Online Supplements to

TFEB activation protects against cardiac proteotoxicity via increasing autophagic flux

Bo Pan[1], Hanming Zhang[1], Taixing Cui[2], Xuejun Wang[1]*

[1]Division of Basic Biomedical sciences, University of South Dakota Sanford School of Medicine, Vermillion, SD 57069, USA

[2]Department of Cell Biology and Anatomy, University of South Carolina School of Medicine, Columbia, SC 29209, USA

Running title: TFEB protects against cardiac proteotoxicity

*Correspondence: Dr. Xuejun Wang, Division of Basic Biomedical Sciences, Sanford School of Medicine of the University of South Dakota, Vermillion, SD 57069, USA. Telephone: 605 658-6345, Fax: 605 677-6381. E-mail: xuejun.wang@usd.edu.

I. Supplementary Tables 1 ~ 3.

Supplementary Table1. Primers used for RT-PCR

Gene Name	Sequence (5'–3')
CtsB	TGGGTTCAGCGAGGACAT
	ATGGTGTAGGGTAAGCAGCC
CtsD	GCGTCTTGCTGCTCATTCT
	AACTCTGACACTGGCTCCTT
Lamp1	GTGGGACTTGCGGTGCC
	GACATTGAGGGCGAGCG
M6PR	GGATAAGGAGTCAAAGAATG
	TGATTCTCCCAACCACCGT
MCOLN1	CGCCGCCGCCTCAAGT
	GCTGCTCCCGTGTGTAGGC
UVRAG	CTTCTGGATACCTACTTCAC
	GACTTTCCACTCTATCAACAGC
VPS18	GCTCCGCATTGACTTGGG
	GCCTTCTGTCCATTGCGGT
RAB7A	CAGTCTCTTGGTGTGGC
	CGAAGTAAGGAATGTTG
P62	GCCACCTCTCTGATAGC
	AGGTTTGCTGACTTCCG
BECN1	TTACTTACCACAGCCCAGG
	TGCTCCAACATCTCCAAAC
TFEB	GGTGTTGAAGGTGCAGTCC
	GGGTAGCGTGTTGGGCATCTG
E-CryAB	GGTGTTGAAGGTGCAGTCC
	GGGTAGCGTGTTGGGCATCTG
T-CryAB	TTCTTCGGAGAGCACCTGTT
	TCTGGGACGTCGTATGGGTA
GAPDH	ATGACATCAAGAAGGTGGTG
	CATACCAGGAAATGAGCTTG

Supplementary Table 2. **Echocardiographic parameters of FVB/N mice at ~6 months of Age**

	NTG (n=8)	CryAB^{R120G} TG (n=8)
Heart rate (bpm)	473±25	336±26**
LV end-diastolic posterior wall thickness (mm)	0.76±0.08	1.33±0.09**
LV end-diastolic volume (mm^3)	77.69±5.59	64.82±7.15**
LV Ejection Fraction (EF, %)	57.47±4.61	54.27±10.53
LV Fractional Shortening (FS, %)	29.98±3.07	28.00±6.71
Stroke Volume (SV, μl)	44.6±+3.7	34.8±6.2**
Cardiac Output (CO, ml/min)	21.2±2.2	12.0±2.2**
Estimated LV Mass (mg)	99.2±16.4	192.3±20.3**

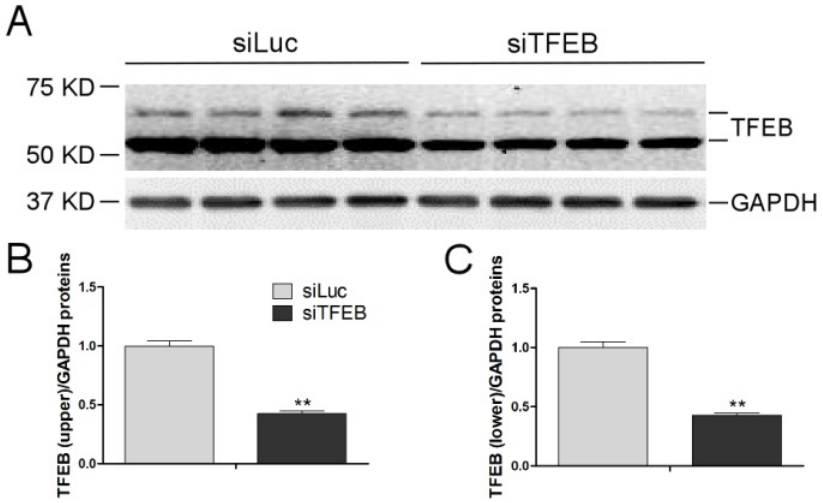
LV, left ventricle; TG, transgenic; NTG, non-TG; ***p*<0.01 vs. NTG; Student's t-tests. Mean ± STD

Supplementary Table 3. Gravimetric analysis of FVB/N mice at ~6 months of Age

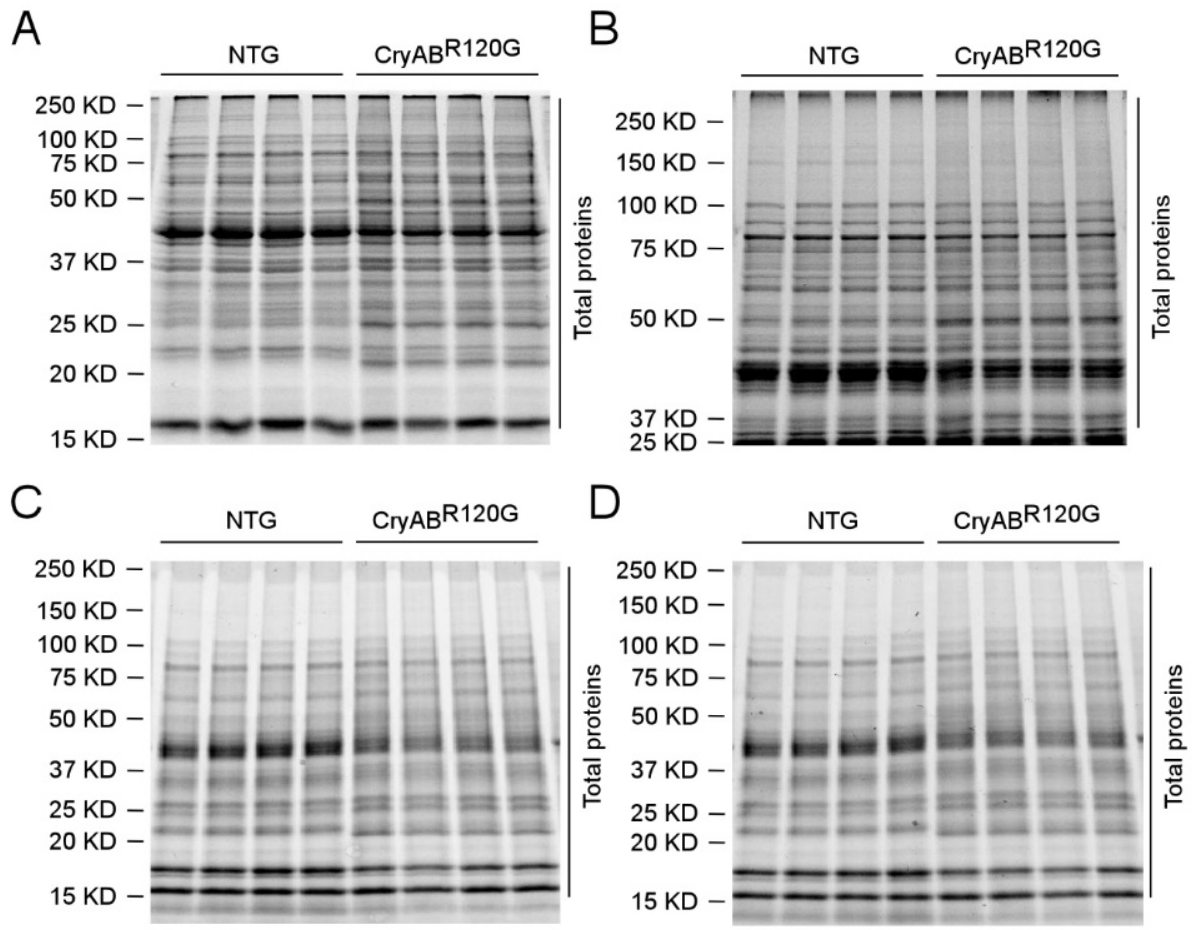
	NTG (n=6)	CryAB^{R120G} TG (n=6)
Heart Weight (mg)	122.2±15.8	212.2±14.0**
Tibial Length (mm)	18.6±0.9	18.7±0.6
Heart weight/Tibial length (mg/mm)	6.57±0.73	11.36±0.61**

***p*<0.01 vs. NTG; Student's *t*-tests. Mean ± STD

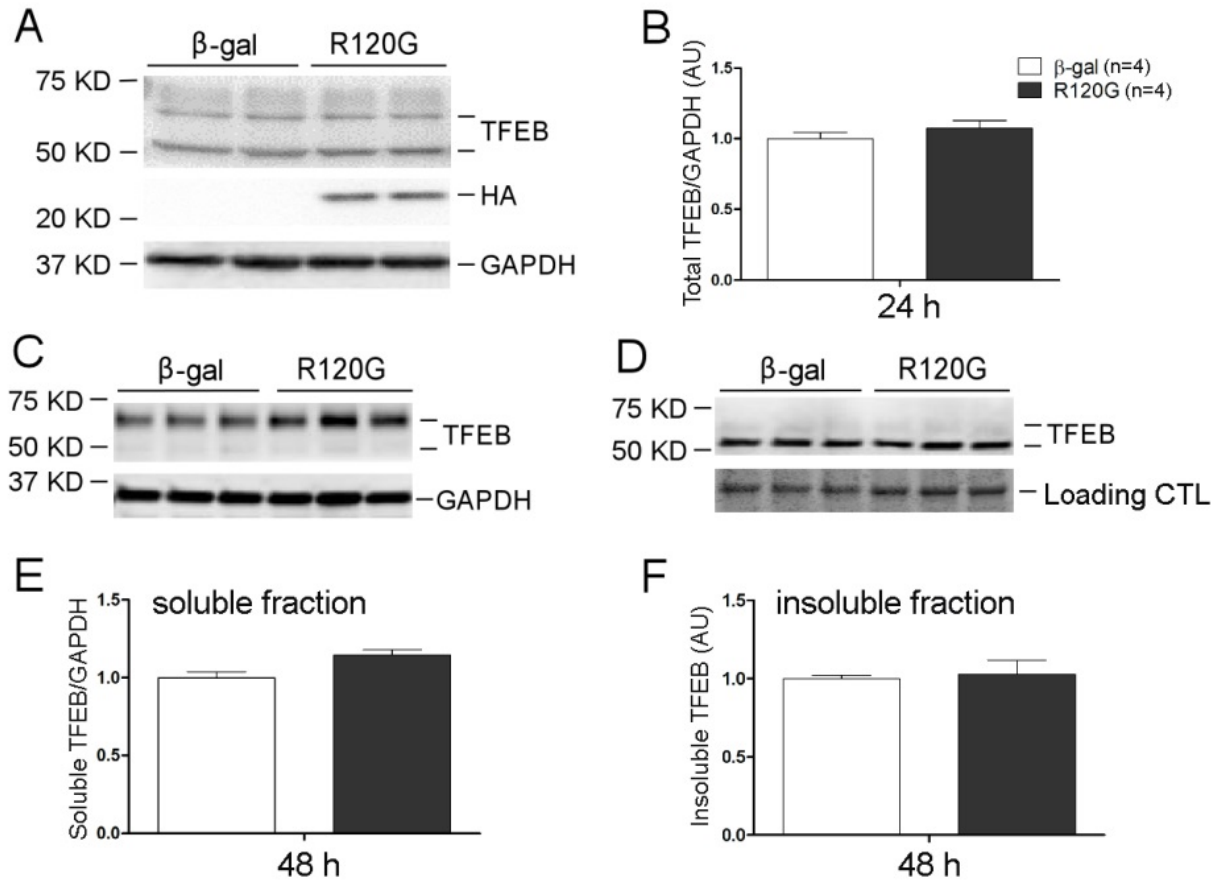
II. Supplementary Figures 1 ~ 8



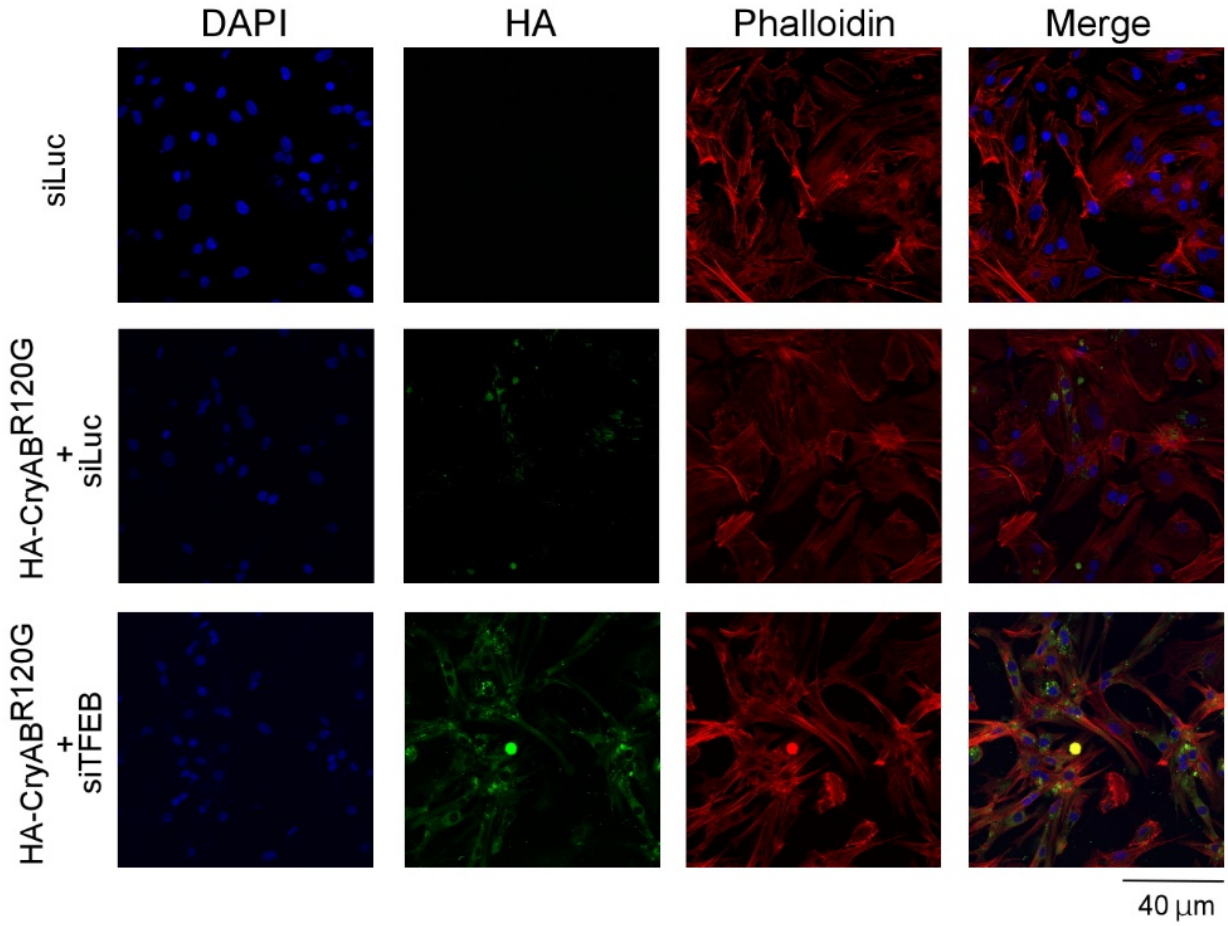
Supplementary Figure 1. Verification of the efficacy of siTFEB transfection. Culturing NRVMs, transfection of siTFEB and siLuc were done in the same way as described in main text Figure 3. Total cell lysates were used for western blot analysis for TFEB and GAPDH. Representative images (**A**) and quantitative comparison of protein levels of the higher molecular weight (**B**) and the lower molecular species (**C**) of TFEB between the siTFEB and the siLuc groups are shown. Mean+SEM, n=4 biological repeats/treatment; ** $p<0.01$ vs. siLuc, student's unpaired *t*-test.



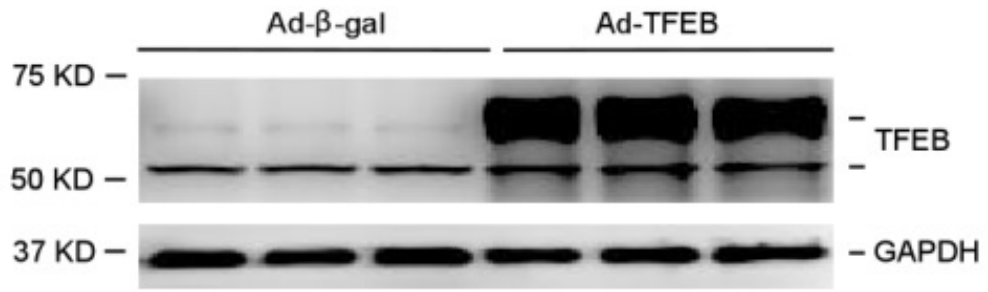
Supplementary Figure 2. **Stain-free total protein images of the gels used for the western blot analyses presented in Figure 2D (mTORC1 activation) of the main text**. Panel **A** was from a 12% gel, **B** from an 8% gel and both **C** and **D** were from 4% to 20% gradient gels. Gels A and D were used for 4E-BP1 and p-4E-BP1, respectively. Gel B was for mTOR and p70 S6 kinase, and Gel C was for phospo-mTOR and phospo-p70 S6 kinase.



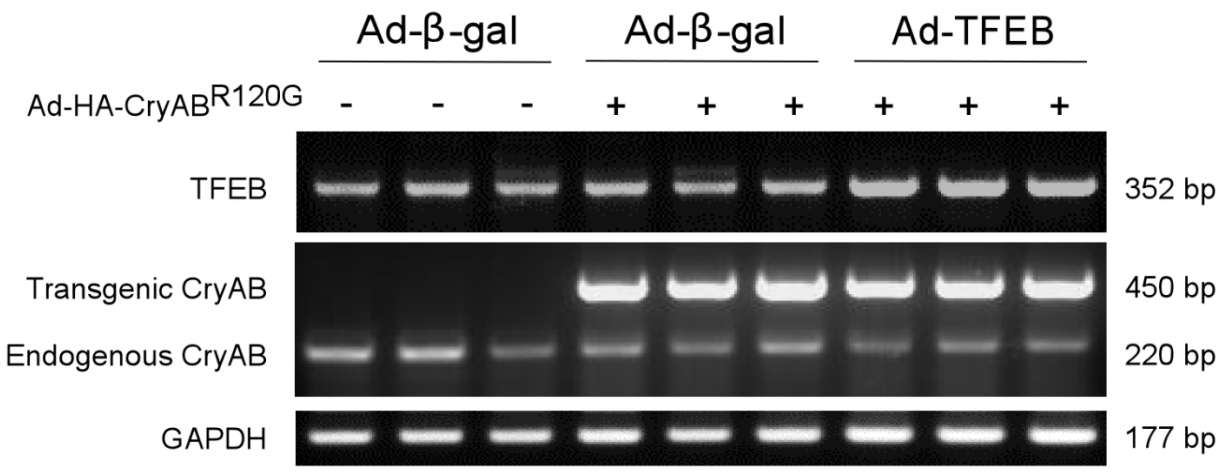
Supplementary Figure 3. The effect of CryAB^{R120G} overexpression on protein expression of TFEB proteins in cultured cardiomyocytes. A and **B,** Western blot analyses for the indicated proteins in the total cell lysates from cultured NRVMs at 24 h after Ad-β-gal or Ad-HA-CryAB^{R120G} (R120G) infection. Overexpression of HA-CryAB^{R120G} was confirmed by the immnoblot for HA-tagged protein (middle image in A). Representative images (A) and pooled densitometry data of two major TFEB bands (B) are shown. N=4 biological repeats/treatment. **C ~ F**, Western blot analyses for the indicated proteins in the soluble (C, E) and insoluble (D, F) fraction of cultured NRVMs at 48 h after Ad-β-gal or Ad-HA-CryAB^{R120G} infection. Note that CryAB^{R120G} overexpression displayed no discernible effect on the expression of TFEB proteins in cultured cardiomyocytes. Mean+SEM, N=3 biological repeats/treatment. p>0.05 for all comparisons between the β-gal and the R120G groups at both 24 h and 48 h time points.



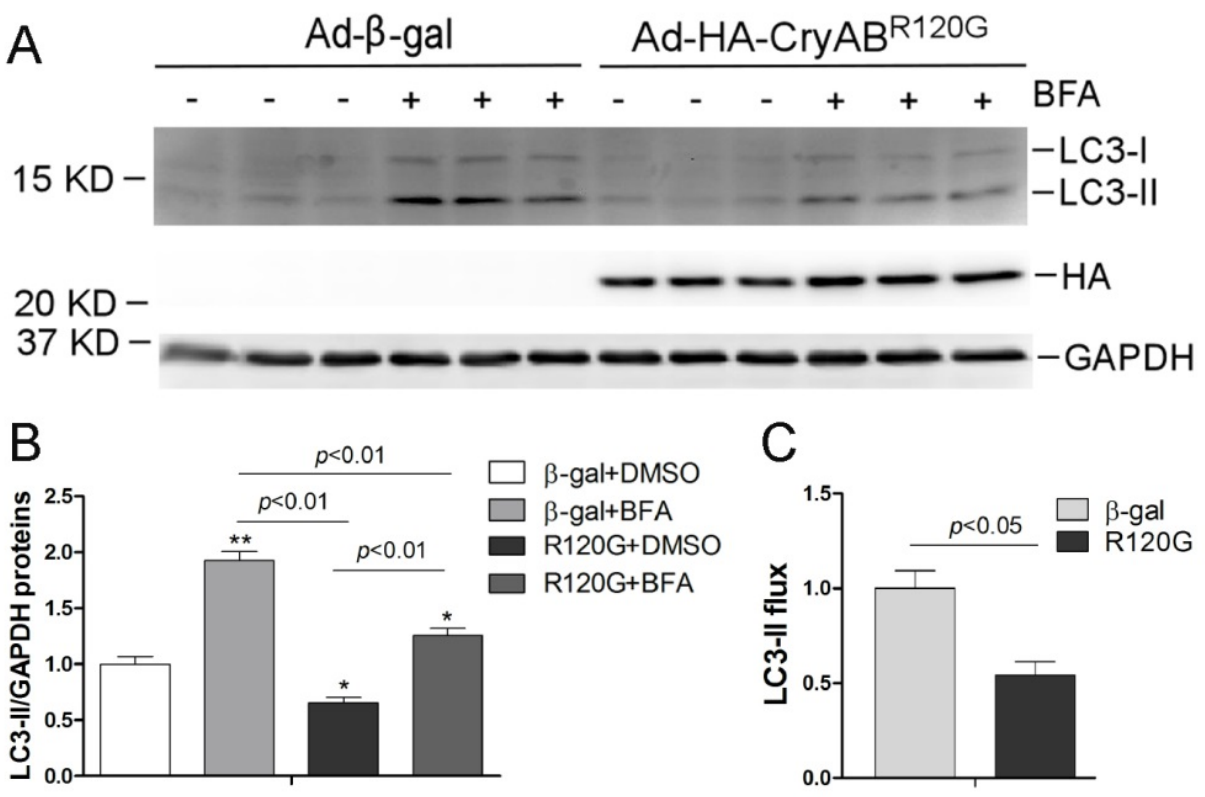
Supplementary Figure 4. Downregulation of TFEB exacerbates the accumulation of aberrant protein aggregates in NRVMs expressing CryAB^{R120G}. Culturing NRVMs, transfection of siTFEB and siLuc, and infection of Ad-HA-CryAB^{R120G} were done in the same way as described in main text Figure 3. And the cell fixation, fluorescence staining, and confocal microscopy were all performed as described for main text Figure 5.



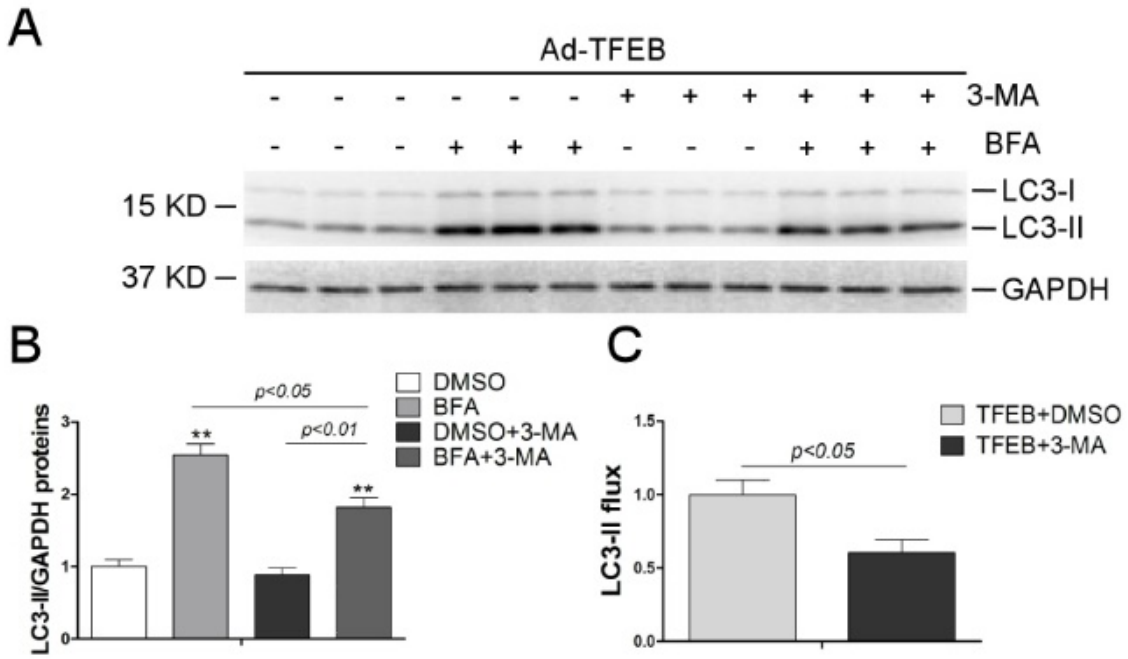
Supplementary Figure 5. Overexpressed TFEB proteins by Ad-TFEB mediated shows a size closer to the as the higher molecular weight band of the endogenous TFEB in NRVMs.



Supplementary Figure 6. TFEB overexpression does not discernibly alter transgenic CryAB^{R120G} transcript levels. NRVMs cultures and adenoviral gene delivery were performed as described in main text Figure 5. Total RNA was extracted and used for RT-PCR analyses of the indicated genes as described in the main text *Method* section. Shown are representative PCR gel images.



Supplementary Figure 7. Overexpression of CryAB^{R120G} significantly reduced LC3-II flux in cultured cardiomyocytes. Total proteins extracted from cultured NRVMs harvested at 72 hours after infection of Ad-HA-CryAB^{R120G} or Ad-β-gal were subject to SDS-PAGE and western blot analyses for the indicated proteins. LC3-II flux assays were carried out as described in the main text. Mean+SEM, n=3 biological repeats/treatment; *p<0.05, **p<0.01 vs. the β-gal+DMSO group, Two way ANOVA followed by Tukey's test for pair-wise comparison.



Supplementary Figure 8. **3-MA is capable of suppressing autophagic flux in presence of TFEB overexpression**. NRVMs were infected with Ad-HA-CryAB^{R120G} at 24 hours before they were treated with 3-MA (2.5 mM) for 12 hours, and then the cells were treated with BFA (6 nM) or vehicle control (DMSO) and harvested 24 hours later for extraction of total proteins. Western blot images (**A**), pooled LC3-II densitometry data (**B**), and the LC3-II flux (**C**) are presented. ***p*<0.01 vs. the DMSO group. Two way ANOVA followed by Tukey's test for pair-wise comparison.