APPENDIX

Control of NAD^+ homeostasis by autophagic flux modulates cardiac function

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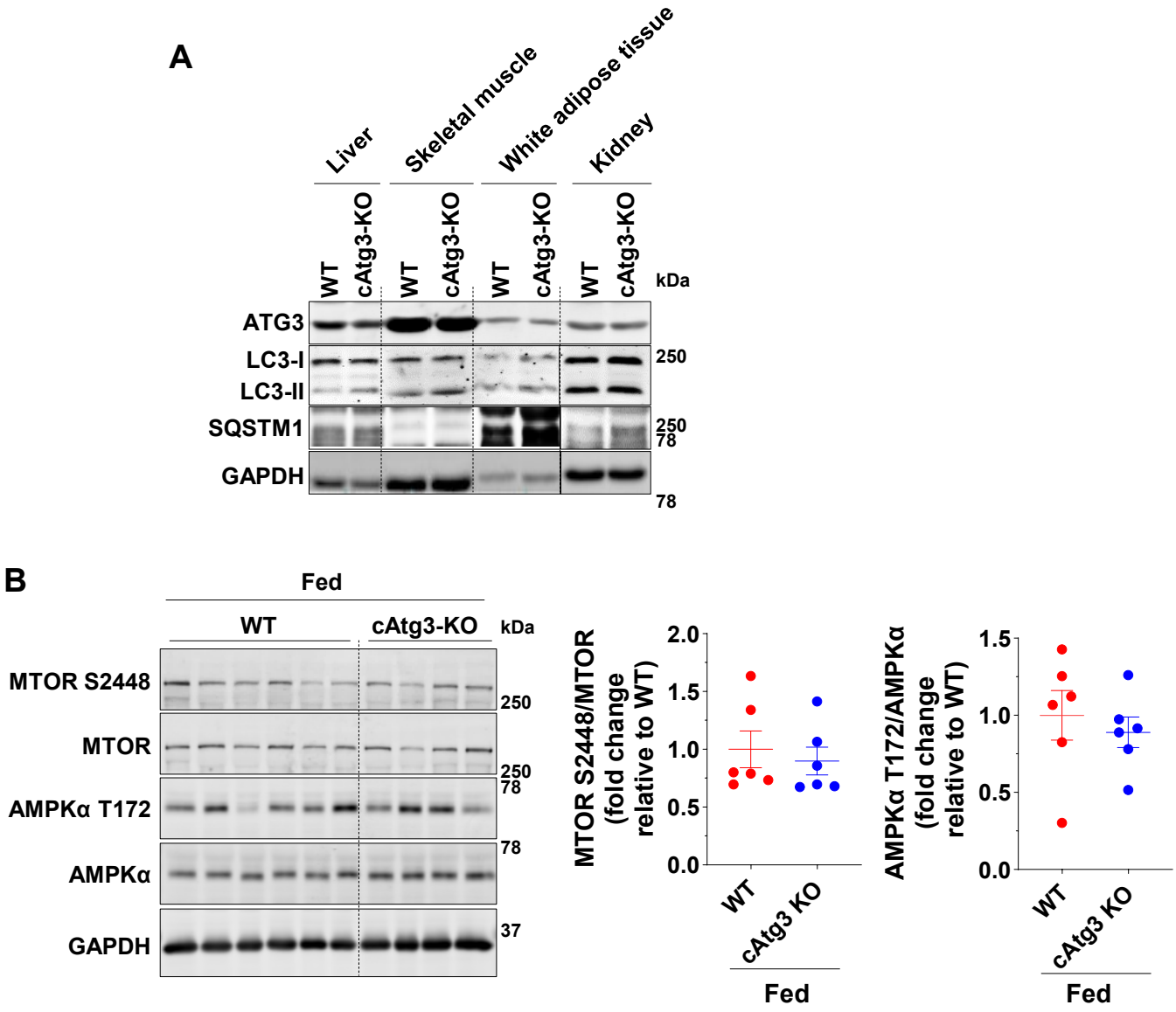
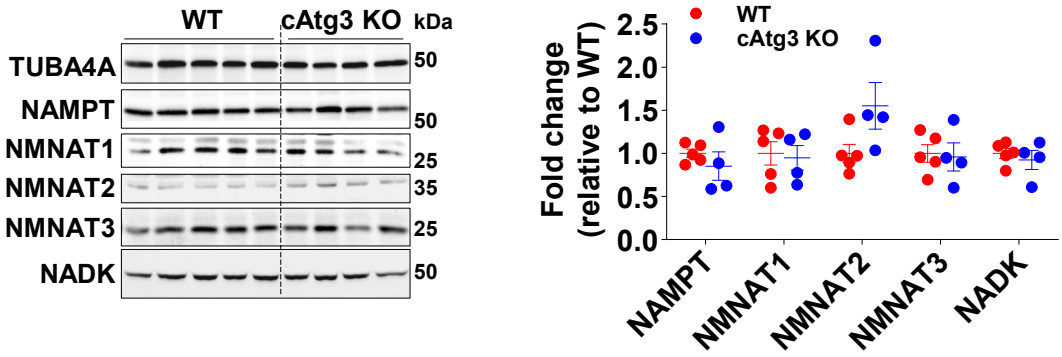


Appendix Figure S1. cAtg3-KO does not alter autophagic flux in non-cardiomyocyte tissues and does not influence MTOR and AMPK signaling in hearts, Related to Figure 1.

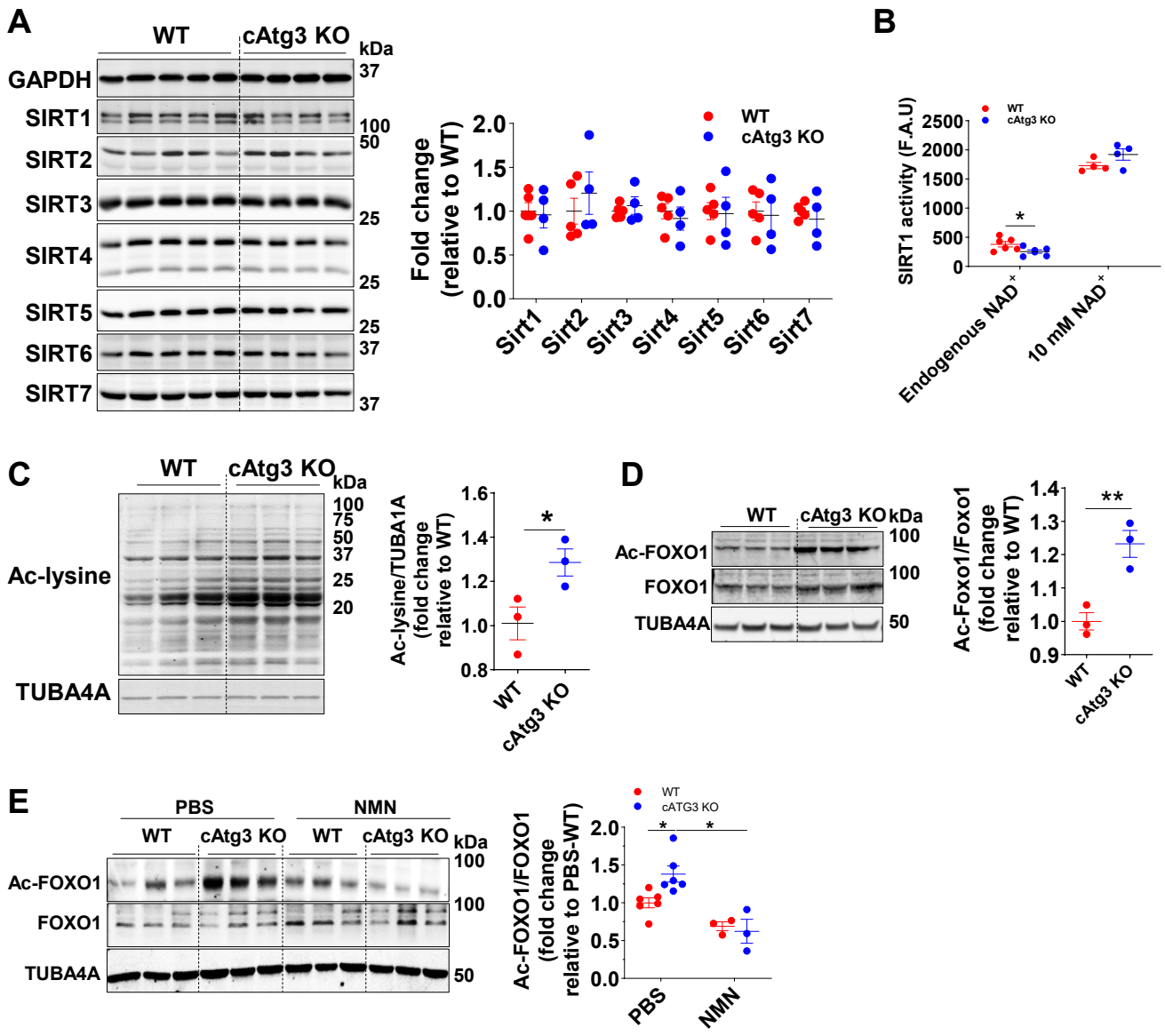
A Protein levels of ATG3, LC3 (MAP1LC3A), SQSTM1, and GAPDH in liver, skeletal muscle, white adipose tissue, and kidney of WT and cAtg3-KO mice. Representative blots are shown, n=2 per group.

B Quantification of MTOR S2448 and AMPKα T172 phosphorylation. Mice were randomly fed. n=6 per group. Data are mean±SEM.



Appendix Figure S2. cAtg3-KO does not alter protein expression of NADK, NAMPT or NAMNAT1-3 in hearts, Related to Figure 5.

Protein levels of NADK, NAMPT, and NAMNAT1-3, TUBA4A (alpha-tubulin) in WT and cAtg3 KO mouse hearts. Mice were randomly fed, n=4 to 5 per group. Data are mean±SEM. Representative section images are shown.



Appendix Figure S3. cAtg3-KO inhibited Sirt1 activities and increased protein acetylation in hearts, Related to Figure 5.

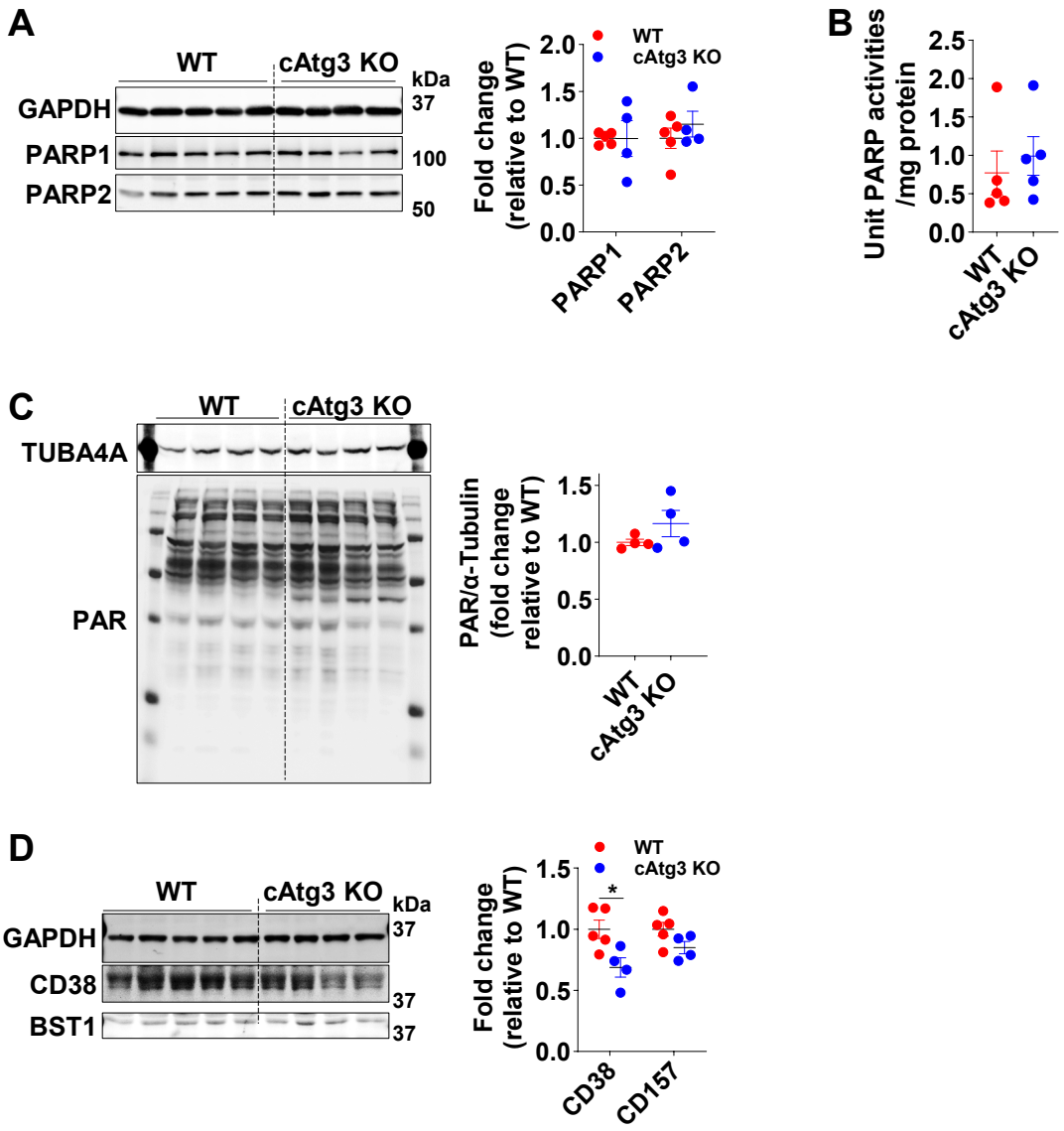
A Protein levels of SIRT1 to 7 and GAPDH in WT and cAtg3 KO mouse hearts. Mice were randomly fed, n=4 to 5 per group. Data are mean±SEM.

B SIRT1 enzymatic activity measurement in 4-week-old WT and Atg3 KO mouse hearts, without or with the addition of 10 mM exogenous NAD^+, n=4 to 6 per group. Data are mean±SEM. Unpaired t-tests were used to determine statistical significance between two groups. *p<0.05.

C Levels of Ac-lysine and TUBA4A (alpha-tubulin) in 4-week-old WT and cAtg3 KO mouse hearts. Mice were randomly fed, n=3 per group. Data are mean±SEM. An unpaired t-test was used to determine statistical significance between two groups. *p<0.05.

D Protein levels of Ac-FOXO1, FOXO1, and TUBA4A in 4-week-old WT and cAtg3 KO mouse hearts. Mice were randomly fed, n=3 per group. Data are mean±SEM. An unpaired t-test was used to determine statistical significance between two groups. **p<0.01.

E Ac-FOXO1 expression levels in hearts from mice that were injected with PBS or NMN, n=3 to 6 per group. Data are mean±SEM. One-way ANOVA followed by Bonferroni's multiple comparison tests was used to determine statistical significance. *p<0.05.



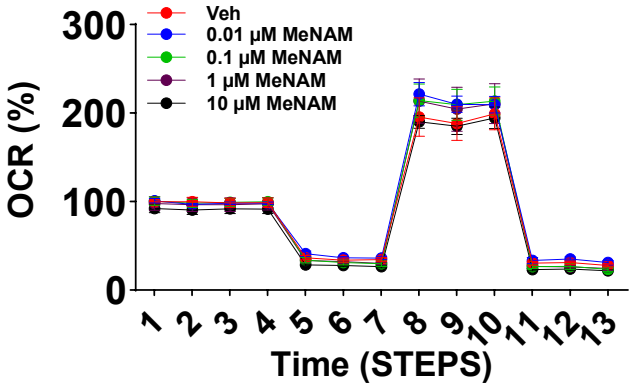
Appendix Figure S4. cAtg3-KO does not alter PARP1/2 protein expression, PARP1/2 activities, or abundance of CD38 and BST1 (CD157) in hearts, Related to Figure 5.

A PARP1/2 protein expression in 4-week-old WT and cAtg3 KO mouse hearts, n=4 to 5 per group. Data are mean±SEM.

B PARP1/2 enzymatic activity measurement in 4-week-old WT and cAtg3 KO mouse hearts, n=5 per group. Data are mean±SEM.

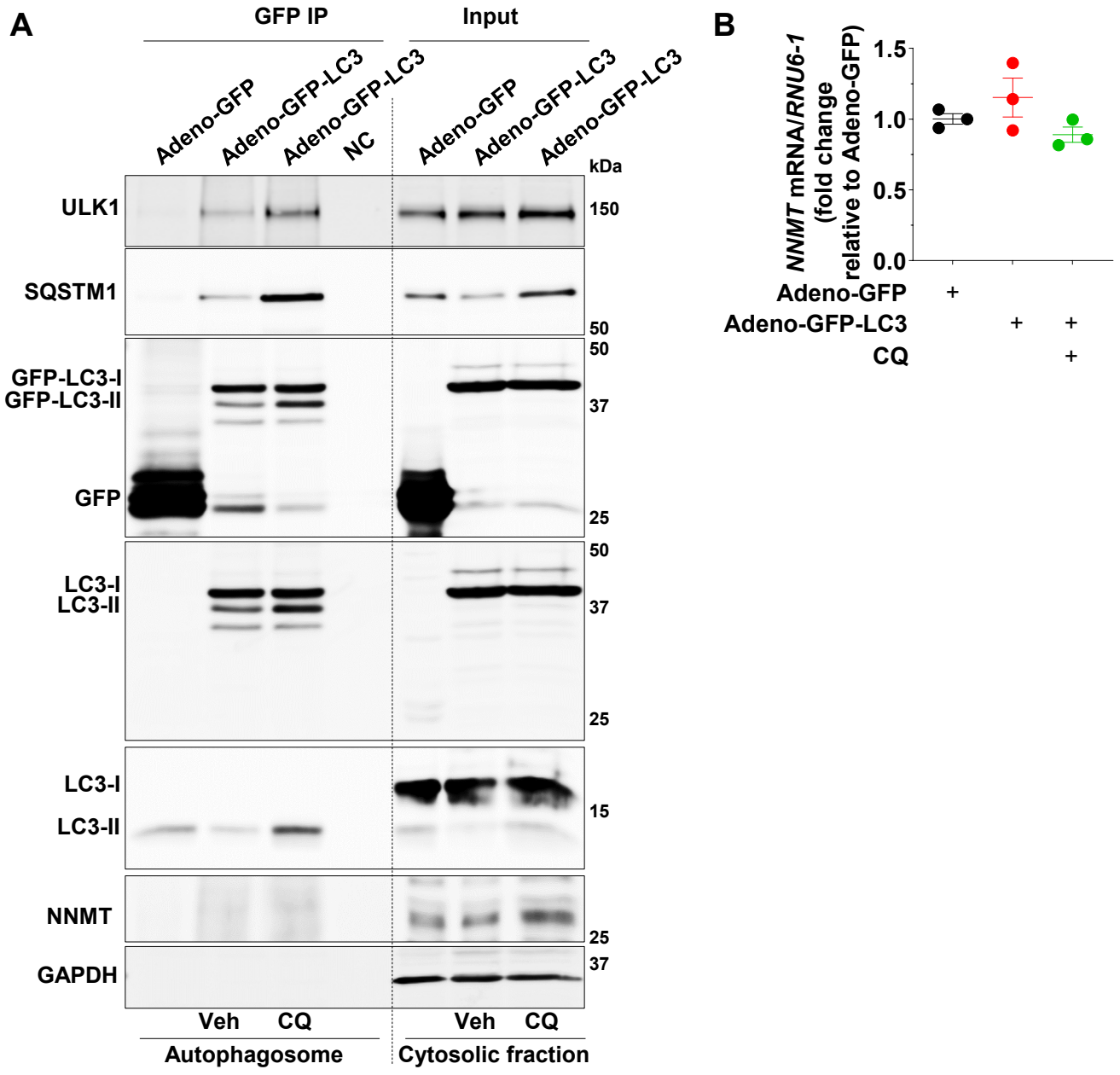
C Abundance of PAR in 16-week-old WT and cAtg3 KO mouse hearts, n=4 per group. Data are mean±SEM.

D Protein levels of CD38 and BST1, and GAPDH in WT and cAtg3 KO mouse hearts. Mice were randomly fed, n-4 to 5 per group. Data are mean±SEM. An unpaired t-test was used to determine statistical significance between two groups. *p<0.05. Representative section images are shown.



Appendix Figure S5. Oxygen consumption in response to increasing concentrations of Me-NAM in H9c2 cardiomyocytes, Related to Figure 6.

Oxygen consumption rate (OCR) in H9c2 cells treated with PBS (Veh) or concentrations of MeNAM as indicated, n=3 to 4 per group. Data are mean±SEM. Step 1 to 4: 20 μM glucose medium, step 5 to 7: oligomycin (1μg/ml), step 8 to 10: FCCP (0.5μg/ml), step 11 to 13: rotenone (1μg/ml).



Appendix Figure S6. Autophagosomes do not engulf NNMT protein or mRNA in H9c2 cardiomyocytes, Related to Figure 7. H9c2 cardiomyocytes were first transfected with adenovirus encoding either GFP or GFP-tagged LC3 (MAP1LC3A), and then cells encoding GFP-LC3 were treated with either vehicle (Veh) or chloroquine (CQ) at 20 uM for 16 h. under nutrient replete conditions.

A The protein levels of ULK1, SQSTM1, GFP, GFP-LC3, LC3, NNMT, and GAPDH in isolated autophagosomes. GFP-LC3 were probed with antibodies against GFP and LC3, and the images of immunoblots were acquired using LI-COR Odyssey DXL system at two independent laser channels, respectively. Therefore, GFP antibody detects GFP and GFP-LC3, and LC3 antibody detects GFP-LC3 only. The experiments were repeated three times. Representative blots are shown. NC indicates the separation buffer with GFP antibody. Input is cytosolic fractions.

B *NNMT* mRNA levels in isolated autophagosomes. n=3 per group. Data are mean±SEM.

Appendix Tables

Table S1. **Percentage of cAtg3 KO mice**

Sex	Genotype	N.	%
M	WT	89	25.72
	cAtg3 KO	85	24.57
F	WT	81	23.41
	cAtg3 KO	91	26.30

Appendix Tables

Table S2. **Primer Sequences**

Gene	Sequences
GAPDH (M)	GCAACAATCTCCACTTTGCCAC AATGGTGAAGGTCGGTGTGAAC
GAPDH (R)	GCTCTCTGCTCCTCCCTGTTC GAGGCTGGCACTGCACAA
RP16S	GATTTGCTGGTGTGGATATC TCTTTGATCTCCTTCTTAGA
ACTB (M)	CGATGCCCTGAGGCTCTT T TGGATGCCACAGGATTCCA
ACTB (R)	GAGACCTTCAACACCCCAGCC TCGGGGCATCGGAACCGCTCA
RPL13A (M)	CTCTGGCCTTTTCCTTTTTG CCGAAGAAGGGAGACAGTTC
RPL13A (R	CCACCCTATGACAAGAAAAAGC ACATTCTTTTCTGCCTGTTTCC
18S (R)	GCCGCTAGAGGTGAAATTCTTA CTTTCGCTCTGGTCCGTCTT
RNU6-1 (R)	GCAAATTCGTGAAGCGTTCC
NPPA (M)	ATGGGCTCCTTCTCCATCA CCTGCTTCCTCAGTCTGCTC
NPPB (M)	GGATCTCCTGAAGGTGCTGT TTCTTTTGTGAGGCCTTGGT
MYH7 (M)	GCCATCATGCACTTTGGAAAC CCCATGAGGTAGGCTGATTTGT
PLN (M)	AACAGGCAGCCAAATGTGA CCCAGCTAAGCTCCCATAAG
NNMT (R)	CAGAGCTGAGACACGATGGA GCAGGCAGAGAGAAGCTGAT
NNMT (M)	GATTGCACGCCTCAACTTCT GAACCAGGAGCCTTTGACTG
NRF1 (M)	CTTCAGAACTGCCAACCACA GCTTCTGCCAGTGATGCTAC
NRF2 (M)	AGTCTTCACTGCCCCTCATC TCTGTCAGTGTGGCTTCTGG
TFAM (M)	GCAAAGGATGATTCGGCTC TCTGCTCTTCCCAAGACTTCA
NMNAT1 (M)	TGAGTCCATGGGGAGAAGTT AGGACTAGGGCCGTTTGG
NMNAT2 (M)	ATCCCGCCAATCACAATAAA GCAGCTTCAATCCCATCACT
NMNAT3 (M)	CAGAAGCACCACAGGGATTC CCTGCAGCACGTTTACAGTC
NMAPT (M)	TCACGGCATTCAAAGTAGGA GCAGAAGCCGAGTTCAACAT
NMRK1 (M)	CTTGAAGCTTGCTCTGCGAC CTCCGTTTGTCACACCACCA
NMRK2 (M)	AAGCCCCAGGACCAAATAGC GCGTGCAAACTTGTGTGGAT

PARP1 (M)	CACCTTCCAGAAGCAGGAGA GCAGCGAGAGTATTCCCAAG
PARP2 (M)	GCAACAGAAGACGACTCTCCT CAGCCATAGGCCCTTTTCTCT
RPL32	ACATCGGTTATGGGAGCAAC GGGATTGGTGACTCTGATGG
mtDNA (M)	CCTATCACCCTTGCCATCAT GAGGCTGTTGCTTGTGTGAC
CHROMOSOME 6 (M)	ATGGAAAGCCTGCCATCATG TCCTTGTTGTTCAGCATCAC
PPARa	GAGAATCCACGAAGCCTACC AATCGGACCTCTGCCTCTTT
VLCAD	AGGCAGTTCTGGACAAGCCA TTCCTCAAAGAACCGGGCCA
LCAD	GCATTGGTGGGGACTTGCTC TGTCATGGCTATGGCACCGA
MCAD	ACTGACGCCGTGCAGATTTT GCTTAGTTACACGAGGGTGATG

Appendix Tables

Table S3. **Antibodies**

Name	Company	Catalog Number
ATG3	Sigma-Aldrich	A3231
LC3	Sigma-Aldrich	L8918
TUBA4A	Sigma-Aldrich	T5168
ACTB	Cell Signaling	3700
SQSTM1	Cell Signaling	5114
Acetylated-Lysine	Cell Signaling	9441
ATG7	Cell Signaling	8858
FOXO1	Cell Signaling	2880
RELA S536	Cell Signaling	3036
RELA	Cell Signaling	6956
MTOR S2448	Cell Signaling	4517
MTOR	Cell Signaling	2971
P70S6K T389	Cell Signaling	9206
p70S6K	Cell Signaling	2708
AMPKα T172	Cell Signaling	2531
AMPKα	Cell Signaling	2793
ULK1	Cell Signaling	8054
GFP	Cell Signaling	55494
Sirtuin antibody sampler kit	Cell Signaling	9787
SIRT4	Cell Signaling	69786
PARP1	Cell Signaling	9532
NADK	Cell Signaling	89833
NRF1	Cell Signaling	69432
GAPDH	Cell Signaling	2118
PRKN	Cell Signaling	4211
CASP3	Cell Signaling	9665
CD38	Proteintech	60006-1-lg
BST1	Proteintech	16337-1-AP
NMNAT1	Proteintech	11399-1-AP
NMNAT3	Proteintech	13236-1-AP
PARP2	Proteintech	20555-1-AP
NMNAT2	ThermoFisher Scientific	PA5-115662
NNMT	Proteintech	15123-1-AP
PINK1	Proteintech	23274-1-AP
NAMPT	Proteintech	11776-1-AP
GAPDH	SCBT	SC-32233
TFAM	SCBT	SC-166965
NRF2	SCBT	SC-722
Ac-FKHR	SCBT	SC-49437
PARGC1A	SCBT	SC-13067
SIRT1	SCBT	SC-74504
TOMM20	SCBT	SC-17764
SDHA	abcam	ab14715

Appendix Tables

Table S4. **siRNA sequence**

Name	Sequences/Company with catalog number
Control siRNA	5´-UAAGGCUAUGAAGAGAUAC-3' 5'-GUAUCUCUUCAUAGCCUUA-3'
ATG3 siRNA	5'-CCCAGAAGAGUUUGUGGCAGCUGGA-3' 5'-UCCAGCUGCCACAAACUCUUCUGGG-3'
ON-TARGETplus SMARTpool *NNMT* siRNA	GE Dharmacon (L-101014-02-0005)
SignalSilence *SQSTM1/p62* siRNA II	Cell Signaling Technology (6399)
SQSTM1/p62	5'-CCUGUGGUGGGAACUCGCUAUAAGU-3' 5'-ACUUAUAGCGAGUUCCCACCACAGG-3'
SignalSilence® *NF-κB p65* (*RELA*) siRNA I	Cell Signaling Technology (6261S)

Appendix Tables

Table S5. **Plasmids and adenovirus**

	Name	Company/Institute	Catalog Number/Provider
Plasmids	*HA-FLAG*	Addgene	10792
	HA-SQSTM1	Addgene	28027
	ATG7	Addgene	24921
Adenovirus	*GFP*	Beth Israel Deaconess Medical Center, Boston, MA	Dr. Pavlos Pissios
	GFP-LC3	Cedars Sinai, Los Angeles, CA	Dr. Roberta A. Gottlieb
	NNMT	Beth Israel Deaconess Medical Center, Boston, MA	Dr. Pavlos Pissios