for LC3B. GAPDH is used as a loading control. (B) LC3-II/LC3-I ratios were calculated from three independent experiments as in (A). (C) Cells were incubated with nanomolar concentrations of AMA for 4 h, in the presence of DMSO (vehicle) or 75 nM BafA1. Cells were subsequently lysed and immunoblotted for LC3B. GAPDH is used as a loading control (D). LC3-II/LC3-I ratios were calculated from three independent experiments as in (C). (E) Cells were treated with increasing concentrations of AMA for 2 h or 24 h and were subsequently lysed and immunoblotted for ATG12–ATG5 conjugate, BECN1 and ATG7. GAPDH is used as a loading control. Data represent three or more independent experiments.

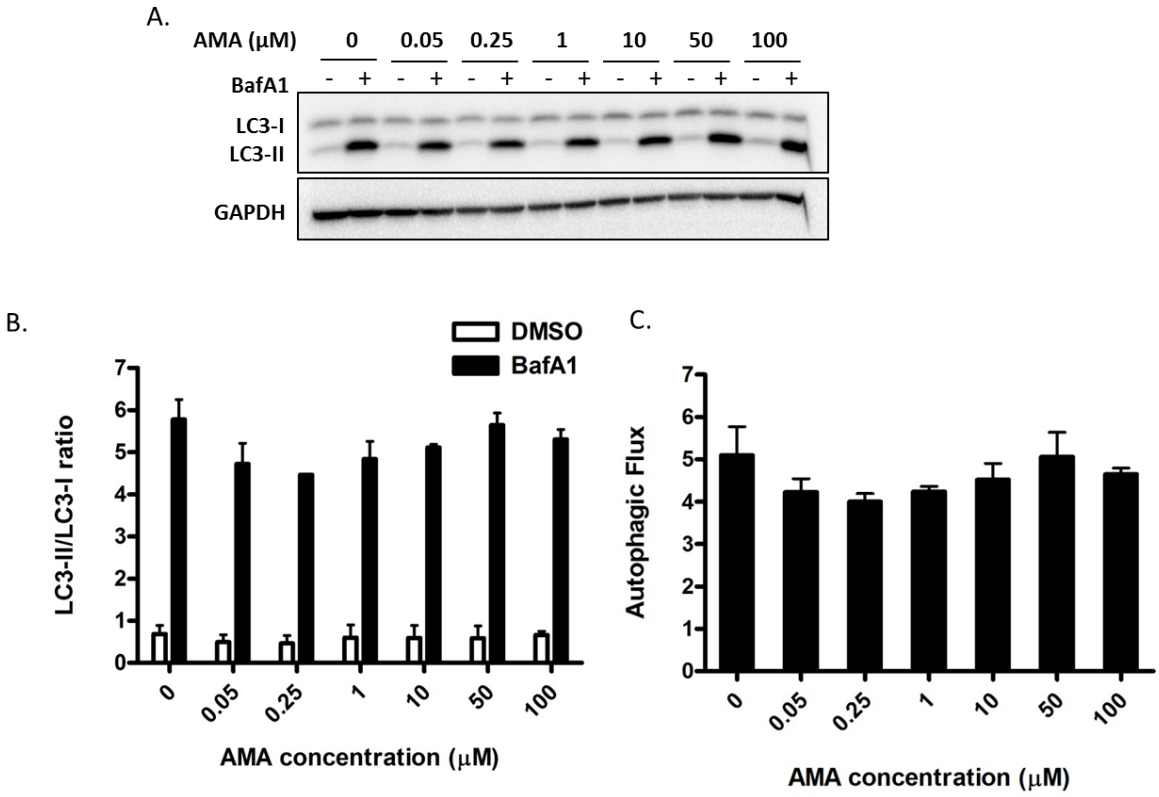

Figure S2. AMA does not induce autophagy in AC16 cells. (A) AC16 cells were treated with increasing concentrations of AMA for 4 h, in the presence of DMSO (vehicle) or 75 nM BafA1. Cells were subsequently lysed and immunoblotted for LC3B. GAPDH is used as a loading control. (B) LC3-II/LC3-I ratios were calculated from two independent experiments as in (A). (C) Autophagic flux in AMA-treated cells as in (A) were calculated by subtracting LC3-II/LC3-I ratios under steady state conditions from that obtained under +BafA1 conditions. Data are derived from two independent experiments.

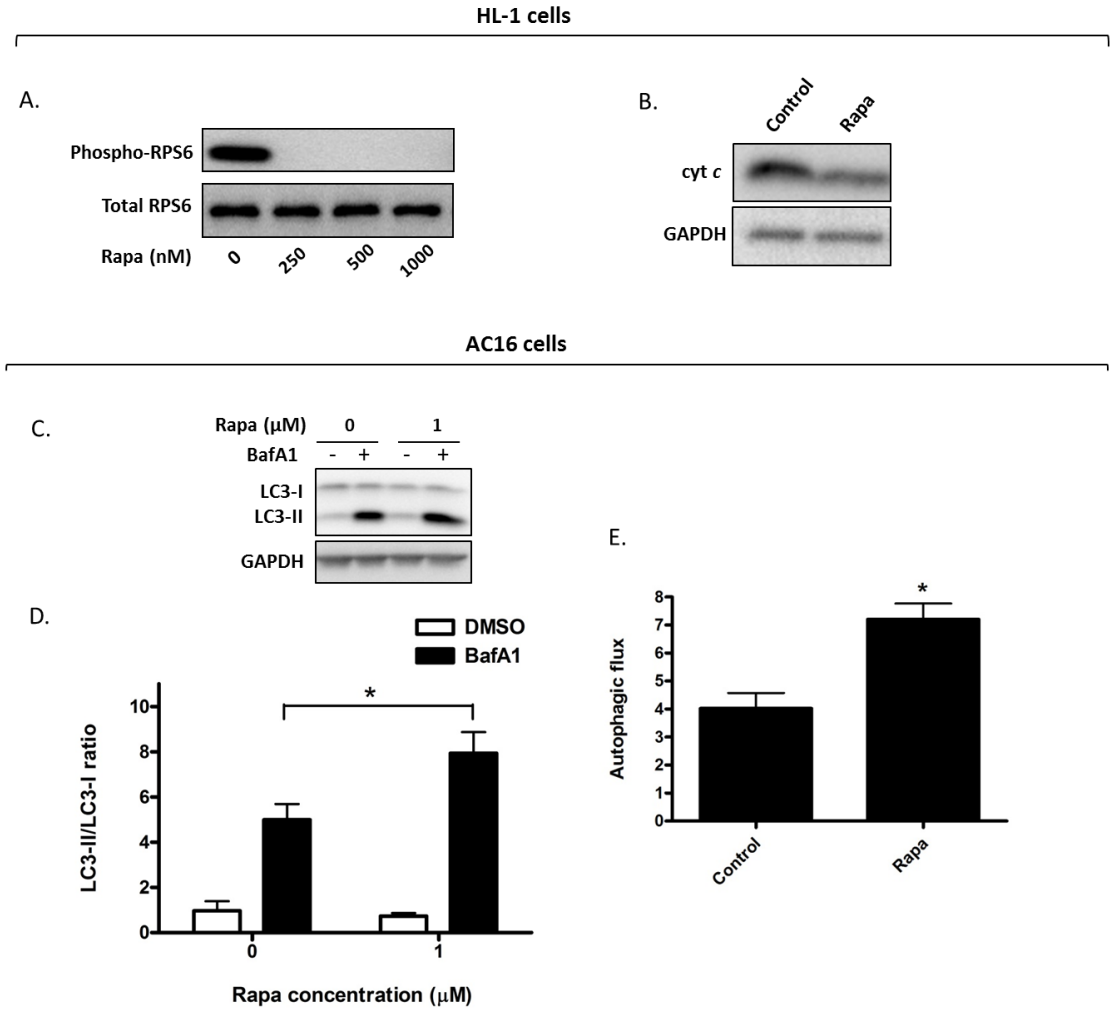

Figure S3. Rapamycin enhances autophagic flux and promotes mitochondrial clearance. (A) HL-1 cells were incubated with increasing concentrations of rapamycin or vehicle control for 16 h and subsequently lysed and immunoblotted for phospho-RPS6 and total RPS6 levels. (B) HL-1 cells were treated with vehicle control or 1 μM rapamycin for 48 h and the cells were subsequently lysed and immunoblotted for cyt *c*. GAPDH is used as a loading control. (C) AC16 cells were treated with vehicle control or 1 μM rapamycin for 4 h in the presence of DMSO (vehicle) or 75 nM BafA1. Cells were subsequently lysed and immunoblotted for LC3B. (D) LC3-II/LC3-I ratios were calculated from three independent experiments as in (C). (F) Autophagic flux in rapamycin-treated cells (C) was calculated by subtracting LC3-II/LC3-I ratios under steady state conditions from that obtained under +BafA1 conditions. Rapa, rapamycin. *$p < 0.05$ vs. control.

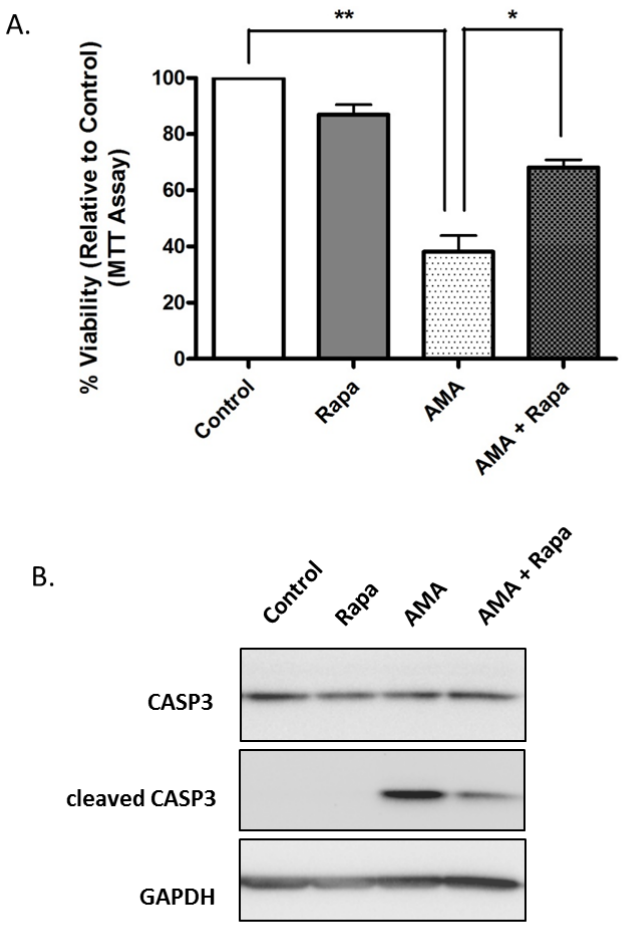

Figure S4. Rapamycin protects against AMA-mediated decreases in cell survival and CASP3 apoptotic signaling. (A, B) HL-1 cells were pretreated with vehicle control or 1 μM rapamycin for 16 h, followed by incubation with 50 μM AMA for an additional 32 h, in the absence or presence of rapamycin. Cell viability was subsequently assessed using MTT assay. Data represent three independent experiments. *$p < 0.05$; **$p < 0.01$ (B) Cells were treated as in (A), cell lysates were subsequently collected and immunoblotted for CASP3. GAPDH is used as a loading control. Data are derived from two to three independent experiments. Rapa, rapamycin.

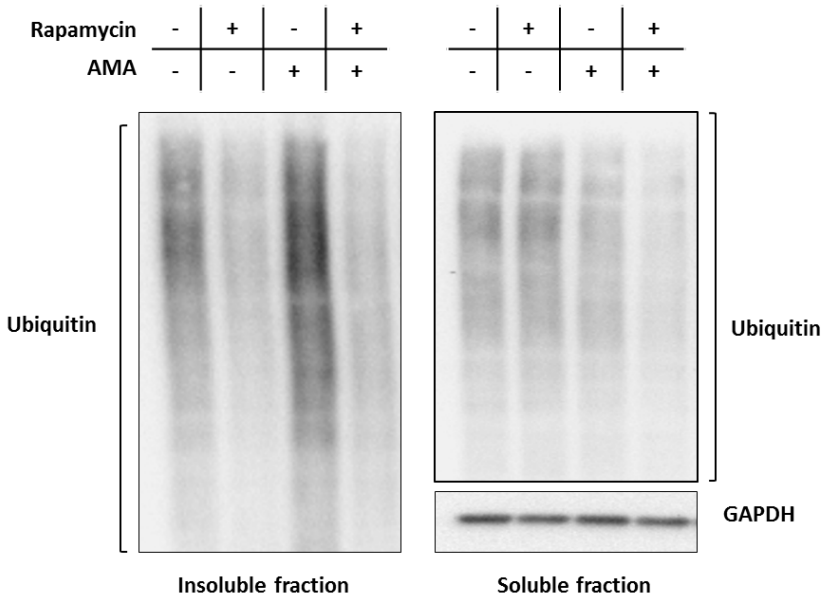

Figure S5. Rapamycin enhances the loss of ubiquitinated proteins induced by AMA. Cells were pretreated with vehicle control or 1 μM rapamycin for 16 h, followed by incubation with 50 μM AMA for an additional 32 h, in the absence or presence of rapamycin. Cell lysates were collected and detergent soluble and insoluble fractions were immunoblotted for uniquitinated proteins. GAPDH is used as a loading control for the soluble fraction.

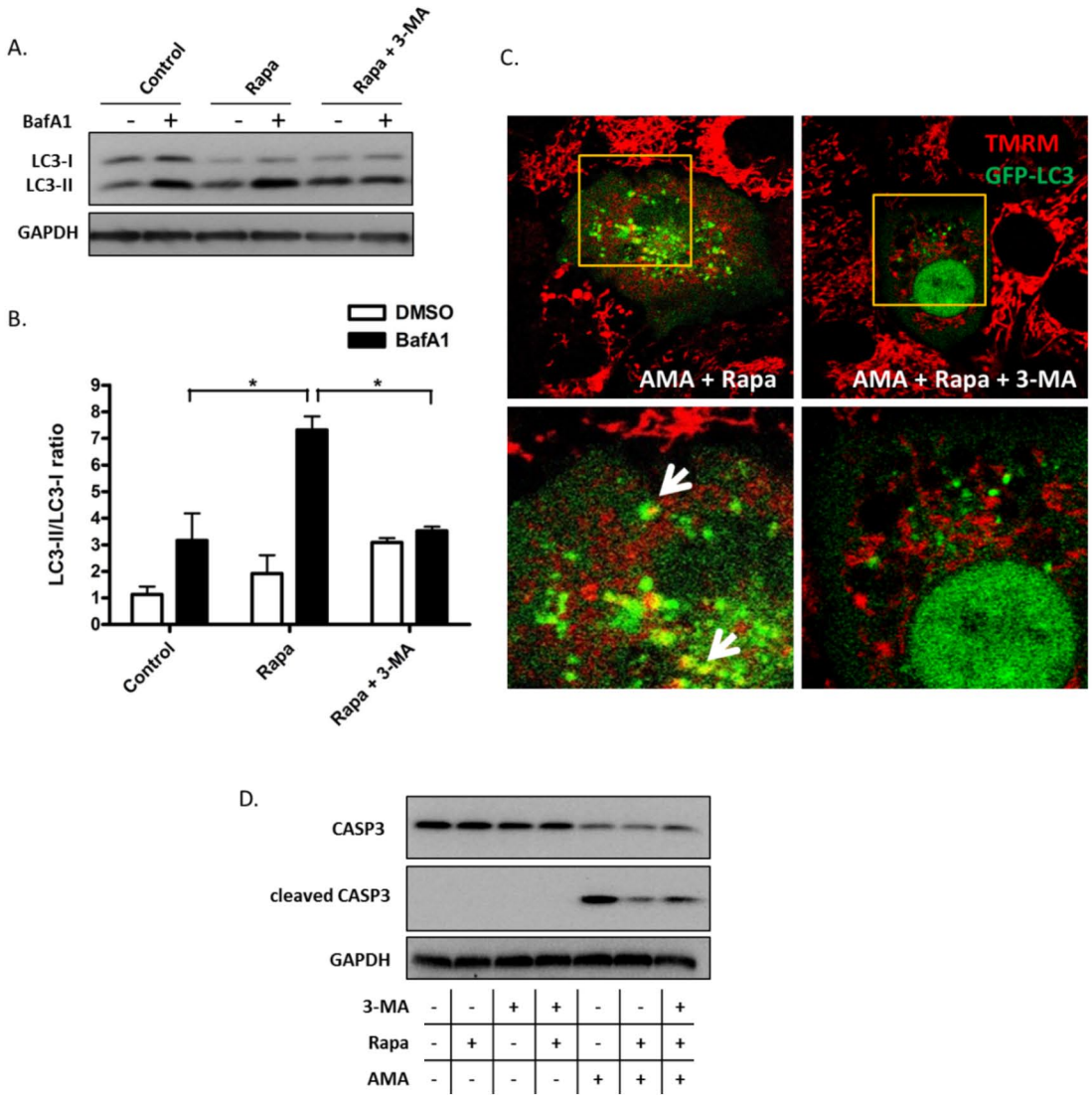

Figure S6. Treatment with 3-MA inhibits rapamycin-induced autophagy and mitophagy and subsequently inhibits rapamycin-mediated protection against AMA-induced CASP3 activation in HL-1 cells. (A) Cells were treated with 1 μM rapamycin in the absence or presence of 5 mM 3-MA for a period of 16 h, in the presence of DMSO or 75 nM BafA1, added during the final 4 h of incubation. Cells were subsequently lysed and immunoblotted for LC3B. (B) LC3-II/LC3-I ratios were calculated from two independent experiments as in (A). *$p < 0.05$ (C) Cells were transfected with GFP-LC3 plasmid and 24 h after transfection, treated with 1 μM rapamycin alone or were coincubated with 5 mM 3-MA for 16 h. Cells were subsequently labeled with 50 nM TMRM for 30 min, followed by treatment with 50 μM AMA. Cells were immediately imaged using confocal microscopy. Higher magnification images of the boxed area are shown in the bottom-panels. Arrow indicates mitophagy. (D) HL-1 cells were pretreated with

1 μM rapamycin in the absence or presence of 5 mM 3-MA for 16 h, followed by treatment with 50 μM AMA for an additional 32 h, in the absence or presence of 1 μM rapamycin and 5 mM 3-MA. Cells were subsequently lysed and immunoblotted for CASP3. GAPDH is used as a loading control. Rapa, rapamycin.

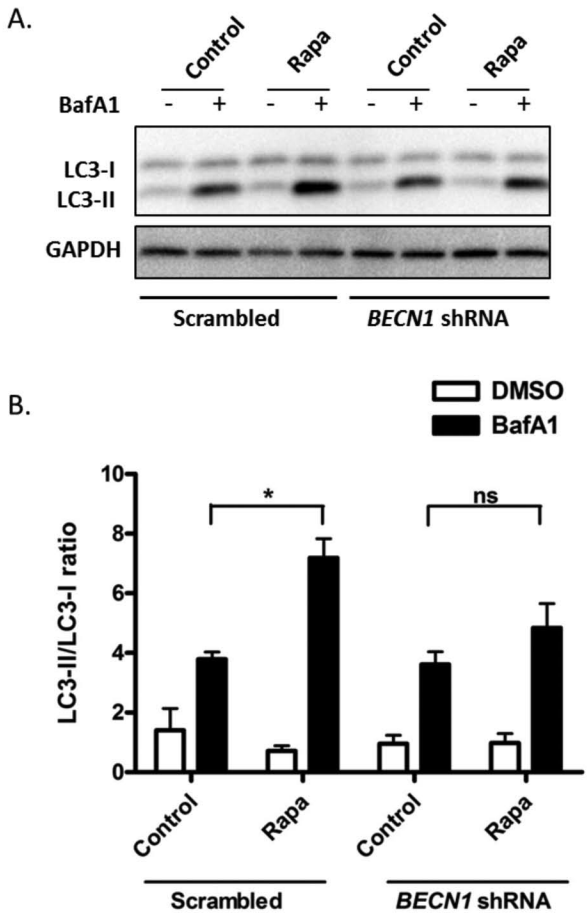

Figure S7. Knockdown of BECN1 inhibits rapamycin-induced autophagy in AC16 cells. Scrambled or *BECN1* shRNA-expressing cells were treated with 1 μM rapamycin for a period of 4 h, in the presence of DMSO (vehicle) or 75 nM BafA1. Cells were subsequently lysed and immunoblotted for LC3B. GAPDH is used as a loading control. (B) LC3-II/LC3-I ratios were calculated from two independent experiments as in (A). Rapa, rapamycin. *$p < 0.05$.

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