Potential Role of BNIP3 in Cardiac Remodeling, Myocardial Stiffness, and Endoplasmic Reticulum
Mitochondrial Calcium Homeostasis in Diastolic and Systolic Heart Failure

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Background—We have shown that BNIP3 expression is significantly increased in heart failure (HF). In this study, we tested the effects of BNIP3 manipulation in HF.

Methods and Results—In a rat model of pressure overload HF, BNIP3 knockdown significantly decreased left ventricular (LV) volumes with significant improvement in LV diastolic and systolic function. There were significant decreases in myocardial apoptosis and LV interstitial fibrosis. Ultrastructurally, BNIP3 knockdown attenuated mitochondrial fragmentation and restored mitochondrial morphology and integrity. On the molecular level, there were significant decreases in endoplasmic reticulum (ER) stress and mitochondrial apoptotic markers. One of the mechanisms by which BNIP3 mediates mitochondrial dysfunction is via the oligomerization of the voltage-dependent anion channels causing a shift of calcium from the ER to mitochondrial compartments, leading to the decrease in ER calcium content, mitochondrial damage, apoptosis, and LV interstitial fibrosis, and hence contributes to both systolic and diastolic myocardial dysfunction, respectively. In systolic HF, the downregulation of SERCA2a (sarcoplasmic-endoplasmic reticulum calcium ATPase), along with an increased BNIP3 expression, further worsens myocardial diastolic and systolic function and contribute to the major remodeling seen in systolic HF as compared with diastolic HF with normal SERCA2a expression.

Conclusions—The increase in BNIP3 expression contributes mainly to myocardial diastolic dysfunction through mitochondrial apoptosis, LV interstitial fibrosis, and to some extent to myocardial systolic dysfunction attributable to the shift of calcium from the ER to the mitochondria and to the decrease in ER calcium content. However, SERCA2a downregulation remains a prerequisite for the major LV remodeling seen in systolic HF. (Circ Heart Fail. 2013;6:572-583.)

Key Words: apoptosis ■ gene therapy ■ heart failure ■ hypertrophy ■ remodeling

In heart failure (HF), the cross-talk between the endoplasmic reticulum–sarcoplasmic reticulum (ER/SR) and the juxtaposed mitochondria is altered, leading to the malfunction of the cardiomyocyte and to the decline in cardiac function. On the SR level, there is hyperphosphorylation of the ryanodine receptors, hypophosphorylation of phospholamban, downregulation and dysfunction of SERCA2a. These changes in the calcium cycling proteins lead to increases in SR Ca2+ release and to decreases in SR Ca2+ uptake, and SR Ca2+ content. On the mitochondrial level, there is an increase in the proapoptotic Bcl-2 and Bcl-2-like family proteins, such as Bax and BNIP3, respectively, in favor of the antiapoptotic marker Bcl-2. BNIP3 is a mitochondrial death and mitophagy marker that has been shown to induce left ventricular (LV) remodeling postmyocardial infarction.1-9 In our previous study, we highlighted the role of c-Jun N-terminal kinases (JNK) in modulating FOXO3a (forkhead box transcription factors) for the expression of BNIP3 in pressure overload hypertrophy (POH) and in HF.10 This signaling pathway was further validated in human samples of HF.10 BNIP3 expression increased the first 2 weeks after POH and peaked at HF development.10 In this study, we show how BNIP3 knockdown, using tail vein injection of adeno-associated virus of serotype 9 (AAV9) Sh BNIP3, reversed cardiac remodeling and improved LV diastolic and systolic function in a pressure overload rat model of diastolic and systolic HF. Moreover, BNIP3 knockdown robustly attenuated LV apoptosis and interstitial fibrosis with major improvements of various cellular components, specifically with regard to mitochondrial morphology and integrity. Mechanistically, we find that BNIP3 exerts its destructive effects on the mitochondria via the oligomerization of the voltage-dependent anion channel (VDAC), leading to mitochondrial Ca2+ overload, release of cytochrome C, and mitochondrial apoptosis as shown below.
**Methods**

**Isolation and Culture of Adult Rat Cardiomyocytes and In Vitro Experiments**

Adult rat ventricular cardiomyocytes were isolated from male Sprague-Dawley rats weighing 250 to 350 g, as previously described.13 The following experiments were performed (n=3 for each experiment in vitro): (1) cell viability, (2) mitochondrial membrane potential, (3) immunofluorescence staining, (4) mitochondrial Ca2+ loading (Figure I in the online-only Data Supplement), and (5) VDAC oligomerization. Details are available in the online-only Data Supplement.

**Western Blotting**

Please refer to the online-only Data Supplement.

**Coimmunoprecipitation**

The coimmunoprecipitation was performed using Pierce Classic IP Kit (Thermo Scientific, Rockford, IL). Details are available in the online-only Data Supplement.

**Transmission Electron Microscopy**

Samples were viewed under a transmission electron microscope (HITACHI H-7650, Tokyo, Japan). Details are available in the online-only Data Supplement.

**Production of Recombinant Adenoviruses and Adeno-Associated Virus**

Recombinant adenoviruses encoding green fluorescent protein was prepared as previously described.13 Ad-BNIP3 and Ad-Sh BNIP3 were done at vector biolabs. We generated a recombinant cardiotropic AA V9, allowing for cardiomyocyte-targeted RNAi against BNIP3 (AAV9 Sh BNIP3) under control of the U6 promoter14 (Figure II in the online-only Data Supplement).

**Apoptosis by Tunnel Staining**

Tunnel staining was performed using the Apoptag red in situ detection kit (Millipore, Billerica, MA). Details are available in the online-only Data Supplement.

**Masson Trichrome Staining**

Cryosections were stained using Masson Trichrome staining kit protocol (Sigma, St. Louis, MO). Details are available in the online-only Data Supplement.

**Measurement of Intracellular Calcium Kinetics and ER Calcium Content**

Fura-2, AM (Molecular Probes, Eugene, OR) was used to detect intracellular Ca2+ concentration [Ca]. Data are expressed as the 340:380 ratio after subtraction of background fluorescence. Details are available in the online-only Data Supplement.

**Experimental Model of Ascending Aortic Banding, Cross-Clamp With Ascending Aortic Banding, and Study Design**

All procedures involving the handling of animals were approved by the Animal Care and Use Committee of the Mount Sinai School of Medicine and adhered with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Sprague-Dawley rats weighing 180 to 200 g underwent ascending aortic banding (AAB), as previously described in detail,14 for the systolic HF model n=7 and for the diastolic HF model n=5 for each group. The cross-clamp surgery with gene transfer and AAB was performed as previously described in detail (n=4 for each group).14,17 Details are available in the online-only Data Supplement.

**Echocardiography**

Transthoracic echocardiography was performed using a vivid 7 echocardiography apparatus with a 14-MHz probe (i13L probe, General Electric, New York, NY). Details are available in the online-only Data Supplement.

**Invasive Pressure–Volume Loop Measurements of the LV**

Hemodynamics were recorded using a Scisense P-V Control Unit (FY897B).14,20 Details are available in the online-only Data Supplement.

**Statistical Analysis**

Results are shown as mean±SD. Statistical significance was determined using 1-way ANOVA followed by Tukey. There was no adjustment for multiple comparisons across the variables being tested. For the pretreatment and 1 month after treatment data, mixed effect models with a random intercept; treatment, time, and the interaction treatment × time were the predictors in the model. A significant interaction means that the change between before and after in either of the noncontrol groups was statistically different than the differences observed in the control group. A P value of <0.05 was considered statistically significant. The P values presented in the figures are 2 sided.

**Results**

**BNIP3 Overexpression Induces Mitophagy and Apoptosis in Cardiomyocytes In Vitro and In Vivo and Impairs Diastolic and Systolic Cardiac Function in a Rat Model of Early POH**

BNIP3 localizes to the mitochondria in cardiomyocytes (Figure IIIA in the online-only Data Supplement). BNIP3 overexpressing cardiomyocytes showed significant increases in cell death, cytoplasmic cytochrome C, and cleaved caspase 3, and significantly decreased mitochondrial membrane potential (Figure IIIB–IIID in the online-only Data Supplement). Gene transfer of Ad-BNIP3 in a rat model of early POH was associated with a significantly lower body weight, LV weight, a significantly lower heart weight/body weight ratio, and a significantly thinner septal and posterior wall, by echocardiography (Figure 1A–1C). There were significant increases in LV end-systolic diameters and volumes and significant decreases in LV fractional shortening and LV ejection fraction (LVEF) in the Ad-BNIP3 group (Figure 1D and 1E). Baseline hemodynamics and tracings are shown in Table I in the online-only Data Supplement and Figure 1F, respectively. The Ad-BNIP3 group had significant increases in their LV end-diastolic pressures, despite having a significantly lower maximum pressure and significantly decreased LVEF (Figure 1G–1I).

On the molecular level, there was no difference in SERCA2a expression among all groups. BNIP3 expression significantly increased in early POH (Ad-Null+AAB) and was further increased in the Ad-BNIP3 group (Figure 1J). The conversion of LC3-1 to LC3-2 and cleaved caspase 3 were significantly decreased in the Ad-Sh BNIP3 group (Figure 1J). Ultrastructurally, BNIP3 overexpression in pressure overload (PO) accentuated mitochondrial fragmentation and cristae destruction. BNIP3 knockdown robustly attenuated mitochondrial fragmentation and the presence of autophagosomes in PO (Figure 1K). Of note,
Figure 1. BNIP3 overexpression increased cardiomyocyte death in vitro and impaired LV systolic and diastolic function in a rat model of early pressure overload hypertrophy (POH). A–E, M-mode images 2 weeks after gene delivery via a cross-clamp technique and ascending aortic banding (AAB). There were significant decreases in left ventricular (LV) weight, interventricular septum thickness (IVSd), left ventricular end-diastolic posterior wall dimension (LVPWd), and left ventricular ejection fraction (LVEF), and significant increase in LV end-systolic volume in the Ad-BNIP3+AAB group, *P<0.05 vs Ad-Null+AAB and Ad-Sh BNIP3+AAB, #P<0.05 vs Ad-Null. There was slight but significant decrease in LV end-systolic volume and increase in LVEF in the Ad-Sh BNIP3+AAB group, &P<0.05 vs Ad-Null+AAB. J, BNIP3 expression as well as cleaved caspase 3 significantly increased in the Ad-Null+AAB and was the highest in Ad-BNIP3+AAB, *P<0.05 vs Ad-Null, #P<0.05 vs all other groups. BNIP3 knockdown significantly attenuated the increase in BNIP3 expression, cleaved caspase 3, and the conversion of LC3-1 to LC3-2 in response to pressure overload (PO), &P<0.05 vs Ad-Null+AAB and Ad-BNIP3+AAB. There was no difference in SERCA2a expression in all groups. K, Ultrastructurally, there was significant decrease in mitochondrial area 2 weeks after PO, *P<0.05 vs Ad-Null and was the worst in Ad-BNIP3 group with significant mitochondrial fragmentation, crista destruction, and myofibrillar damage, #P<0.05 vs all other groups. BNIP3 knockdown prevented mitochondrial damage in PO, &P<0.05 vs Ad-Null+AAB and Ad-BNIP3+AAB. Arrows are showing autophagosomes. Images ×5000 magnified, scale bar 2 μm. LV EDV indicates left ventricular end-diastolic volume; and LV ESV, left ventricular end-systolic volume.
Figure 2. Tail vein delivery of 5E10 vg/mL of adeno-associated virus of serotype 9 (AAV9) Sh BNIP3 reversed cardiac remodeling and improved left ventricular (LV) diastolic function and contractility in a rat model of diastolic heart failure (HF) with preserved ejection fraction (EF). A–C, M-mode images of the above group of animals before and 1 month after treatment with AAV9 Sh Luc vs AAV9 Sh BNIP3. LV volumes were significantly decreased, and the LVEF was significantly increased 1 month after treatment with AAV9 Sh BNIP3, *P<0.05 vs pressure overload hypertrophy (POH) 4M+AAV9 Sh Luc, #P<0.05 vs sham. D, Pressure–volume loop tracings in the different groups at baseline and during inferior vena cava occlusion. E, LVEF significantly improved 1 month after Sh BNIP3 treatment, *P<0.05 vs other 2 groups. F and G, Left ventricular end-diastolic pressure (LVEDP) and end-diastolic pressure–volume relationship (EDPVR) were significantly increased in the Sh Luc group, #P<0.05 vs sham, &P<0.05 vs other 2 groups. Those parameters were significantly decreased 1 month after Sh BNIP3 treatment, *P<0.05 vs POH 4M+AAV9 Sh Luc. H, LV contractility significantly increased in the Sh BNIP3 group, *P<0.05 vs POH 4M+AAV9 Sh Luc. Note that the Sh Luc group has a falsely increased end-systolic pressure–volume relationship (ESPVR) compared with shamm animals as their left ventricular end-systolic pressure (LVESP are higher, but their V0 is significantly shifted to the right as compared with the sham group). I, Western blot analysis of LV tissue lysate showed robust decrease in BNIP3 expression, endoplasmic reticulum (ER) stress (p-eIF2α), and ER stress apoptotic marker (CHOP [C/EBP homologous protein]), as well as in Bax/Bcl-2 ratio and in cleaved caspase 3 in the Sh BNIP3 group, *P<0.05 vs POH 4M+AAV9 Sh Luc, #P<0.05 vs sham and &P<0.05 vs other 2 groups. There was no difference in SERCA2a expression among all groups.
there are differences in each group in terms of mitochondria size, which may be because of the inhomogeneous nature of the gene transfer.

**Therapeutic Gene Delivery of AAV9 Sh BNIP3 Reverses Cardiac Remodeling and Improves LV Diastolic and Systolic Function in a Pressure Overload–Induced HF Rat Model of Diastolic and Systolic Dysfunction**

To examine the effects of knocking down BNIP3 in a chronic model of HF, we used AAV vectors that afford us long-term expression. Echocardiography data of the diastolic HF with preserved ejection fraction animals are shown in the Table III in the online-only Data Supplement. M-mode images are shown in Figure 2A. There were no significant differences in echocardiographic parameters between the Sh Luc and Sh BNIP3 groups before treatment. One month after treatment, there was significant decrease in LV end-systolic diameter and significant increase in LV fractional shortening in the AAV9 Sh BNIP3 group. LV end-diastolic and end-systolic volumes were significantly decreased because of the significant decrease in LV length in diastole and systole and reflected a significant increase in LVEF (Figure 2B and 2C; Figure IV in the online-only Data Supplement). Hemodynamic data are shown in Table IV in the online-only Data Supplement, and tracings are shown in Figure 2D. There were significant decreases in LV end-diastolic pressures and end-diastolic pressure–volume relationship and significant increases in LV ejection fraction and end-systolic pressure–volume relationship in the AAV9 Sh BNIP3 group (Figure 2E–2H). Western blotting of LV tissue lysate showed significant decreases in BNIP3 expression, as well as in Bax/Bcl-2 ratio, ER stress marker (p-eIF2a), and ER stress apoptotic marker (CHOP [C/EBP homologous protein]) and cleaved caspase 3 in the AAV9 Sh BNIP3 group (Figure 2I). However, there was no difference in SERCA2a expression between the AAV9 Sh Luc and AAV9 Sh BNIP3 groups, respectively (Figure 2I). Myocardial apoptosis and interstitial fibrosis were significantly decreased in the AAV9 Sh BNIP3
Figure 3. Tail vein delivery of 5E10 vg/mL of adeno-associated virus of serotype 9 (AAV9) Sh BNIP3 reversed cardiac remodeling and improved left ventricular (LV) diastolic function and contractility in a rat model of systolic heart failure (HF). A–C, M-mode images of the above group of animals before and 1 month after treatment with AAV9 Sh Luc vs AAV9 Sh BNIP3. LV volumes were significantly decreased, and the LV ejection fraction (EF) was significantly increased 1 month after treatment with AAV9 Sh BNIP3, $P<0.05$ vs HF+AAV9 Sh Luc, $P<0.05$ vs sham. D, Pressure–volume loop tracings in the different groups at baseline and during inferior vena cava occlusion. E, LVEF significantly improved 1 month after Sh BNIP3 treatment, $P<0.05$ vs HF+AAV9 Sh Luc and $P>0.05$ vs sham. F and G, Left ventricular end-diastolic pressure (LVEDP) and end diastolic pressure–volume relationship (EDPVR) were significantly increased in the Sh Luc group, $P<0.05$ vs sham, $P>0.05$ vs other 2 groups. Those parameters were significantly decreased 1 month after Sh BNIP3 treatment, $P<0.05$ vs HF+AAV9 Sh Luc. H, LV contractility significantly increased in the Sh BNIP3 group, $P<0.05$ vs HF+AAV9 Sh Luc. Note that the Sh Luc group has a falsely increased end-systolic pressure–volume relationship (ESPVR) compared with sham animals as their left ventricular end-systolic pressure (LVESP) are higher, but their V0 is significantly shifted to the right as compared with the sham group. I, Western blot analysis of LV tissue lysate showed robust decrease in BNIP3 expression, endoplasmic reticulum (ER) stress (p-eIF2a) and ER stress apoptotic marker (CHOP [C/EBP homologous protein]) as well as in Bax/Bcl-2 ratio and in cleaved caspase 3 in the Sh BNIP3 group, $P<0.05$ vs HF+AAV9 Sh Luc, $P>0.05$ vs sham and $P<0.05$ vs other 2 groups. There was a significant decrease in SERCA2a expression in the HF groups, $P<0.05$ vs sham.
group (Figure 2J and 2K). Ultrastructurally, autophagosomes, mitochondrial fragmentation, and myofibrillar damage were noted in the AA V9 Sh Luc group. BNIP3 knockdown robustly improved the aforementioned ultrastructural findings (Figure 2L; Figure V in the online-only Data Supplement). Similar results were noted for the systolic HF animals treated with AA V9 Sh Luc versus AA V9 Sh BNIP3 except for that the systolic HF animals had much higher LV volumes and significantly lower SERCA2a expression compared with sham-operated animals (Figure 3I). Echocardiography and hemodynamic data are presented in Figure 3A–3H and Figure VI in the online-only Data Supplement, and Tables V and VI in the online-only Data Supplement. Myocardial apoptosis, interstitial fibrosis, and ultrastructure data are presented in Figure 3J–3L and Figure VII in the online-only Data Supplement.

**Figure 3 (Continued).** J and K. There were robust decreases in myocardial apoptosis and in LV interstitial fibrosis in the Sh BNIP3 group, *P*<0.05 vs HF+AAV9 Sh Luc; #P<0.05 vs sham and &P<0.05 vs other 2 groups. L. Ultrastructurally, there was significant mitochondrial fragmentation and cristae destruction with dilated T tubules and damaged myofibrils. BNIP3 knockdown robustly attenuated mitochondrial fragmentation and cristae destruction with the mitochondrial area almost back to control level and attenuated myofibrillar damage, #P<0.05 vs sham and *P<0.05 vs HF+AAV9 Sh Luc. Arrows showing the presence of autophagosomes. Above images are ×5000 magnified, scale bar 2 μm. Lower images are ×12000 magnified, scale bar 1 μm. CAF indicates collagen area of fibrosis.

**Increase in BNIP3 Expression Decreases ER Calcium and Increases Mitochondrial Calcium**

BNIP3 overexpressing cardiomyocytes had significantly decreased beat-to-beat Ca\(^{2+}\) release due to the significant decrease in ER Ca\(^{2+}\) with marked impairment in cardiomyocyte relaxation and 3-fold increase in mitochondrial Ca\(^{2+}\) (Figure 4A and 4B). Hypertrophic cardiomyocytes showed significant increase in beat-to-beat Ca\(^{2+}\) release and no change in ER Ca\(^{2+}\) content, 2 weeks after PO. However, 5 weeks after PO, beat-to-beat Ca\(^{2+}\) transients were significantly decreased attributable to a significant decrease in ER Ca\(^{2+}\) content. Beat-to-beat Ca\(^{2+}\) transients and ER Ca\(^{2+}\) content were significantly higher and mitochondrial Ca\(^{2+}\) was significantly lower in hypertrophic cardiomyocytes isolated from animals, 5 weeks after PO treated with AAB and tail vein injection of AAV9 Sh BNIP3 (Figure 4C and 4D).

**BNIP3 Regulates Mitochondrial Calcium Via the Modulation of the VDAC Channels**

Coimmunoprecipitation did not show a complex formation between BNIP3 and VDAC channels (Figure 5A). There was modulation of the VDAC channels by BNIP3. The increase in BNIP3 expression increases the oligomerization of the VDAC channels, mainly in the form of a dimer, with and without the presence of a cross-linking reagent, leading to
the increase in mitochondrial Ca\(^{2+}\) and to cytochrome C release (Figure 5B). DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid), an anion channel inhibitor, inhibited the oligomerization of the VDAC channels and significantly attenuated mitochondrial Ca\(^{2+}\) and cell death in Ad-BNIP3–infected cardiomyocytes. B, There was 3-fold increase in mitochondrial Ca\(^{2+}\) in the Ad-BNIP3 group, \(P<0.05\) vs Ad-Null and Ad-Sh BNIP3. C, In hypertrophic cardiomyocytes, there is robust increase in beat-to-beat Ca\(^{2+}\) release with no change in ER Ca\(^{2+}\) content 2 weeks after pressure overload (PO), \(P<0.05\) vs sham. However, 5 weeks after PO beat-to-beat Ca\(^{2+}\) transients are significantly decreased attributable to a significant decrease in ER Ca\(^{2+}\) content, \(P<0.05\) vs all other groups. BNIP3 knockdown significantly increased beat-to-beat Ca\(^{2+}\) transients and ER Ca\(^{2+}\) content in hypertrophic cardiomyocytes 5 weeks after PO, \(P<0.05\) vs pressure overload hypertrophy (POH), W5+AAV9 Sh Luc. D, Mitochondrial Ca\(^{2+}\) significantly increased in hypertrophic cardiomyocytes 5 weeks after PO, \(P<0.05\) vs other 2 groups.

Expression of a Constitutively Active FOXO3a Increases Cell Death In Vitro and Impairs Cardiac Diastolic and Systolic Function in a Rat Model of Early POH

Cardiomyocytes overexpressing constitutively active FOXO3a (Ad-FX3) showed significant increases in BNIP3 expression\(^{10}\) and 4-fold increases in cell death (Figure 5F; Figure VIII in the online-only Data Supplement). Gene transfer of Ad-FX3 in a rat model of early POH was associated with a significantly lower body weight, LV weight, a significantly lower heart weight/body weight ratio and a significantly thinner septal and posterior wall by echocardiography (Figure 6A–6C; Table VII in the online-only Data Supplement). There were significant increases in LV end-diastolic pressures, despite having a significantly lower maximum pressure and significant decrease in LVEF in the Ad-FX3+AAB group (Figure 6D and 6E). Baseline hemodynamics and tracing are shown in Table VIII in the online-only Data Supplement and Figure 6F, respectively. The Ad-FX3+AAB group had significant increases in LV end-diastolic pressures, despite having a significantly lower maximum pressure and significant decreases in LV fractional shortening and LVEF (Figure 6G–6I). On the molecular level, Ad-DN-FX3 significantly attenuated the increase in BNIP3 expression in response to PO (Figure 6J).

Discussion

BNIP3 is a mitochondrial death and mitophagy marker located at the outer mitochondrial membrane, with the N
terminus oriented into the cytoplasm and the C terminus inside the mitochondria. It has been shown that an increase in BNIP3 expression under hypoxemic conditions increases cell death and mitochondrial autophagy and attributes to LV remodeling and myocardial apoptosis postmyocardial infarction.1–4,6–9,21 Also, it has been shown how BNIP3 and NIX play a role in ER/SR-mitochondrial Ca\(^{2+}\) homeostasis.22,23 In our previous study, we have shown that BNIP3 expression increases in POH and peaks at the time of HF development and highlighted the critical role of JNK in the modulation of FOXO3a for the expression of BNIP3. We also validated the JNK-FOXO3a-BNIP3 pathway in human samples of HF.10 In this study, we show the critical role of BNIP3 in inducing LV remodeling and myocardial stiffness in PO rat model of AD.

Figure 5. BNIP3 modulates the voltage-dependent anion channels (VDAC) and shifts the Ca from the endoplasmic reticulum (ER) into the mitochondria. A, Coimmunoprecipitation did not show that BNIP3 and VDAC channels form a complex. B, Rather there is modulation of the VDAC channels by BNIP3. The increase in BNIP3 expression causes oligomerization of the VDAC channels, mainly in the form of a dimer, with and without the presence of a cross-linking reagent (EGS) leading to the increase in mitochondrial Ca\(^{2+}\). C and D, DIDS, an anion channel inhibitor, inhibited the oligomerization of the VDAC channels in Ad-BNIP3 infected cardiomyocytes. There was a 3-fold decrease in mitochondrial Ca\(^{2+}\) in all groups with significant decrease in the Ad-BNIP3 infected cardiomyocytes treated with DIDS, \#P<0.05 vs Ad-Null+DIDS and Ad-Sh BNIP3+DIDS. E, DIDS significantly attenuated cell death in Ad-BNIP3 infected cardiomyocytes, \#P<0.05 vs Ad-Null+DIDS and *P<0.05 vs other 2 groups. F, Ultrastructurally, DIDS significantly attenuated mitochondrial fragmentation and autophagosome formation in Ad-BNIP3 infected cardiomyocytes. The mitochondrial area was significantly higher in cardiomyocytes treated with DIDS compared with no DIDS treatment. Images ×12000 magnified, scale bar 1 \(\mu\)m.
HF, and we highlight a novel mechanism through which the chronic increase in BNIP3 expression induces mitochondrial damage, cytochrome C release, apoptosis, and thus myocardial fibrosis. In cardiac myocytes, BNIP3 is the effector of the transcription factor FOXO3a. The expression of constitutively active FOXO3a in cardiac myocytes, in vitro, was associated with significant increase in BNIP3 expression and cell death.10 Gene transfer of constitutively active FOXO3a in cardiac myocytes, in vitro, was associated with significant increase in BNIP3 expression and cell death.10

Figure 6. The expression of a constitutively active FOXO3a impairs cardiac diastolic and systolic function in a rat model of early pressure overload hypertrophy (POH). A. M-mode images 2 weeks after gene delivery via a cross-clamp technique and ascending aortic banding (AAB). B–E. There were significant decreases in left ventricular (LV) weight, interventricular septum thickness (IVSd), left ventricular end-diastolic posterior wall dimension (LVPWd), and LV ejection fraction (EF) and significant increase in LV end-systolic volume in the Ad-FX3a+AAB group, *P<0.05 vs Ad-Null+AAB and Ad-DN-FX3a+AAB, #P<0.05 vs Ad-Null. There was slight but significant decrease in LV end-systolic volume (LVESV) and increase in LVEF in the Ad-DN-FX3a+AAB group, &P<0.05 vs Ad-Null+AAB. F–I. Pressure-volume loop measurements showed significant decreases in LV maximum pressure and EF, and significant increases in left ventricular end-diastolic pressure (LVEDP) in the Ad-FX3a+AAB group, *P<0.05 vs Ad-Null+AAB and Ad-DN-FX3a+AAB, #P<0.05 vs Ad-Null. There was significant decrease in LVEDP and a slight but significant increase in LVEF in the Ad-DN-FX3a+AAB group, &P<0.05 vs Ad-Null+AAB. J. FOXO3a expression increased in the Ad-FX3a and Ad-DN-FX3a groups, #P<0.05 vs Ad-Null and Ad-DN-FX3a+AAB. BNIP3 expression increased in the Ad-Null+AAB and in the Ad-FX3a+AAB groups, #P<0.05 vs Ad-Null and *P<0.05 vs all other groups. The delivery of Ad-DN-FX3a significantly attenuated the increase in BNIP3 expression in response to pressure overload, &P<0.05 vs Ad-Null+AAB and Ad-FX3a+AAB. LVEDP indicates LV end-diastolic pressure.
The increase in BNIP3 expression, which is highest in HF, primarily contributes to diastolic dysfunction through the intracellular destruction exerted by BNIP3 on the mitochondria leading to mitophagy, apoptosis, decline in cardiac energetics and myocardial fibrosis, and stiffness, and to the systolic dysfunction via the ER-mitochondria Ca\textsuperscript{2+} shift, and decline in ER Ca\textsuperscript{2+} content. However, the major remodeling of the LV chamber, which is seen in systolic HF, takes place when SERCA2a expression is downregulated, which along with the increase in BNIP3 expression contribute to the systolic and diastolic dysfunction of the cardiomyocyte with significant increases in LV end-diastolic and end-systolic volumes and significant decline in LV contractile function and LVEF.

In conclusion, the increase in BNIP3 expression, which is clearly linked to mitochondrial calcium overload and decline in cardiac energetics, SERCA2a malfunction and to the increase in LV apoptosis and interstitial fibrosis. SERCA2a downregulation worsens the deleterious effect exerted by BNIP3 on the mitochondria, as well as ER stress, and becomes the core of 2 vicious cycles as shown in Figure 7. SERCA2a overexpression in systolic HF improved LV systolic function and attenuated BNIP3-mediated mitochondrial destruction and ER stress by shuffling the Ca\textsuperscript{2+} from the cytoplasm into the ER (Figure X in the online-only Data Supplement). The simultaneous overexpression of SERCA2a and BNIP3 in cardiomyocytes in vitro attenuated mitochondrial fragmentation compared with BNIP3 overexpression alone. However, DIDS was superior in preventing BNIP3-induced mitochondrial destruction in BNIP3 overexpressing cardiomyocytes. Morphologically, the largest mitochondria were seen in cardiomyocytes in vitro by simultaneous overexpression of SERCA2a and BNIP3 knockdown and with BNIP3 knockdown plus DIDS treatment (Figure X in the online-only Data Supplement).

In addition, mitochondrial fragmentation and ER stress–induced apoptosis (systolic dysfunction) with significant increases in ER stress and apoptosis, decline in cardiac energetics and myocardial fibrosis, and stiffness, and to the systolic dysfunction via the ER-mitochondria Ca\textsuperscript{2+} shift, and decline in ER Ca\textsuperscript{2+} content. However, the major remodeling of the LV chamber, which is seen in systolic HF, takes place when SERCA2a expression is downregulated, which along with the increase in BNIP3 expression contribute to the systolic and diastolic dysfunction of the cardiomyocyte with significant increases in LV end-diastolic and end-systolic volumes and significant decline in LV contractile function and LVEF.

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### Disclosures
A patent application has been submitted for this work.

### References
15. The increase in BNIP3 expression correlated with diastolic dysfunction, mitochondrial apoptosis, and autophagy that were evident as early as 2 weeks after pressure overload hypertrophy. Those parameters worsened with BNIP3 overexpression, in vivo, and peaked at HF development, whether diastolic or systolic. The downregulation of SERCA2a contributed to worsening in left ventricular systolic function and cardiac remodeling leading to systolic HF. BNIP3 knockdown in HF robustly improved left ventricular end-diastolic pressure, myocardial relaxation, myocardial contractility, and cardiac remodeling, and significantly decreased myocardial apoptosis, and left ventricular interstitial fibrosis. The effects of increased BNIP3 expression on cardiomyocyte diastolic dysfunction is mediated by the calcium shift from the endoplasmic reticulum to the mitochondria, leading to mitochondrial calcium overload, mitochondrial dysfunction, and decline in cardiac energetics. This study highlights a novel role of BNIP3 as a potential therapeutic target for the treatment of diastolic HF.

**CLINICAL PERSPECTIVE**

Heart failure (HF), is a syndrome with complex pathophysiological disturbances and with major alterations in myocardial signaling pathways leading to changes in calcium handling proteins, mitochondrial dysfunction, and heightened levels of myocardial apoptotic and autophagic cell death. Moreover, although there is a clear mortality benefit with β-blockers, angiotensin-converting enzyme inhibitors and mineralocorticoid receptor antagonists in systolic HF, no therapies have shown promise in treating diastolic HF with preserved ejection fraction in different randomized clinical trials. In this study, we showed that the increase in BNIP3 expression correlated with diastolic dysfunction, mitochondrial apoptosis, and autophagy that were evident as early as 2 weeks after pressure overload hypertrophy. Those parameters worsened with BNIP3 overexpression, in vivo, and peaked at HF development, whether diastolic or systolic. The downregulation of SERCA2a contributed to worsening in left ventricular systolic function and cardiac remodeling leading to systolic HF. BNIP3 knockdown in HF robustly improved left ventricular end-diastolic pressure, myocardial relaxation, myocardial contractility, and cardiac remodeling, and significantly decreased myocardial apoptosis, and left ventricular interstitial fibrosis. The effects of increased BNIP3 expression on cardiomyocyte diastolic dysfunction is mediated by the calcium shift from the endoplasmic reticulum to the mitochondria, leading to mitochondrial calcium overload, mitochondrial dysfunction, and decline in cardiac energetics. This study highlights a novel role of BNIP3 as a potential therapeutic target for the treatment of diastolic HF.
Potential Role of BNIP3 in Cardiac Remodeling, Myocardial Stiffness, and Endoplasmic Reticulum: Mitochondrial Calcium Homeostasis in Diastolic and Systolic Heart Failure
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SUPPLEMENTAL MATERIAL

Materials and Methods

Isolation and Culture of adult rat Cardiomyocytes and the design of the in vitro experiments

Male Sprague-Dawley rats weighing (250–350 g) were given sodium heparin (100 U) and sodium pentobarbital (15 mg) for anesthesia by intraperitoneal injection. Sigma reagents were used for isolation. All solutions were prepared with deionized water. After decapitation, the hearts were immediately removed and placed into an ice-cold isolation medium. The hearts were cannulated as quickly as possible and perfused using Krebs—Henseleit buffer containing (in mM): 118 NaCl, 4.7 KCl, 1.25 KH2PO4, 1.3 MgSO4, 10 glucose, and 10 HEPES (pH 7.4) for 4 min at 35°C. The buffer was pumped through the heart by means of peristaltic pump at a rate of 4 ml/min. Then the heart was perfused with Krebs—Henseleit buffer with low Ca²⁺ content (<0.25 mM), pH 7.4, with collagenase (0.2 mg/ml) for 15-20 min. After 15 min, the heart was removed from the apparatus, and the ventricles were separated below the atrioventricular junction, then the heart was cut into 1-2 mm² fragments and carefully resuspended for 1 min with a large automated pipette. The cells were filtered through nylon mesh and allowed to settle by gravity for 5 min. The cell pellet was subsequently suspended in an incubation buffer (in mM: 118 NaCl, 25 NaHCO3, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 10 glucose, 30 N-2 hydroxyethylpiperazine-N-2 ethanesulfonic acid (HEPES), 60 taurine, 20 creatine, 1% bovine serum albumin, vitamins and amino acids (Sigma Chemicals, St. Louis, MO) at pH 7.4, 37°C. Calcium concentration was gradually increased to a 1.2 mM over 20 min. After centrifugation at 20 g for 30 sec, myocytes were resuspended in Medium 199 containing 10 units/mL penicillin, 10 μg/mL streptomycin, 5% fetal calf serum and 100 nM insulin. Using this method, each heart yielded 6-7 x 10⁹ rod-shaped myocytes with viability greater than 80%. The adult cardiac myocytes (ACM) were placed on a laminin coated culture dishes in full nutrient M199 medium as mentioned above and were allowed to attach for 1 h in humidified 5% CO₂, 95% air at 37°C and then washed once to remove unattached cells. All reagents were purchased from (Sigma Chemicals,
St. Louis, MO, USA). After cardiomyocyte isolation, the following experiments were performed (n=3 for each experiment in vitro):

(1) Cell viability was determined using the vital dyes calceinacetoxymethyl ester (green fluorescence, 2μM) and ethidium homodimer-1 (red fluorescence, 2 μM) to determine the number of live and dead cells, respectively (Molecular Probes, Eugene, Oregon, USA). Images taken at 10X magnification using fluorescent microscope, Olympus 1X71. At least 9 fields were analyzed from each condition and were obtained from three independent experiments.

(2) Mitochondrial membrane potential: cardiomyocytes were incubated in 50 nM tetra-methylrhodamine methyl ester perchlorate (TMRM), (Molecular Probes, Eugene, OR, USA). Images taken at 40X magnification using fluorescent microscope, Olympus 1X71. At least 9 fields were analyzed from each condition and were obtained from three independent experiments.

(3) Immunofluorescence staining: cardiomyocytes were placed on laminin precoated coverslips and were labeled with 100 nM MitoTracker Red (Invitrogen, Eugene, OR, USA) for 10 min at 37°C. After washing with PBS, cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for 30 min at room temperature. Cells were then exposed to blocking solution (20% goat serum in PBS), followed by overnight incubation with a rabbit monoclonal anti-BNIP3 primary antibody at 4°C (Abcam Cambridge, MA, USA, 1/200 dilution). Cells were then rinsed with PBS 3 times, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Coverslips were then mounted onto glass slides, and images were acquired and analyzed using a Zeiss LSM 510 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany) equipped with a 63X oil-immersion objective.

(4) Mitochondrial calcium: cells were loaded with the low-affinity Ca^{2+} dye Rhod-2AM (2 μM) for 20 min (Invitrogen, Eugene, OR, USA). Specificity of mitochondria staining with Rhod-2AM was confirmed by VDAC immunofluorescence staining with a mouse monoclonal anti-VDAC primary antibody (Abcam Cambridge, MA, USA, 1/200 dilution) Supplement Figure 1. Images were acquired and analyzed using a
Zeiss LSM 510 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany) equipped with a 63X oil-immersion objective.

(5) VDAC oligomerization: cells treated with the cross linking reagent Ethylene glycol-bis (succinic acid N-hydroxysuccinimide ester), EGS, for 20 min room temperature (Sigma Chemicals, St. Louis, MO, USA, 250 µM) before being lysed with RIPA buffer. 4,4'-Diisothiocyanatostilbene-2, 2'-disulfonic acid, disodium salt (DIDS) was used as an anion channel inhibitor (Thermo Scientific, Barrington, IL, USA, 200 µM). Afterwards, cells were lysed with RIPA buffer. Lysates were loaded on an SDS-Page Gel and blotted as described below.

**Western Blotting**

Protein extraction was obtained by lysing cells and 0.2g of mechanically crushed tissue in a RIPA lysis buffer containing protease inhibitor (Roche Diagnostics Inc., Indianapolis, IN, USA) and phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Protein extracts for western blotting were obtained by centrifuging at 13,000 rpm for 10 min at 4ºC and aspirating the supernatant. Protein concentrations were measured using bradford protein assay (Bio-Rad, Hercules, CA, USA). For cytoplasmic fraction extraction, the mitochondrial isolation kit was used (Thermo Scientific, Barrington, IL, USA). 30 ug of proteins from each sample were loaded and electrophoresed using SDS-PAGE gels then transferred to a PVDF membrane. The membrane was blocked for 1 hr using blocking solution containing 0.15M sodium chloride, 3 mM potassium chloride, 25 mM tris-base, 5% skim milk, and 0.05% tween-20. Blots were incubated with the primary Ab overnight at 4ºC. The following primary antibodies were used: GAPDH (Sigma, St. Louis, MO, USA 1:10000 dilution), LC 3, BNIP3, FOXO3a, Bax, Bcl-2, CHOP, p-eIF2a, eIF2a, Caspase 3/ cleaved caspase 3 and VDAC (Cell signaling, Danvers, MA, USA 1:1000 dilution). The second day, after three washing steps with TBS-0.05% Tween-20, the blot was incubated with secondary horseradish peroxidase conjugated antibody (Thermo Scientific, Barrington, IL, USA 1:10000 dilution) for 45 min. the blot was washed three times with TBS- 0.05% Tween-20 then a supersignal west pico chemiluminescent substrate (Thermo Scientific, Barrington, IL, USA) was used for the detection of
protein bands using the film method. Bands densities were quantified using Photoshop program and were normalized to GAPDH to correct for variations in protein loading.

**Co-Immunoprecipitation**

The co-immunoprecipitation was performed using Pierce Classic IP Kit (Thermo scientific, Rockford, IL, USA). Briefly, 1 mg of protein lysate from normal and hypertrophic adult cardiomyocytes were incubated with mouse monoclonal anti-BNIP3 primary antibody (Abcam, Cambridge, MA, USA, 1/500 dilution) following the protocol supplied by the IP kit. IgG2a was used as control (Abcam, Cambridge, MA, USA, 1/500 dilution). Proteins were analyzed on SDS-PAGE gel and immunoblotted with mouse monoclonal anti-BNIP3 (Abcam, Cambridge, MA, USA, 1/1000 dilution) and rabbit monoclonal anti-VDAC primary antibody (Cell signaling, Danvers, MA, USA 1:1000 dilution). The immune complexes were detected by horseradish conjugated secondary antibody and developed using a supersignal west pico chemiluminescent substrate (Thermo Scientific, Barrington, IL, USA) via the film method.

**Transmission Electron Microscopy**

Cells and fractions (1mm³) from fresh ventricles were pre-fixed in a solution of 3% glutaraldehyde overnight at 4°C, post-fixed in 1% osmium tetroxide (OsO₄), dehydrated in an ascending series of alcohols, and embedded in epoxy resin. Ultrathin sections were stained with uranylacetate and lead citrate. Samples were viewed under a transmission electron microscope (HITACHI H-7650, Japan). Images were taken at 1K, 5K and 12K magnification.

**Image Analysis Using Image J**

Mitochondrial area analysis as well as fluorescence intensity were done using Image-J, a public domain Java image-processing program inspired by NIH. Scale setting and calibration were done using the “Set Scale and Calibration Menu,” and measurement parameters were selected. The mitochondrial area was summed for each image using 12,000 X magnified images and the average mitochondrial area per image was calculated in µm² (total area/number of mitochondria) after correction by the magnification factor.
Production of Recombinant Adenoviruses and Adeno-Associated virus (AAV)

Recombinant adenoviruses encoding green fluorescent protein (Ad-GFP) was prepared as described previously 1. Briefly, The Ad-Easy Adenoviral vector system (Stratagene, USA) was used to generate recombinant adenoviruses. Full length EGFP gene was subcloned into the pShuttle vector (containing the cDNA for enhanced GFP) under the control of CMV promoter. Viral titers were determined by the plaque assay and the absence of replication-competent adenovirus was confirmed by polymerase chain reaction (PCR) to assess for the wild type E1 region. Ad-Null, Ad-DN-FX3a and Ad-FX3a were purchased from vector biolabs. Ad-BNIP3 and Ad-Sh BNIP3 were done at vector biolabs. A multiplicity of infection of 100 has been used in all infection experiments in vitro (n=3 for each experiment in vitro). For the in vivo experiments, the adenovirus was delivered via a cross clamp technique as described below with an infectious dose of 200 pfu/cell.

We generated a recombinant cardiotropic adeno-associated virus of serotype 9 (AAV9), allowing for cardiomyocyte-targeted RNAi against BNIP3 (AAV9 Sh BNIP3) under control of the U6 promoter. An AAV9 encoding Sh RNA directed against luciferase served as a negative control (AAV9 Sh Luc). AAV cis plasmid “pds-AAV-Sh BNIP3” was created by removing the Sh PLB sequence, from the AAV9-Sh PLB 975 bp construct 2, and replaced it with the Sh BNIP3 sequence extracted from the RNAi-ready pSIREN-DNR-DsRed-Express Sh BNIP3 vector Supplement Figure 2. BamHI and HindIII restriction sites were used for removing and replacing sequences. AAV was produced by the method described in Rapti et al 3 except that the PEG precipitation step was omitted. Also, after the first iodixanol gradient, a modified 2nd and 3rd gradient step was performed to concentrate the virus prior to dialysis. The modified gradient contained from the bottom to top: 4 ml 60% iodixanol, 4 ml 40% iodixanol, and 22ml AAV collected from the previous gradient diluted to 20% iodixanol content.

Apoptosis by Tunnel Staining

Tunnel staining was performed using the Apoptag red in situ detection kit and following the protocol provided (Millipore, Billerica, MA, USA). Images obtained using a Zeiss LSM 510 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany) equipped with a 40X oil-immersion objective.
Results were quantified using Image J software (NIH). At least 10 fields per animal were quantified from at least 4 animals per group.

**Masson Trichrome Staining**

8 µm left ventricular cryosections were stained using Masson’s Trichrome staining kit (Sigma, cat# HT15-1KT). Briefly, slides were fixed in Bouin solution (Sigma, cat# HT10132) 15 minutes at 56°C. After washes in tap water (15 minutes) and deionized water, slides were stained with Hematoxylin 10 minutes (Fisher Scientific, cat# CS400-1D). After 5 minutes wash in tap water, slides were dipped in a mix solution of phosphotungstic acid and phosphomolybdic acid solutions (2.5%) for 5 minutes, then in Aniline solution 5 minutes and finally in Acetic acid solution (1%) 2 minutes. Slides were washed in deionized water and then dehydrated by Ethanol 95% and 100% bath 2 minutes each. After 2 baths of xylene, slides were mounted with Cyotseal 60 (Thermo Scientific, cat# 8310-16). 20x magnification pictures were taken using software Spot v 3.5.7.1 (Diagnostics Instruments, Inc) and LV interstitial fibrosis was quantified using Image J software (NIH). At least 10 fields per animal were quantified from at least 4 animals per group.

**Measurement of Intracellular Calcium Kinetics and ER Calcium Content**

Cells were placed on laminin-coated coverslips (Deckgläser, Germany) and were loaded with Fura-2 AM (Molecular probes, Eugene, OR, USA, 1 µM) to detect intracellular Ca²⁺ concentration [Ca]i. After cells were loaded for 10 min, they were washed twice with Hepes buffer containing 1 mM Ca and then coverslips were placed into the bottom of a perfusion chamber connected with Myopace Cell Stimulator (Ionoptix Inc, MA). The cells were perfused with Hepes containing 0.6 mM Ca²⁺ and were paced at a frequency of 0.5 Hz. Fura-2 fluorescence was recorded with a dual-excitation fluorescence photo multiplier system (PTI, NJ) with an Olympus IX-70 inverted microscope, a Fluor 40x oil objective and a CCD camera. Cells were exposed to light emitted by a 75W lamp and passed through either a 340 or a 380 filter, while being stimulated to contract at 0.5 Hz. Changes in [Ca²+]i were monitored by Fura-2 excitation at 340 and 380 nm and emission at 510 nm at baseline conditions and during rapid application of 10 mM of caffeine administration (Sigma Chemicals, St. Louis, MO, USA). Data are expressed as the
340/380 ratio following subtraction of background fluorescence. After a steady Ca\textsuperscript{2+} transient was obtained (around 2 min of pacing) cell pacing was stopped and 30 sec later 10 mM of caffeine was added rapidly to the chamber for ER Ca\textsuperscript{2+} content release. At least 6 cells were analyzed in each group and were obtained from three independent experiments.

**Experimental Model of Ascending Aortic Banding, Cross Clamp with Ascending Aortic Banding and Study Design**

All procedures involving the handling of animals were approved by the Animal Care and Use Committee of the Mount Sinai School of Medicine and adhered with the Guide for the Care and Use of laboratory Animals published by the National Institutes of Health. The aortic banding model was used to generate pressure overload induced hypertrophy and heart failure. Sprague-Dawley rats weighing 180-200 g underwent ascending aortic banding (AAB), as previously described in detail \textsuperscript{4}. Briefly, animals were sedated by intraperitoneal administration of ketamine (65 mg/kg) plus xylazine (5 mg/kg) and intubated using a 16-gauge catheter and mechanically ventilated with tidal volumes of 2 ml at 50 cycles/min and FIO2 of 21%. A 1 cm incision was made in the right axilla and the thoracic cage was approached at the level of the second intercostal space. The thymus gland was dissected, then the underlying ascending aorta was separated from the superior vena cava and a 1mm vascular clip was placed around the ascending aorta, right before the right brachiocephalic artery. Pressure overload developed right after the placement of the vascular clip. For the heart failure experiment, animals that developed systolic heart failure were randomized to receive AAV9 Sh BNIP3 (n=7) vs AAV9 Sh Luc (n=7) for one month. Age matched sham operated animals were used as control (n=4). The diastolic HF animals were included only if they remained compensated four months after PO, had a significantly thick septal and posterior wall and more importantly an LA/Ao diameter ratio of 1.5 or above by echocardiography. Also prerequisite was a 20%-30% decrease in LVFS and LVEF from what those parameters were two weeks after PO (on average LVFS decreased from 80% to 55-60% and LVEF decreased from 87-90% to 70-75%). Animals were randomized to receive AAV9 Sh Luc (n=5) vs AAV9 Sh BNIP3 (n=5). Age matched sham operated animals were used as control (n=4). The cross clamp surgery with gene transfer and AAB was performed
as previously described in detail\textsuperscript{,5,6}. Briefly, the chest was opened at midline between the 2\textsuperscript{nd} and the fifth intercostals space. The aorta and the pulmonary arteries were cross-clamped simultaneously and the adenovirus was injected into the left ventricle. The cross clamp duration was 45 seconds and the dose used was 200 PFU/cell. After adenoviral delivery, AAB was performed. Two weeks later, the animals underwent echocardiography and hemodynamic study prior to their sacrifice. \textit{n}=4 in each group.

\textbf{Echocardiography}

Transthoracic echocardiography was performed using a vivid 7 echocardiography apparatus with a 14 MHZ probe (i13L probe, General Electric, New York, NY). Animals were sedated with ketamine 80-100 mg/kg injected intraperitoneally. Long axis parasternal views and short axis parasternal two dimensional (2D) views, at the mid-papillary level, of the left ventricle (LV) were obtained to calculate the LV end diastolic (LVEDV) and end systolic (LVESV) volumes as well as the ejection fraction of the LV (LVEF). Volumes were calculated by using the formulae of the area length method ($V=\frac{5}{6} \times A \times L$, where \textit{V}: is the volume in ml, \textit{A}: is the cross sectional area of the LV cavity in cm\textsuperscript{2}, obtained from the mid-papillary short parasternal image in diastole and in systole, and \textit{L}: is the length of the LV cavity in cm, measured from the long parasternal axis image as the distance from the endocardial LV apex to the mitral-aortic junction in diastole and in systole). M-mode images were obtained by 2D guidance from the parasternal short axis view for the measurements of LV wall thickness of the septum (IVSd, cm) and the posterior wall (LVPWd, cm), LV end diastolic diameter (LVIDd, cm) and LV end systolic diameter (LVIDs, cm) as well as to calculate the LV fractional shortening (LVFS, %).

\textbf{Invasive Pressure-Volume Loop Measurements of the Left Ventricle}

At end point, LV pressure-volume loops measurements were obtained as previously described\textsuperscript{7}. Briefly, rats were anesthetized with inhaled 5\% (volume/volume) isoflurane for induction, and subsequently intubated and mechanically ventilated as noted above in the surgery section. Isoflurane was lowered to 2 - 3\% (volume/volume) for surgical incision. The chest was opened through a median sternotomy. A 1.9F rat P-V catheter (Scisense, London, Ontario, Canada) was inserted into the LV apex through an apical stab performed with a 25G needle. Hemodynamic recordings were performed after 5 minutes of stable
heart rate. Anesthesia was maintained at 0.75-1% isoflurane to keep the animal sedated and maintain a stable heart rate around 350 beats/minute. Hemodynamics were recorded subsequently through a Scisense P-V Control Unit (FY897B). The intrathoracic inferior vena cava was transiently occluded to decrease venous return during the recording to obtain load-independent P-V relationships. Linear fits were obtained for end-systolic pressure volume relationships (ESPVR) and end-diastolic pressure-volume relationships (EDPVR). At the end of the experiment, 50 µl of 30% NaCl were slowly injected into the external jugular vein for ventricular parallel conductance (Gp) measurement as previously described \(^7,\,8\). Blood resistivity was measured using a special probe (Scisense). Volume measurements were initially obtained as blood conductance and calibrated using the Baan equation \(^9\), and pressure sensors were calibrated as per manufacturer’s instructions.

**Statistical Analysis**

Results are shown as mean ± Standard Deviation. Statistical significance was determined using one-way ANOVA followed by Tukey. There was no adjustment for multiple comparisons across the variables being tested. For the pretreatment and one month after treatment data, mixed effect models with a random intercept; treatment, time and the interaction treatment * time were the predictors in the model. A significant interaction means that the change between before and after in either of the non-control groups was statistically different than the difference observed in the control group. A p-value of < 0.05 was considered statistically significant. The p-values presented in the figures are two sided.

**References**


Supplement Table 1. Echocardiography data two weeks after gene transfer of Ad-BNIP3 and Ad-Sh BNIP3 via a cross clamp technique in a rat model of early pressure overload.
<table>
<thead>
<tr>
<th>Echo data</th>
<th>Ad-Null (n=4)</th>
<th>Ad-Null + AAB (n=4)</th>
<th>Ad-BNIP3 + AAB (n=4)</th>
<th>Ad-Sh BNIP3 + AAB (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HW/BW (mg/g)</td>
<td>3.51 ± 0.21</td>
<td>4.89 ± 0.12 ^</td>
<td>4.17 ± 0.08 ^</td>
<td>4.48 ± 0.19 ^</td>
</tr>
<tr>
<td>LVW (mg)</td>
<td>0.66 ± 0.03</td>
<td>0.89 ± 0.07 ^</td>
<td>0.69 ± 0.05 ^</td>
<td>0.87 ± 0.07</td>
</tr>
<tr>
<td>BW (g)</td>
<td>287.33 ± 7.51</td>
<td>265.5 ± 17.94</td>
<td>231 ± 11.34 ^</td>
<td>271.14 ± 17.87</td>
</tr>
<tr>
<td>IVSd (cm)</td>
<td>0.18 ± 0.00</td>
<td>0.27 ± 0.002 ^</td>
<td>0.23 ± 0.005 ^</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>LVPWd (cm)</td>
<td>0.2 ± 0.03</td>
<td>0.28 ± 0.005 ^</td>
<td>0.23 ± 0.007 ^</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>LVIDd (cm)</td>
<td>0.69 ± 0.04</td>
<td>0.61 ± 0.04</td>
<td>0.60 ± 0.05</td>
<td>0.62 ± 0.04</td>
</tr>
<tr>
<td>LVIDs (cm)</td>
<td>0.24 ± 0.04</td>
<td>0.12 ± 0.006 ^</td>
<td>0.20 ± 0.03 ^</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>64.78 ± 4.37</td>
<td>80.47 ± 1.26 ^</td>
<td>66.69 ± 2.82 ^</td>
<td>82.89 ± 1.88</td>
</tr>
<tr>
<td>LVEDV (ul)</td>
<td>536.75 ± 79.07</td>
<td>464.27 ± 15.02</td>
<td>505.72 ± 19.43</td>
<td>400.38 ± 50.26</td>
</tr>
<tr>
<td>LVESV (ul)</td>
<td>104.99 ± 18.58</td>
<td>59.55 ± 4.43 ^</td>
<td>160.77 ± 27.98 ^</td>
<td>32.4 ± 12.07 ^</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>80.47 ± 1.33</td>
<td>87.98 ± 2.08 ^</td>
<td>68.29 ± 4.51 ^</td>
<td>92.05 ± 2.21 ^</td>
</tr>
</tbody>
</table>

^ P<0.002 vs Ad-Null  
^ B P<0.01 vs Ad-Null + AAB and Ad-Sh BNIP3 + AAB  
^ c P<0.05 vs Ad-Null + AAB
Supplement Table 2. Baseline hemodynamic data two weeks after gene transfer of Ad-BNIP3 and Ad-Sh BNIP3 via a cross clamp technique in a rat model of early pressure overload.

<table>
<thead>
<tr>
<th>Baseline Hemodynamic</th>
<th>Maximum pressure (mmHg)</th>
<th>End diastolic pressure (mmHg)</th>
<th>End systolic pressure (mmHg)</th>
<th>Ejection fraction (%)</th>
<th>Heart rate (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-Null (n=4)</td>
<td>130.78 ± 6.8</td>
<td>6.88 ± 0.51</td>
<td>129.11 ± 7.04</td>
<td>58.22 ± 8.00</td>
<td>400.33 ± 27.54</td>
</tr>
<tr>
<td>Ad-Null + AAB (n=4)</td>
<td>209.5 ± 13.32&lt;sup&gt;ᴬ&lt;/sup&gt;</td>
<td>13 ± 0.81&lt;sup&gt;ᴬ&lt;/sup&gt;</td>
<td>115.25 ± 23.48</td>
<td>79.58 ± 5.48&lt;sup&gt;ᴬ&lt;/sup&gt;</td>
<td>388.04 ± 17.37</td>
</tr>
<tr>
<td>Ad-BNIP3 + AAB (n=4)</td>
<td>177.79 ± 19.18&lt;sup&gt;ᴮ&lt;/sup&gt;</td>
<td>15 ± 0.34&lt;sup&gt;ᴮ&lt;/sup&gt;</td>
<td>129.66 ± 14.20</td>
<td>55.22 ± 6.11&lt;sup&gt;ᴮ&lt;/sup&gt;</td>
<td>401.87 ± 20.19</td>
</tr>
<tr>
<td>Ad-Sh BNIP3 + AAB (n=4)</td>
<td>209.91 ± 21.47&lt;sup&gt;ᶜ&lt;/sup&gt;</td>
<td>7.66 ± 1.86&lt;sup&gt;ᶜ&lt;/sup&gt;</td>
<td>136.44 ± 22.23</td>
<td>90.75 ± 3.09&lt;sup&gt;ᶜ&lt;/sup&gt;</td>
<td>419.64 ± 20.99</td>
</tr>
</tbody>
</table>

<sup>ᴬ</sup> P<0.002 vs Ad-Null  
<sup>ᴮ</sup> P<0.01 vs Ad-Null + AAB and Ad-Sh BNIP3 + AAB  
<sup>ᶜ</sup> P<0.01 vs Ad-Null + AAB

Supplement Table 3. Echocardiography data of the diastolic HF animals with preserved EF treated with tail vein injection of 5E10 vg/ml of AAV9 Sh Luc vs AAV9 Sh BNIP3.
<table>
<thead>
<tr>
<th>Echo data</th>
<th>Pre-treatment</th>
<th>One Month Post Rx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham (n=4)</td>
<td>POH 4M + AAV9 Sh Luc (n=5)</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVW (mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW (g)</td>
<td>537.50 ± 32.27</td>
<td>532.00 ± 76.73</td>
</tr>
<tr>
<td>IVSd (cm)</td>
<td>0.21 ± 0.01 ^A</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>LVPWd (cm)</td>
<td>0.22 ± 0.01 ^A</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>LVIDd (cm)</td>
<td>0.68 ± 0.04</td>
<td>0.70 ± 0.05</td>
</tr>
<tr>
<td>LVIDs (cm)</td>
<td>0.27 ± 0.02</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>59.41 ± 2.35</td>
<td>61.11 ± 3.68</td>
</tr>
<tr>
<td>LVEDV (ul)</td>
<td>455.55 ± 38.37 ^A</td>
<td>565.49 ± 58.36</td>
</tr>
<tr>
<td>LVESV (ul)</td>
<td>84.15 ± 12.79 ^A</td>
<td>160.26 ± 21.04</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>81.38 ± 3.52 ^A</td>
<td>71.71 ± 1.61</td>
</tr>
</tbody>
</table>

^A_P<0.005 vs POH 4M + AAV9 Sh Luc and POH 4M + AAV9 Sh BNIP3
^B_P<0.003 vs POH 4M + AAV9 Sh Luc
^C_P<0.005 vs Sham and POH 4M + AAV9 Sh BNIP3
Supplement Table 4. Hemodynamic data of the diastolic HF animals with preserved EF treated with tail vein injection of 5E10 vg/ml of AAV9 Sh Luc vs AAV9 Sh BNIP3.

<table>
<thead>
<tr>
<th>Hemodynamic data</th>
<th>Maximum pressure (mmHg)</th>
<th>End diastolic pressure (mmHg)</th>
<th>End systolic pressure (mmHg)</th>
<th>Ejection Fraction (%)</th>
<th>Heart Rate (bpm)</th>
<th>ESPVR (mmHg/µl)</th>
<th>V0 (µl)</th>
<th>EDPVR (mmHg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sham (n=4)</strong></td>
<td>115.22 ± 13.93 (^c)</td>
<td>8.08 ± 1.64 (^c)</td>
<td>119.20 ± 36.70</td>
<td>58.67 ± 8.18</td>
<td>340.00 ± 30.25</td>
<td>0.15 ± 0.04</td>
<td>-400 ± 130 (^c)</td>
<td>0.016 ± (^c) 0.006</td>
</tr>
<tr>
<td><strong>POH 4M + AAV9 Sh Luc (n=5)</strong></td>
<td>274.92 ± 20.32 (^A)</td>
<td>20.3 ± 3.27 (^A)</td>
<td>165.27 ± 24.18 (^A)</td>
<td>50.92 ± 9.42</td>
<td>353.73 ± 41.23</td>
<td>0.31 ± 0.03 (^A)</td>
<td>-104.76 ± 85.69 (^A)</td>
<td>0.090 ± 0.016 (^A)</td>
</tr>
<tr>
<td><strong>POH 4M + AAV9 Sh BNIP3 (n=5)</strong></td>
<td>294.11 ± 25.24 (^B)</td>
<td>15.59 ± 0.60 (^B)</td>
<td>135.44 ± 19.71</td>
<td>78.33 ± 6.01 (^B)</td>
<td>330.61 ± 17.42</td>
<td>0.52 ± 0.11 (^B)</td>
<td>-280.45 ± 27.72 (^B)</td>
<td>0.026 ± 0.006 (^B)</td>
</tr>
</tbody>
</table>

\(^A\)P<0.001 vs Sham  
\(^B\)P<0.01 vs POH 4M + AAV9 Sh Luc  
\(^c\)P<0.001 vs POH 4M + AAV9 Sh Luc and POH 4M + AAV9 Sh BNIP3
Supplement Table 5. Echocardiography data of the systolic HF animals treated with tail vein injection of 5E10 vg/ml of AAV9 Sh Luc vs AAV9 Sh BNIP3

<table>
<thead>
<tr>
<th>Echo data</th>
<th>Sham (n=4)</th>
<th>HF + AAV9 Sh Luc (n=7)</th>
<th>HF + AAV9 Sh BNIP3 (n=7)</th>
<th>Sham (n=4)</th>
<th>HF + AAV9 Sh Luc (n=7)</th>
<th>HF + AAV9 Sh BNIP3 (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HW/BW (mg/g)</td>
<td></td>
<td></td>
<td></td>
<td>2.59 ± 0.13 ᴬ</td>
<td>4.80 ± 0.58</td>
<td>5.10 ± 0.54</td>
</tr>
<tr>
<td>LVW (mg)</td>
<td></td>
<td></td>
<td></td>
<td>1.17 ± 0.03 ᴬ</td>
<td>1.62 ± 0.18</td>
<td>1.73 ± 0.22</td>
</tr>
<tr>
<td>BW (g)</td>
<td>537.50 ± 32.27</td>
<td>492.71 ± 82.31</td>
<td>492.57 ± 51.58</td>
<td>656.5 ± 19.98</td>
<td>539.00 ± 84.82</td>
<td>517.57 ± 64.60</td>
</tr>
<tr>
<td>IVSd (cm)</td>
<td>0.21 ± 0.01 ᴬ</td>
<td>0.26 ± 0.03</td>
<td>0.27 ± 0.02</td>
<td>0.23 ± 0.005 ᴬ</td>
<td>0.26 ± 0.03</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>LVPWd (cm)</td>
<td>0.22 ± 0.01 ᴬ</td>
<td>0.28 ± 0.01</td>
<td>0.29 ± 0.03</td>
<td>0.23 ± 0.005 ᴬ</td>
<td>0.29 ± 0.03</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>LVIDd (cm)</td>
<td>0.68 ± 0.04 ᴬ</td>
<td>0.86 ± 0.05</td>
<td>0.86 ± 0.04</td>
<td>0.72 ± 0.03 ᴬ</td>
<td>0.91 ± 0.04</td>
<td>0.89 ± 0.07</td>
</tr>
<tr>
<td>LVIDs (cm)</td>
<td>0.27 ± 0.02 ᴬ</td>
<td>0.49 ± 0.04</td>
<td>0.51 ± 0.07</td>
<td>0.29 ± 0.03 ᴬ</td>
<td>0.57 ± 0.07</td>
<td>0.45 ± 0.05 ᴮ</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>59.41 ± 2.35 ᴬ</td>
<td>42.17 ± 2.89</td>
<td>40.41 ± 5.05</td>
<td>59.47 ± 3.01 ᴬ</td>
<td>38.14 ± 5.77</td>
<td>49.65 ± 2.74 ᴮ</td>
</tr>
<tr>
<td>LVEDV (ul)</td>
<td>455.55 ± 38.37 ᴬ</td>
<td>958.46 ± 122.81</td>
<td>953.97 ± 136.49</td>
<td>495.30 ± 43.31 ᴬ</td>
<td>999.57 ± 135.64</td>
<td>815.12 ± 143.37 ᶜ</td>
</tr>
<tr>
<td>LVESV (ul)</td>
<td>84.15 ± 12.79 ᴬ</td>
<td>461.74 ± 81.46</td>
<td>452.94 ± 108.09</td>
<td>96.17 ± 14.40 ᴬ</td>
<td>550.19 ± 113.87</td>
<td>271.86 ± 79.97 ᴮ</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>81.38 ± 3.52 ᴬ</td>
<td>52.03 ± 2.86</td>
<td>52.96 ± 4.91</td>
<td>80.66 ± 1.55 ᴬ</td>
<td>45.22 ± 5.85</td>
<td>67.08 ± 5.51 ᴮ</td>
</tr>
</tbody>
</table>

ᴬP<0.001 vs HF + AAV9 Sh luc and HF + AAV9 Sh BNIP3  
ᴮP<0.001 vs HF + AAV9 Sh Luc  
ᶜP<0.01 vs HF + AAV9 Sh Luc
Supplement Table 6. Hemodynamic data of the systolic HF animals treated with tail vein injection of 5E10 vg/ml of AAV9 Sh Luc vs AAV9 Sh BNIP3.

| Hemodynamic data       | Maximum pressure (mmHg) | End diastolic pressure (mmHg) | End systolic pressure (mmHg) | Ejection Fraction (%) | Heart Rate (bpm) | ESPVR (mmHg/µl) | V0 (µl) | EDPVR (mmHg/µl) |
|------------------------|-------------------------|--------------------------------|-----------------------------|-----------------------|------------------|----------------|---------|----------------|------------------|
| Sham (n=4)             | 115.22 ± 13.93          | 8.08 ± 1.64                    | 119.20 ± 36.70              | 58.67 ± 8.18          | 340.00 ± 30.25   | 0.15 ± 0.04    | -400 ± 130 | 0.016 ± 0.006  |
| HF + AAV9 Sh Luc (n=7) | 262.52 ± 22.47<sup>A</sup> | 24.14 ± 4.29<sup>A</sup>       | 191.60 ± 24.54<sup>A</sup>  | 31.25 ± 3.83<sup>A</sup> | 330.24 ± 16.48<sup>A</sup> | 0.27 ± 0.07<sup>A</sup> | 207.23 ± 213.89<sup>A</sup> | 0.070 ± 0.026<sup>A</sup> |
| HF + AAV9 Sh BNIP3     | 267.54 ± 23.91<sup>B</sup> | 11.76 ± 3.24<sup>B</sup>       | 184.45 ± 12.45              | 56.52 ± 10.64<sup>B</sup> | 338.49 ± 29.62<sup>B</sup> | 0.46 ± 0.07<sup>B</sup> | -120.79 ± 66.06<sup>B</sup> | 0.022 ± 0.007<sup>B</sup> |

<sup>A</sup>P<0.001 vs Sham  
<sup>B</sup>P<0.001 vs HF + AAV9 Sh Luc
Supplement Table 7. Echocardiography data two weeks after gene transfer of Ad-FX3a and Ad-DN-FX3a via a cross clamp technique in a rat model of early pressure overload.

<table>
<thead>
<tr>
<th>Echo data</th>
<th>Ad-Null (n=4)</th>
<th>Ad-Null + AAB (n=4)</th>
<th>Ad-FX3a + AAB (n=4)</th>
<th>Ad-DN-FX3a + AAB (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HW/BW (mg/g)</td>
<td>3.51 ± 0.21</td>
<td>4.89 ± 0.12 ^</td>
<td>4.38 ± 0.13 ^</td>
<td>4.60 ± 0.12 ^</td>
</tr>
<tr>
<td>LVW (mg)</td>
<td>0.66 ± 0.03</td>
<td>0.89 ± 0.07 ^</td>
<td>0.73 ± 0.10 ^</td>
<td>0.91 ± 0.02</td>
</tr>
<tr>
<td>BW (g)</td>
<td>287.33 ± 7.51</td>
<td>265.5 ± 17.94</td>
<td>233 ± 19.91 ^</td>
<td>284.25 ± 5.19</td>
</tr>
<tr>
<td>IVSd (cm)</td>
<td>0.18 ± 0.00</td>
<td>0.27 ± 0.002 ^</td>
<td>0.20 ± 0.01 ^</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>LVPWd (cm)</td>
<td>0.2 ± 0.03</td>
<td>0.28 ± 0.005 ^</td>
<td>0.22 ± 0.03 ^</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>LVIDd (cm)</td>
<td>0.69 ± 0.04</td>
<td>0.61 ± 0.04</td>
<td>0.61 ± 0.04</td>
<td>0.63 ± 0.06</td>
</tr>
<tr>
<td>LVIDs (cm)</td>
<td>0.24 ± 0.04</td>
<td>0.12 ± 0.006 ^</td>
<td>0.22 ± 0.03 ^</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>64.78 ± 4.37</td>
<td>80.47 ± 1.26 ^</td>
<td>64.33 ± 6.36 ^</td>
<td>82.49 ± 1.76</td>
</tr>
<tr>
<td>LVEDV (ul)</td>
<td>536.75 ± 79.07</td>
<td>464.27 ± 15.02</td>
<td>424.58 ± 32.49</td>
<td>471.56 ± 80.15</td>
</tr>
<tr>
<td>LVESV (ul)</td>
<td>104.99 ± 18.58</td>
<td>59.55 ± 4.43 ^</td>
<td>120.04 ± 21.71 ^</td>
<td>37.01 ± 8.60 ^</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>80.47 ± 1.33</td>
<td>87.98 ± 2.08 ^</td>
<td>71.89 ± 2.92 ^</td>
<td>92.19 ± 0.71 ^</td>
</tr>
</tbody>
</table>

^ P<0.002 vs Ad-Null  
^ P<0.01 vs Ad-Null + AAB and Ad-DN-FX3a + AAB  
^ P<0.05 vs Ad-Null + AAB
Supplement Table 8. Baseline hemodynamic data two weeks after gene transfer of Ad-FX3a and Ad-DN-FX3a via a cross clamp technique in a rat model of early pressure overload.

<table>
<thead>
<tr>
<th>Baseline Hemodynamic</th>
<th>Maximum pressure (mmHg)</th>
<th>End diastolic pressure (mmHg)</th>
<th>End systolic pressure (mmHg)</th>
<th>Ejection fraction (%)</th>
<th>Heart rate (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-Null (n=4)</td>
<td>130.78 ± 6.8</td>
<td>6.88 ± 0.51</td>
<td>129.11 ± 7.04</td>
<td>58.22 ± 8.00</td>
<td>400.33 ± 27.54</td>
</tr>
<tr>
<td>Ad-Null + AAB (n=4)</td>
<td>209.5 ± 13.32 ^</td>
<td>13 ± 0.81 ^</td>
<td>115.25 ± 23.48</td>
<td>79.58 ± 5.48 ^</td>
<td>388.04 ± 17.37</td>
</tr>
<tr>
<td>Ad-FX3a + AAB (n=4)</td>
<td>181.00 ± 11.78 ^</td>
<td>15.00 ± 1.00 ^</td>
<td>113.56 ± 15.61</td>
<td>58.33 ± 4.36 ^</td>
<td>420.22 ± 8.34</td>
</tr>
<tr>
<td>Ad-DN-FX3a + AAB (n=4)</td>
<td>220.94 ± 7.61 ^</td>
<td>9.00 ± 1.00 ^</td>
<td>126.17 ± 23.82</td>
<td>90.17 ± 2.25 ^</td>
<td>377.28 ± 19.25</td>
</tr>
</tbody>
</table>

^ P<0.002 vs Ad-Null  
^ P<0.01 vs Ad-Null + AAB and Ad-DN-FX3a + AAB  
^ P<0.01 vs Ad-Null + AAB
Supplemental Figure Legends

**Supplement Figure 1.** VDAC immunofluorescence staining colocalizes with mitochondria loaded with Rhodamine-2AM. Also note that the VDAC channels are also found at the plasma membrane.

**Supplement Figure 2.** A: Showing the map of the AAV cis plasmid “pds-AAV-ShBNIP3” and the sequence of the Sh BNIP3 plasmid used (blue arrows). B: Showing the restriction map and the cloning site of the RNAi-ready pSIREN-DNR-DsRed-Express vector.

**Supplement Figure 3.** BNIP3 overexpression increases cell death in cardiomyocytes in vitro.

**S1-A:** BNIP3 localizes to the mitochondria in cardiomyocytes. **S1-B:** BNIP3 overexpression significantly decreased mitochondrial membrane potential, *P<0.05 vs Ad-Null and Ad-Sh BNIP3.** S1 C-D:** BNIP3 overexpression significantly increased cytoplasmic cytochrome C, cleaved caspase 3 and cell death, #P<0.05 vs Ad-Null and Ad-Sh BNIP3. BNIP3 knockdown significantly attenuated cell death, *P<0.05 vs Ad-Null.

**Supplement Figure 4.** Long parasternal axis view of sham and diastolic HF animals with preserved EF treated with AAV9 Sh Luc vs AAV9 Sh BNIP3. S3 A-C: The length of the left ventricle, in diastole and in systole, significantly increased in HF. BNIP3 knockdown significantly decreased LV length in diastole and in systole.

**Supplement Figure 5.** Larger size images that are presented in the manuscript. Images on the left are 5,000X magnified, scale bar 2µm. Images on the right are 12,000X magnified, scale bar 1µm. Arrows are showing the presence of autophagosomes.

**Supplement Figure 6.** Long parasternal axis view of sham and systolic HF animals treated with AAV9 Sh Luc vs AAV9 Sh BNIP3. S5 A-C: The length of the left ventricle, in diastole and in systole, significantly increased in HF. BNIP3 knockdown significantly decreased LV length in diastole and in systole.

**Supplement Figure 7.** Larger size images that are presented in the manuscript. Images on the left are 5,000X magnified, scale bar 2µm. Images on the right are 12,000X magnified, scale bar 1µm. Arrows are showing the presence of autophagosomes.
Supplement Figure 8. Larger size images of the ones presented in the manuscript. Arrows are indicating the presence of autophagosomes. Images 12,000X magnified, scale bar 1 µm. The mitochondrial area was significantly higher in cardiomyocytes treated with DIDS compared to no DIDS treatment, @P<0.05 vs Ad-Null, #P<0.05 vs Ad-BNIP3, &P<0.05 vs Ad-Sh BNIP3 and *P<0.05 vs all other groups. Images 12,000X magnified, scale bar 1 µm.

Supplement Figure 9. The expression of a constitutively active FOXO3a increases cardiomyocyte death in vitro. Cell death increased by fourfold in Ad-FOXO3a and decreased by 2 fold in the Ad-DN-FOXO3a infected cardiomyocytes, #P<0.05 vs Ad-GFP and *P<0.05 vs other two groups.

Supplement Figure 10. SERCA2a overexpression in systolic HF improves LV systolic function and attenuates ER stress and BNIP3 mediated mitochondrial destruction. S 10 A-B: SERCA2a overexpression in systolic HF improved LVEF and LV efficiency, *P<0.05 vs HF + Empty. S 10-C: Western blotting of LV tissue lysate showed significant increase in SERCA2a expression and significant decreases in ER stress and ER stress apoptotic markers (p-eIF2a and CHOP), respectively; as well as in Bax/Bcl-2 ratio in the HF + SERCA2a group, *P<0.05 vs HF + Empty and #P<0.05 vs other two groups. There was slight increase in BNIP3 expression in the HF + SERCA2a but was not statistically significant compared to HF + Empty. S 10-D: Ultrastrasturally, there was significant improvement in mitochondrial morphology in the HF + SERCA2a group compared to HF + Placebo. However, SERCA2a did not affect the formation of autophagosomes which were abundant in both groups. Arrows are showing the presence of autophagosomes. Upper images are 5,000X magnified, scale bar 2µm. Lower images are 12,000X magnified, scale bar 1µm. S 10-E: Western blotting analysis of cell lysates from cardiomyocytes infected with Ad-Null vs Ad-Null + Ad-SERCA2a (Ad-S2a) vs Ad-S2a + Ad-BNIP3 vs Ad-Sh BNIP3. &P<0.05 vs other groups and *P<0.05 vs Ad-Null and Ad-Null + Ad-S2a. S 10-F: SERCA2a overexpression attenuated mitochondrial fragmentation in BNIP3 overexpressing cardiomyocytes, but was not superior to DIDS treatment. The largest mitochondria are seen in the Ad-S2a + Ad-ShBNIP3 and Ad-Sh BNIP3 + DIDS infected cardiomyocytes. Images 12,000X magnified, scale bar 1 µm. Arrows are showing the presence of autophagosomes.
Figures

Supplement Figure 1
Supplement Figure 2
Supplement Figure 3
Supplement Figure 3 continued
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Supplement Figure 4 continued
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Supplement Figure 6 continued
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Supplement Figure 9
Supplement Figure 10
Supplement Figure 10 continued
Supplement Figure 10 continued.