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Supplementary Materials for

The immunoproteasome catalytic β5i subunit regulates cardiac hypertrophy by targeting the autophagy protein ATG5 for degradation

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Supplementary Materials

Table S1. Clinical characteristics of HF cohort cases.

	Control (n=38)	HF (n=38)	p value
Median ages, yrs	61.97±0.3724	68.58±1.243***	<0.001
Male	18 (47.3)	24(63.1)	0.166
Laboratory results			
Chymotryptic-like activity (RLU)	516.1±46.34	822.6±80.83**	0.002
β5i (Psmb8, ng/ml)	1.665±0.2448	3.428±0.6244*	0.0122
NT-proBNP (pg/ml)	67.22±7.294	1626.4±242.1***	<0.001
Cardiovascular risk factor			
Alcohol drinking	12	8	0.297
Smoke	11	14	0.464
Diabetes	4	3	0.692
Dyslipidemia	10	19*	0.034
Obesity	8	9	0.783
Medications			
ACE inhibitor	0	31	<0.001
Diuretics	0	28	<0.001
Digitalis	0	26	<0.001
Beta-blockers	0	16	<0.001
Examination			

HR (beats/min)	68.42±1.964	70.92±2.625	0.4481
Systolic BP (mm Hg)	127.2±1.94	129.2±3.43	0.956
LVEF (%)	63.34±0.8162	32.11±5.568***	<0.001
LVFS (%)	34.34±0.62	32.16±1.162	0.1014

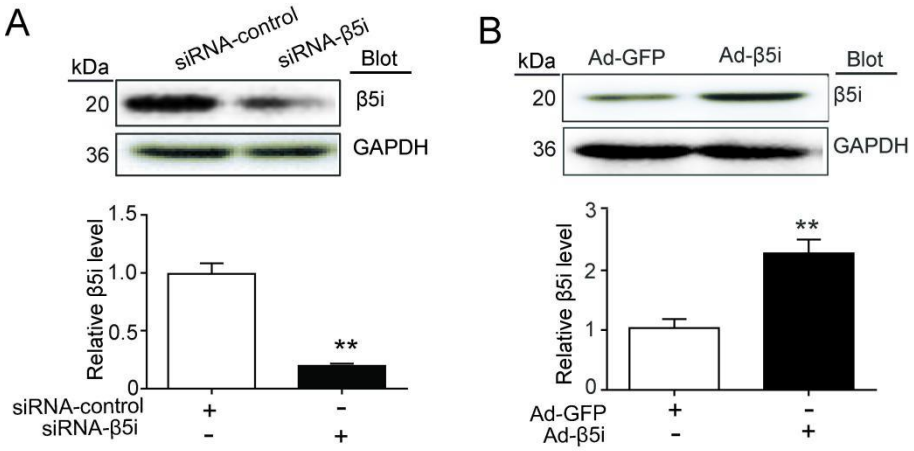
Values are mean ± SEM, n=38 per group.

RLU, relative light units; NT-proBNP,N-terminal pro-B-type natriuretic peptide; HR, heart rate; Systolic BP , systolic blood pressure; LVEF, left ventricular ejection fraction; LVFS, left ventricular fraction shortening. **P < 0.05, **P <0.01, ***P < 0.001 versus control.*

Table S2. Multivariable logistic regression analysis of β5i and chymotrypsin-like activity associated with HF (*n* = 38).

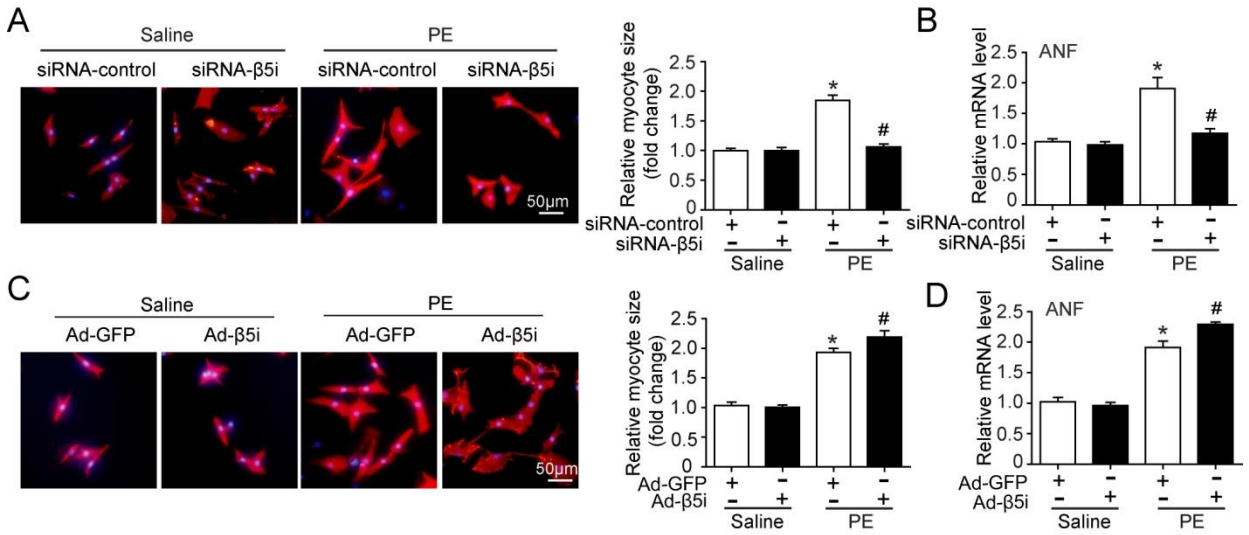
Model	OR(95%CI)	P value
β5i (Psmb8)	12.153 (2.38-62.08)	0.003
Chymotryptic-like activity	1.005 (1.002-1.009)	0.003

All models adjusted for age, sex, alcohol drinking, smoke, diabetes, dyslipidemia and obesity.



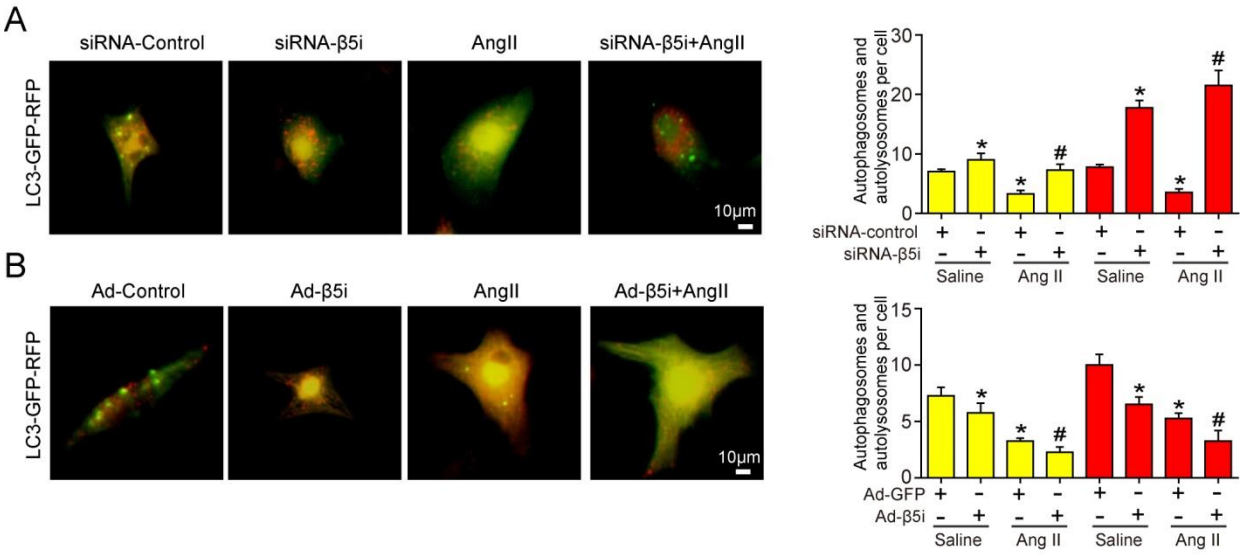
Supplementary Figure 1

Fig. S1. Analysis of the β5i protein levels in rat neonatal cardiomyocytes. (**A**) Representative western blot analysis of β5i protein expression in neonatal rat cardiomyocytes (NRCMs) infected with adenovirus vector expressing siRNA-control or siRNA-β5i for 24 hours (upper). Quantification of the relative protein levels (lower, n=3). (**B**) Representative western blot analysis of β5i protein expression in NRCMs infected with adenovirus vector expressing GFP (Ad-GFP) or β5i (Ad-β5i) for 24 hours (upper). Quantification of the relative protein levels (lower, n=3). Data are presented as mean ± SEM, and n represents number of samples per group. ***P* < 0.01 versus siRNA-control or Ad-GFP.



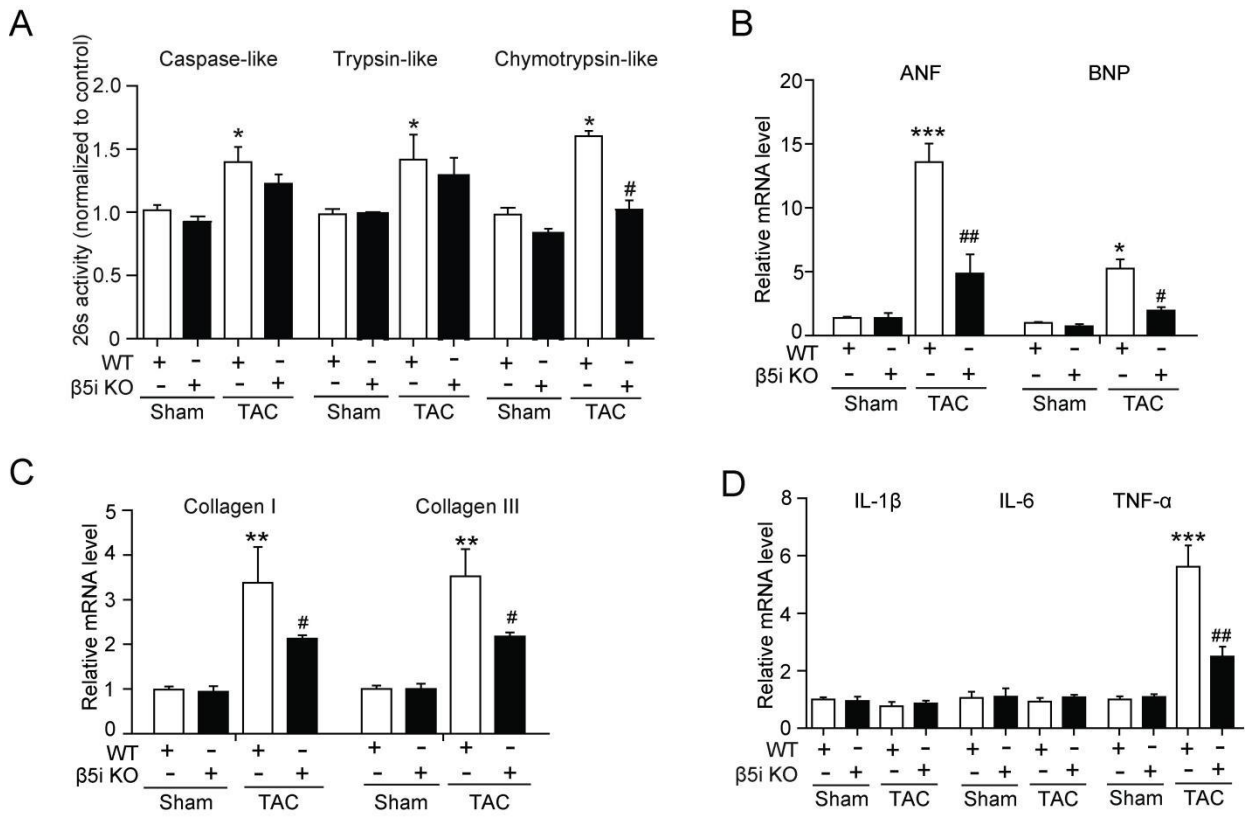
Supplementary Figure 2

Fig. S2. KO of β5i inhibits phenylephrine (PE)–induced cardiomyocyte hypertrophy. (**A**) Representative images of double immunostaining (green for α-actinin, blue for DAPI) of NRCMs transfected with Ad-siRNA-β5i or siRNA-control after PE stimulation. Scale bar 50 μm. Quantification of cell surface area (at least 150 cells counted per experiment; right). (**B**) qPCR analyses of ANF mRNA expression (right, n=5). (**C**) Immunostaining of cardiomyocyte size with α-actinin (red) and DAPI (blue) infected with Ad-GFP or Ad-β5i after PE stimulation. Scale bar 50 μm. Quantification of cell surface area (at least 150 cells counted per experiment; right). (**D**) qPCR analyses of ANF mRNA expression (right, n=5). For A and B, **P*< 0.05 versus siRNA-control+Saline; $^{\#}$*P*< 0.05 versus siRNA-control+PE. For C and D, **P*< 0.05 versus Ad-GFP+Saline; $^{\#}$*P*< 0.05 versus Ad-GFP+PE.



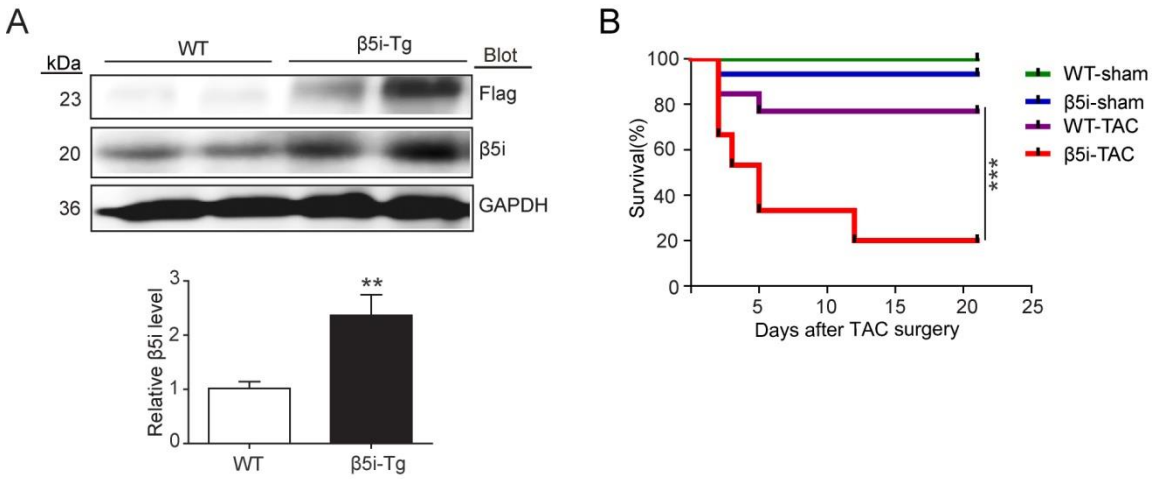
Supplementary Figure 3

Fig. S3. Analysis of autophagic influx in rat neonatal cardiomyocytes. (A) Rat neonatal cardiomyocytes (NRCMs) infected with adenovirus expressing siRNA-control or siRNA-β5i together with mRFP-GFP-LC3-PE, and then treated with Ang II for 24 hours. **(B)** NRCMs infected with adenovirus overexpressing GFP-control or β5i together with mRFP-GFP-LC3-PE, and then treated with Ang II for 24 hours. The autophagic flux was detected with Live Cell Imaging Microscopy. Red coloure represents autophagolysosomes, and red/green double-coloure shows autophagosomes (left). Scale bar 10 μm.. Quantification of autophagosomes and autophagolysosomes (right, n=3). Data are presented as mean ± SEM, and n represents number of samples per group. **P* < 0.05 versus siRNA-control+Saline or Ad-GFP+Saline; #*P* < 0.05 versus siRNA-control+Ang II or Ad-GFP+Ang II.



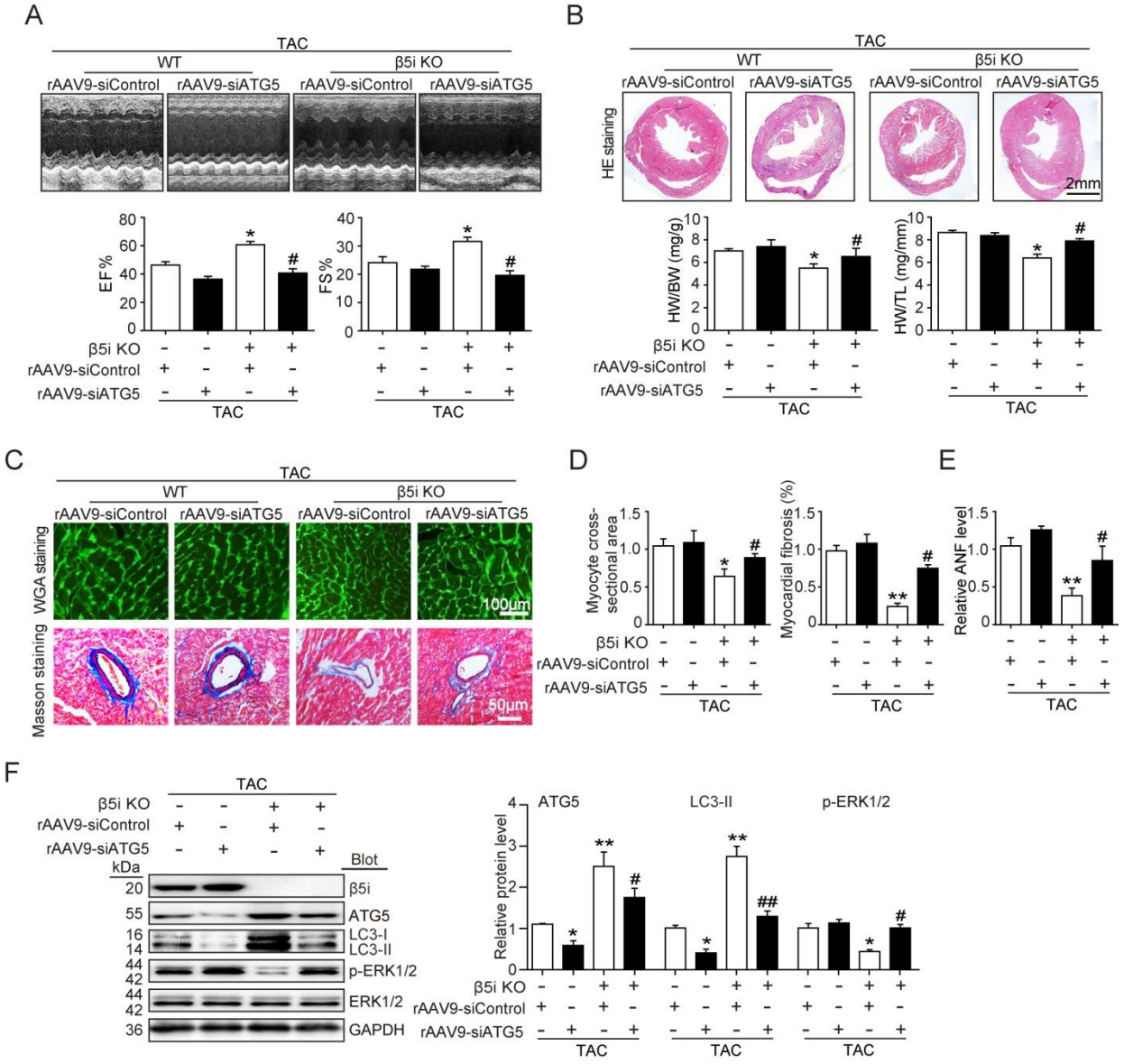
Supplementary Figure 4

Fig. S4. KO of β5i decreases chymotrypsin-like activity in mice after TAC operation. (**A**) Proteasome activities, including caspase-like, trypsin-like or chymotrypsin-like in the heart tissues of WT or β5i KO mice after 6 weeks of sham or TAC (n=5). (**B，C and D**) qPCR analysis of hypertrophic markers (ANF and BNP), fibrotic markers (collagen I and III) and inflammatory cytokines (IL-1β, IL-6 and TNF-α) mRNA expression in the heart tissues of wild-type (WT) and knockout (β5i KO) mice after 6 weeks of sham or TAC (n=5). Data are presented as mean ± SEM, and n represents number of animals per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus WT+sham; $^{\#}$*P* < 0.05, $^{\#\#}$*P* < 0.01 versus WT+TAC.



Supplementary Figure 5

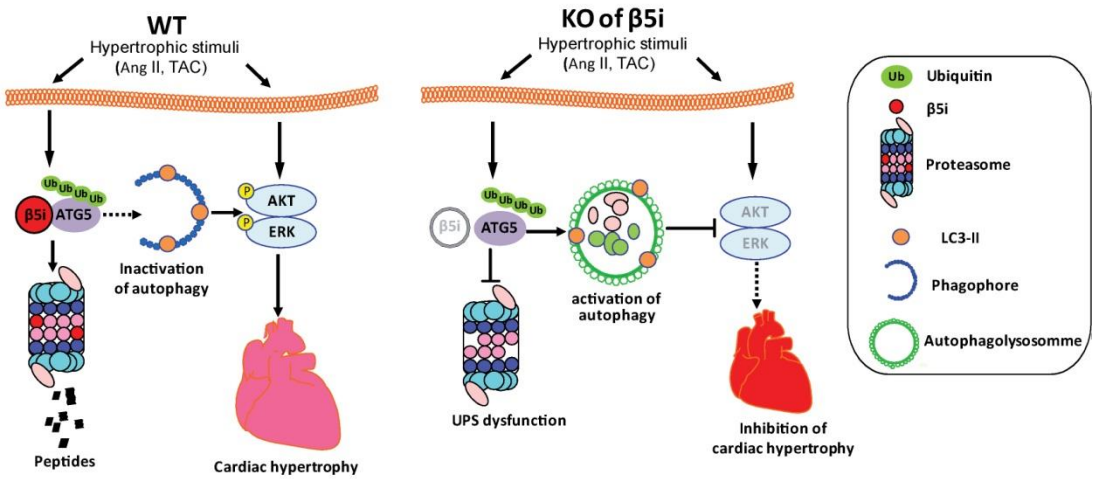
Fig. S5. Analysis of the β5i expression and survival rate in WT and β5i-Tg mice. (**A**) Representative western blot analysis of β5i expression with anti-Flag or β5i antibody in the heart tissues of wild-type (WT) and β5i transgenic (β5i-Tg) mice (left). Quantification of the relative β5i protein level (down, n=6). (**B**) WT and cardiac-specific β5i transgenic (β5i-Tg) mice were subjected to sham or TAC operation for 3 weeks, then the survival rate was calculated (n=12). Data are presented as mean ± SEM, and n represents number of animals per group. For (A), **P < 0.01 versus WT. For (B), ***P < 0.001 versus WT+TAC.



Supplementary Figure 6

Fig. S6. Knockdown of ATG5 by rAAV9-siATG5 in mice abolishes the cardioprotective effect of β5i KO after pressure overload. WT and β5i KO mice were injected with rAAV9-siAGT5 or rAAV9-GFP (1.04×10^{12} mg/mg) for 3 weeks and then subjected to TAC operation for additional 6 weeks. (**A**) Representative M-mode echocardiography of the left ventricle (upper). Measurement of ejection fraction (EF%) and fractional shortening (FS%) (lower, n=5). (**B**) Heart size were detected by H&E staining, and heart weight to body weight (HW/BW) and heart weight to tibia length (HW/TL) ratios were calculated (n=5). (**C**) Cardiac myocyte size and fibrosis were detected by FITC-labeled

wheat germ agglutinin (WGA) staining and Masson's Trichrome staining. Scale bar 100 μm. (**D**) Quantification of the relative myocyte cross-sectional area (200 cells counted per heart) and fibrosis. qPCR analyses of ANF mRNA expression (n=5). (**E**) The protein levels of β5i, ATG5, LC3, p-ERK1/2 and ERK1/2 in the heart tissues and quantification (n=5). GAPDH as an internal control. Data are presented as mean ± SEM, and n represents number of animals per group. **P*< 0.05, ***P* < 0.01 versus WT-rAAV9-siControl+TAC, $^{\#}P$<0.5, $^{\#}P$<0.01 versus β5i KO-rAAV9-siATG5+TAC.



Supplementary Figure 7

Fig. S7. The summarized diagram showing that the proposed mechanisms underlying β5i regulate cardiac hypertrophy.