Muscle-Specific RING Finger 1 Negatively Regulates Pathological Cardiac Hypertrophy Through Downregulation of Calcineurin A

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Background—Muscle-specific RING finger protein-1 (MuRF1) is an E3 ligase that inhibits cardiac hypertrophy. However, how MuRF1 regulates cardiac hypertrophy and function during pressure overload (PO) remains poorly understood. We investigated the role of endogenous MuRF1 in regulating cardiac hypertrophy in response to PO in vivo.

Methods and Results—Transverse aortic constriction (TAC) for 4 weeks significantly reduced expression of MuRF1 in the mouse heart. After 2 and 4 weeks of TAC, MuRF1 knockout (Murf1−/−) mice exhibited enhanced cardiac hypertrophy and left ventricular (LV) dysfunction compared with that of nontransgenic (NTg) mice. Histological analyses showed that Murf1−/− mice exhibited more severe fibrosis and apoptosis than NTg mice after TAC. TAC-induced increases in the activity of a nuclear factor of activated T cells (NFAT) luciferase reporter were significantly greater in Murf1−/− than in NTg mice. TAC-induced increases in calcineurin A (CnA) expression were also significantly enhanced in Murf1−/− compared with that in NTg mice. Coimmunoprecipitation assays showed that endogenous MuRF1 and CnA interact with one another. Polyubiquitination of CnA was attenuated in Murf1−/− mouse hearts at baseline and in response to TAC, and the protein stability of CnA was enhanced in cardiomyocytes, in which MuRF1 was downregulated in vitro. Furthermore, MuRF1 directly ubiquitinated CnA in vitro. Cardiac-specific overexpression of ZAKI-4β, an endogenous inhibitor of CnA, significantly suppressed the enhancement of TAC-induced cardiac hypertrophy and dysfunction, as well as increases in cardiac fibrosis and apoptosis, in Murf1−/− mice.

Conclusions—Endogenous MuRF1 negatively regulates cardiac hypertrophy and dysfunction in response to PO through inhibition of the calcineurin–NFAT pathway. (Circ Heart Fail. 2014;7:479-490.)

Key Words: calcineurin ■ cardiomegaly ■ ubiquitin protein ligases ■ muscular atrophy ■ ubiquitination

Cardiac hypertrophy is an adaptive response of the heart to hemodynamic overload, as a means of both augmenting systolic function and reducing wall stress. Pathological hypertrophy, which is accompanied by cardiac dysfunction and pathological changes in the myocardium, develops in response to both pressure and volume overload or mutations in sarcomeric genes. The prolonged presence of cardiac hypertrophy is associated with an increased risk of arrhythmia, sudden death, and the development of heart failure. Among a plethora of signaling pathways participating in the development of pathological hypertrophy, one of the most intensely characterized hypertrophic signaling cascades is the one involving calcineurin, a calcium/calmodulin-dependent serine/threonine phosphatase. The primary targets of calcineurin A (CnA) in the heart include the nuclear factor of activated T cells (NFAT) family. CnA dephosphorylates NFATs in the cytoplasm, resulting in their translocation to the nucleus and the subsequent development of cardiac hypertrophy. Although the activity of CnA is regulated primarily at the level of protein expression and by endogenous inhibitors of CnA, including MCIP1 and CIB1, other mechanisms may also exist.

The muscle-specific RING finger (MuRF) family consists of RING finger E3 ubiquitin ligases expressed specifically in cardiac and skeletal muscles. MuRF1 is upregulated during skeletal muscle atrophy, and mice lacking MuRF1 showed a reduction in denervation-induced skeletal muscle atrophy. MuRF1 is also expressed in the heart and the cardiomyocytes therein. Previous studies have shown that MuRF1 negatively...
regulates phenylephrine (PE)-induced cardiac hypertrophy in vitro and pressure overload (PO)-induced cardiac hypertrophy in vivo. Overexpression of MuRF1 in the heart induces wall thinning and cardiac dysfunction, suggesting that MuRF1 may promote heart failure when overexpressed. Recently, mutations of TRIM63, the gene encoding MuRF1, were found in patients with hypertrophic cardiomyopathy. Inducible expression of those TRIM63 mutants in the mouse heart causes cardiac hypertrophy associated with activation of mTOR-S6K and CnA signaling. Important, however, neither the molecular mechanism by which MuRF1 negatively regulates cardiac hypertrophy in response to PO nor the direct link between the TRIM63 mutation and CnA signaling has been elucidated. Furthermore, whether endogenous MuRF1 plays a protective or detrimental role in cardiac function during PO is not well understood.

In this study, we investigated the role of endogenous MuRF1 in mediating cardiac hypertrophy in response to PO. In particular, we asked whether MuRF1 plays a protective or detrimental role in PO-induced cardiac hypertrophy. In addition, we investigated the downstream signaling mechanism through which MuRF1 regulates cardiac hypertrophy. Surprisingly, contrary to the results obtained from gain-of-function animal models, our results obtained from a loss-of-function mouse model suggest that MuRF1 plays a protective role against PO-induced cardiac hypertrophy through downregulation of the CnA–NFAT pathway. The fact that MuRF1 is downregulated during PO suggests that downregulation of the protective mechanism may contribute to the development of pathological hypertrophy.

Methods
An expanded Methods section is available in the Data Supplement.

Animals
MuRF1 knockout (Murf1−/−) mice have been described previously. NFAT-luciferase reporter mice and ZAKI-β transgenic mice were generated using the α-myosin heavy chain (α-MHC) promoter. All animal protocols were approved by the review board of the Institutional Animal Care and Use Committee of the New Jersey Medical School.

Isolation of Ubiquitinated Proteins
Heart homogenate (500 μg) was incubated with Glutathione S Transferase-Tandem Ubiquitin Binding Entity 2 (GST-TUBE2, 20 μg, Life Sensors) at 4°C overnight. After adding glutathione sepharose beads, the samples were incubated at 4°C for 1 hour and then washed with radio immunoprecipitation assay (RIPA) buffer 5 times. Samples were boiled and subjected to SDS-PAGE analyses.

In Vitro Ubiquitylation Assay
In vitro ubiquitylation was performed as described previously. In brief, 2 μmol/L CnA (Non-tagged, Enzo Life Sciences), 100 μmol/L Biotin-ubiquitin, 100 nmol/L E1 enzyme, 5 μmol/L Hist-UBCH5c (E2), and 1 μmol/L GST-MuRF1 (Boston Biochem) or 1 μmol/L bacterially expressed GST were incubated in 20 μmol/L 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 7.2), 100 μmol/L KCl, 5 μmol/L MgCl2, 5 μmol/L ATP, 10 μmol/L DTT, and 1 μmol/L phenylmethylsulfonyl fluoride for 1 hour at 37°C. Samples were analyzed by SDS-PAGE and immunoblotting.

Statistical Analysis
All values are expressed as mean±SEM. Statistical comparisons between groups were performed by exact permutation test or 1-way ANOVA followed by a post hoc Bonferroni–Dunn comparison test. A value of P<0.05 was accepted as significant. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc).

Results
MuRF1 Negatively Regulates Cardiac Hypertrophy
We examined the effect of PO on protein expression of MuRF1 in the mouse heart. PO applied by transverse aortic constriction (TAC) for 2 and 4 weeks decreased MuRF1 protein in a time-dependent manner (Figure 1A). PO also decreased mRNA expression of MuRF1 (Figure 1B). We then examined the role of endogenous MuRF1 in regulating PO-induced cardiac hypertrophy. Three-month-old nontransgenic (NTg) and Murf1−/− mice were subjected to TAC for 2 to 4 weeks. Murf1−/− mice exhibited a greater increase in left ventricular (LV) weight/tibia length (TL) than NTg mice (Figure 1C and Tables I and II in the Data Supplement). The TAC-induced increase in cardiomyocyte cross-sectional area was also significantly greater in Murf1−/− than in NTg mice (Figure 1D). Quantitative PCR analysis showed that TAC increased mRNA expression of fetal-type genes, including atrial natriuretic factor (ANF) and α-skeletal actin (ASA), in both Murf1−/− and NTg mice. However, mRNA expression of ANF and ASA after TAC was significantly greater in Murf1−/− than in NTg mice (Figure 1E). These data suggest that a lack of MuRF1 enhances PO-induced cardiac hypertrophy. We examined the role of MuRF1 in regulating hypertrophy induced by PE, an α1-adrenergic receptor agonist, in cultured cardiomyocytes. Adenovirus-mediated upregulation of MuRF1 significantly attenuated PE-induced cardiac hypertrophy (Figure IA in the Data Supplement). On the contrary, MuRF1 had no effect on IGF-1-induced cardiac hypertrophy (Figure IA in the Data Supplement). These results are consistent with previously reported data, indicating that MuRF1 plays an essential role in mediating α1-adrenergic receptor-induced, but not IGF-1-induced, cardiac hypertrophy. Treatment with CK59, a CAMKII inhibitor, did not alter the PE-induced downregulation of MuRF1 protein levels in cultured cardiomyocytes, suggesting that the expression level of MuRF1 is not regulated by CAMKII (Figure IB in the Data Supplement).

Lack of MuRF1 Exacerbates Cardiac Dysfunction and Histopathologic Changes in the Heart in Response to PO
Lung weight/TL, an index of lung congestion, was significantly greater in Murf1−/− mice than in NTg mice 4 weeks after TAC (Figure 2A and Tables I and II in the Data Supplement). Echocardiographic measurements indicated that the ejection fraction (EF) and fractional shortening (FS) were significantly lower in Murf1−/− mice than in NTg mice 2 and 4 weeks after TAC (Figure 2B and Tables III and IV in the Data Supplement). The number of TdT-mediated dUTP nick-end labeling (TUNEL)-positive nuclei was increased in both NTg and Murf1−/− mice 2 weeks after TAC compared with that in sham-operated mice. However, the increase in the percentage of TUNEL-positive nuclei was significantly greater in Murf1−/− than that in NTg mice (Figure 2C). Similarly, cardiac fibrosis was increased in both NTg and Murf1−/− mice, but the increase in percentage fibrosis after
2 weeks of TAC was significantly greater in Murf1−/− than in NTg mice (Figure 2D). These results suggest that more severe cardiac dysfunction was induced after TAC in the absence of MuRF1.

MuRF1 Regulates the CnA–NFAT Pathway

We evaluated how MuRF1 negatively affects cardiac hypertrophy in response to PO. As previously reported, the protein levels of the β-isoform of myosin heavy chain (β-MYH),
creatine kinase-myocardial band isoenzyme (MB) fraction (CK-MB), and Troponin I, known targets of MuRF1, were increased by TAC in NTg mice, and the TAC-induced increase in these proteins was significantly enhanced in Murf1−/− compared with that in NTg mice (Figure 3A). Interestingly, the protein level of CnA was also increased by TAC in NTg mice, and the level of CnA after TAC was significantly higher in Murf1−/− than in NTg mice (Figure 3A and 3B). mRNA expression of CnA was also increased significantly after TAC in both NTg and Murf1−/− hearts, but there was no significant difference between NTg and Murf1−/− hearts (Figure 3C). Based on these results, we hypothesized that MuRF1 promotes ubiquitination-mediated degradation of CnA, thereby negatively regulating cardiac hypertrophy in response to PO. To test this hypothesis, we next addressed whether MuRF1 affects the CnA–NFAT pathway in the heart under PO. To this end, we first evaluated NFAT protein expression by immunoblotting and immunostaining. Neither

**Figure 2.** Pressure overload in Murf1−/− mice causes myocardial apoptosis, cardiac fibrosis, and cardiac dysfunction after transverse aortic constriction (TAC). A, Lung weight/TL in Murf1−/− and nontransgenic (NTg) mice 2 and 4 weeks after TAC. #P<0.05 compared with sham-operated mice with the same genotype, *P<0.05 (N=4–8). B, Statistical analysis of echocardiographically measured LV ejection fraction (LVEF) is shown. #P<0.05 compared with sham-operated mice with the same genotype, *P<0.05 (N=5–7). C, Left, Representative images of TdT-mediated dUTP nick-end labeling (TUNEL) staining of cardiac sections 2 weeks after TAC. Right, Percentage of TUNEL-positive myocytes. *P<0.05 (N=3). D, Left, Masson Trichrome (MT) staining of cardiac sections 2 weeks after TAC. Right, Percentage of area that is MT-positive. *P<0.05 (N=4).
Figure 3. Muscle-specific RING finger 1 (MuRF1) negatively regulates the calcineurin–nuclear factor of activated T cells (NFAT) pathway. A, The protein levels of calcineurin A (CnA), β-isoform of myosin heavy chain (β-MYH), CK-MB, and Troponin-I at baseline and in response to transverse aortic constriction (TAC; 4 weeks) in Murf1−/− and nontransgenic (NTg) mice. B, The results of the quantitative analysis of the CnA protein level shown in Figure 3A. *P<0.05 (N=6). C, mRNA expression of the CnA gene was measured by quantitative RT-polymerase chain reaction. *P<0.05 (N=5). CnA, D, Murf1−/− enhances NFAT activity at baseline and in response to TAC. Transgenic NFAT-luciferase reporter gene (Tg-NFAT-Luc) mice were crossed with Murf1−/− or control mice, and each mouse group was subjected to either sham or TAC operation. After 1 week, NFAT-Luc activity in the heart homogenates was evaluated. *P<0.05 (N=4–6). D, Calcineurin phosphatase activity in the hearts of Murf1−/− or NTg mice subjected to either sham or TAC operation. *P<0.05 (N=6). E, Representative images of neonatal rat cultured cardiomyocytes treated with PE and the indicated adenoviruses. G, G, Left, Representative images of neonatal rat cultured cardiomyocytes treated with PE and the indicated adenoviruses. Right, Relative cell size of myocytes treated with the indicated adenoviruses in the presence or absence of PE for 72 hours was examined. *P<0.05 (N=3).
TAC nor ablation of MuRF1 affected the NFAT protein level (Figure IIA in the Data Supplement). However, TAC significantly increased nuclear staining of NFAT in NTg hearts, and the TAC-induced increase in nuclear staining of NFAT was significantly enhanced in Murf1−/− hearts (Figure IIB in the Data Supplement). Similarly, PE-induced upregulation of NFAT in the nucleus was enhanced in the presence of MuRF1 short hairpin RNA (sh-MuRF1) in cultured cardiomyocytes (Figure IIC and IID in the Data Supplement). We next crossed transgenic mice harboring an NFAT-luciferase reporter gene (Tg-NFAT-Luc) with Murf1−/− mice (Murf1−/−-Tg-NFAT-Luc). Tg-NFAT-Luc mice showed a significant increase in the NFAT-Luc reporter activity in the heart 1 week after TAC compared with that after sham operation (Figure 3D). The NFAT-Luc reporter activity was significantly enhanced at baseline and in response to TAC in Murf1−/−-Tg-NFAT-Luc mice compared with that in Tg-NFAT-Luc mice (Figure 3D). We also evaluated the phosphatase activity of CnA, which was normalized to the amount of total protein in the heart that changes in activity reflect changes in CnA protein abundance. NTg mice showed a significant increase in CnA activity in the heart 2 weeks after TAC compared with that after sham operation (Figure 3E). The CnA activity was significantly enhanced at baseline and after TAC in Murf1−/− mice compared with that in NTg mice (Figure 3E). Knockdown of MuRF1 through transduction of adenovirus harboring sh-MuRF1 (Ad-sh-MuRF1) induced greater NFAT activity (Figure 3F) and a larger cell size (Figure 3G) in cultured cardiomyocytes in response to PE in vitro, suggesting that the effect of MuRF1 downregulation on CnA activity and hypertrophy is cell-autonomous. Taken together, these results suggest that the lack of MuRF1 enhances CnA–NFAT signaling.

MuRF1 Plays an Important Role in Mediating Ubiquitination of CnA

We hypothesized that MuRF1 affects the protein amount of CnA through ubiquitination. To this end, the extent of CnA ubiquitination was evaluated with the GST-Tandem Ubiquitin Binding Entity assay. Ubiquitination of CnA in the presence of PO was significantly attenuated in Murf1−/− mouse hearts (Figure 4A), suggesting that endogenous MuRF1 regulates ubiquitination of CnA in the presence of PO. To examine whether MuRF1 and CnA interact with one another, coimmunoprecipitation assays were performed. Heart homogenates prepared from NTg and Murf1−/− mice were subjected to immunoprecipitation with either anti-CnA antibody, anti-MuRF1 antibody, or control IgG. MuRF1 was found in the CnA immunoprecipitate prepared from NTg but not Murf1−/− mouse hearts (Figure 4B). MuRF1 was not found in the control IgG immunoprecipitate. Similarly, CnA was found in the MuRF1 immunoprecipitate prepared from NTg but not Murf1−/− mouse hearts (Figure 4C). These results suggest that endogenous MuRF1 and CnA interact with one another. In addition, less MuRF1 was immunoprecipitated with CnA after TAC than after sham operation, suggesting that the interaction between CnA and MuRF1 decreases in response to TAC (Figure 4B). We next evaluated the effect of MuRF1 on the stability of CnA, using CHX, an inhibitor of protein synthesis. Degradation of CnA was significantly slower when MuRF1 was downregulated with Ad-sh-MuRF1 in cultured cardiomyocytes, suggesting that endogenous MuRF1 is involved in CnA degradation (Figure 4D). To test whether MuRF1 directly ubiquitines CnA, in vitro ubiquitination assays were performed using recombinant proteins. Glutathione-S-transferase (GST)-MuRF1 and UBCH5c, an E2 ubiquitin-conjugating enzyme, were incubated with biotin-ubiquitin, a ubiquitin-activating enzyme (E1), and CnA, and the reaction mixture was subjected to immunoblotting with antiubiquitin and anti-CnA antibodies. Ubiquitination of CnA, as assessed by the detection of high-molecular-weight polyubiquitin chains of ubiquitin and monoubiquitinated CnA, was detected only in a reaction containing E1, UBCH5c, MuRF1, and ubiquitin (Figure 4E). Elimination of any 1 of the 4 components in this reaction abolished ubiquitination of CnA. These results suggest that MuRF1 directly polyubiquitinates CnA.

Inhibition of CnA Suppresses Cardiac Hypertrophy and Dysfunction Induced by MuRF1 Gene Disruption

To evaluate the involvement of CnA in mediating the enhancement of TAC-induced cardiac hypertrophy in Murf1−/− mice, transgenic mice with cardiac-specific overexpression of ZAKI-4β (Tg-ZAKI),13 an endogenous inhibitor of CnA, were crossed with Murf1−/− mice (Murf1−/−-Tg-ZAKI). To evaluate whether ZAKI-4β abrogates the enhancement of CnA activity caused by MuRF1 deletion, these mice were further crossed with Tg-NFAT-Luc. One week after TAC, the NFAT-Luc reporter activity was significantly lower in Murf1−/−-Tg-ZAKI mice than that in mice with Murf1−/− alone (Figure 4F). We also evaluated the phosphatase activity of CnA in the heart. The CnA activity was significantly lower in Murf1−/−-Tg-ZAKI mice than that in mice with Murf1−/− alone (Figure 4G). These results indicate that ZAKI-4β effectively inhibits the enhancement of TAC-induced increases in CnA activity in Murf1−/− mice.

Two weeks after TAC, the enhancement of the increases in LV weight/TL and lung weight/TL in Murf1−/− mice compared with that in NTg mice was normalized by ZAKI-4β expression in the Murf1−/− mice (Figure 5A and 5B). Echocardiographic analysis also demonstrated that ZAKI-4β expression in Murf1−/− mice significantly attenuated the reduction in LVEF and the enhanced increase in wall thickness after TAC observed in mice with Murf1−/− alone (Figure 5C–E). Histological analyses indicated that ZAKI-4β expression in Murf1−/− mice significantly attenuated the enhanced increase in TUNEL-positive myocytes and interstitial fibrosis after TAC seen in mice with Murf1−/− alone (Figure 6A and 6B). These results suggest that Murf1−/− enhances cardiac hypertrophy and cardiac dysfunction after TAC through CnA-dependent mechanisms.

Discussion

Although previous studies have suggested that MuRF1 is a negative regulator of cardiac hypertrophy in response to PO,10 the mechanism by which the E3 ligase negatively regulates
Figure 4. Muscle-specific RING finger 1 (MuRF1) plays a critical role in mediating ubiquitination of calcineurin A (CnA). A. Left, Heart homogenates were incubated with agarose-conjugated glutathione S transferase-tandem ubiquitin binding entity 2 (GST-TUBE2) to obtain ubiquitinated proteins. The samples were then subjected to immunoblot analyses with anti-CnA antibody. Right, An immunoblot of the input with antiubiquitin antibody. The results are representative of 3 experiments. B. Heart homogenates were subjected to immunoprecipitation with anti-CnA antibody or control IgG. The immunoprecipitates were immunoblotted with anti-MuRF1 antibody. C. Heart homogenates were subjected to immunoprecipitation with anti-MuRF1 antibody or control IgG. The immunoprecipitates were immunoblotted with anti-CnA antibody. D. Cultured cardiomyocytes were transduced with either Ad-sh-MuRF1 or Ad-sh-scramble. Ninety-six hours after transduction, cells were treated with cycloheximide (CHX) and then harvested after the indicated times. Immunoblot analysis was performed using anti-CnA antibody. The amount of CnA was normalized with that of α-tubulin at each time point and expressed as a percentage of the value at time 0 h. *P<0.05 vs Ad-sh-scramble at that time point (N=3). E, In vitro ubiquitination reactions were performed using anti-CnA antibody. The accumulation of slower-migrating species of CnA with concomitant loss of the nonubiquitinated protein. The ubiquitination of CnA is represented by the accumulation of slower-migrating species of CnA with concomitant loss of the nonubiquitinated protein. The results are representative of 3 experiments. F, ZAKI-4 abrogates the enhancement of transverse aortic constriction (TAC)-induced increases in the CnA activity in Murf1−/− mice. Transgenic nuclear factor of activated T cells–luciferase reporter gene (Tg-NFAT-Luc) mice were crossed with Tg-ZAKI, Murf1−/−, Murf1−/−-Tg-ZAKI cross or control mice, and each mouse group was subjected to either sham or TAC operation. After 1 week, NFAT-Luc activity in the heart homogenates was evaluated. *P<0.05 (N=4–7). G, Calcineurin phosphatase activity in the hearts of Tg-ZAKI, Murf1−/−, Murf1−/−-Tg-ZAKI, or nontransgenic (NTg) mice subjected to either sham or TAC operation. *P<0.05 (N=6).
cardiac hypertrophy has been unknown. Our results suggest that endogenous MuRF1 is a critical upstream regulator of the CnA–NFAT pathway in the heart, and that MuRF1 negatively regulates pathological cardiac hypertrophy and cardiac dysfunction in response to PO through direct down-regulation of CnA.

Previous studies have shown that MuRF1 negatively regulates cardiac hypertrophy both in vitro and in vivo, and that overexpression of MuRF1 stimulates cardiac dysfunction at baseline and in response to PO. However, how endogenous MuRF1 affects cardiac function under stress has not been addressed critically to date. We confirmed that downregulation of endogenous MuRF1 enhances cardiac hypertrophy caused by PO. Importantly, however, downregulation of endogenous MuRF1 also exacerbated pathological findings in the heart, including cardiac fibrosis and apoptosis, and decreases in LVEF. Thus, our results are in marked contrast with those of the previous study by

Figure 5. Effects of inhibition of calcineurin A (CnA) on the enhancement of transverse aortic constriction (TAC)-induced cardiac hypertrophy and cardiac dysfunction in Murf1−/− mice. Tg-ZAKI, Murf1−/−, Murf1−/−-Tg-ZAKI, or nontransgenic (NTg) mice were subjected to TAC for 4 weeks. A, Postmortem measurements of LV weight/TL. *P<0.05 (N=4–7). B, Postmortem measurements of Lung weight/TL. *P<0.05 (N=4–7). C, Representative recordings of the M-mode echocardiography are shown. D, Statistical analysis of echocardiographically measured LVEF is shown. * P<0.05 (N=5–8). E, Statistical analysis of echocardiographically measured interventricular septum thickness (IVST) is shown. *P<0.05 (N=5–8).
Willis et al., in which transgenic overexpression of MuRF1 in the heart induced both pathological hypertrophy and cardiac dysfunction at baseline and under PO. Although we do not yet know the reason for the discrepancy, it is important to point out that expression of MuRF1 is decreased during PO. Thus, it is possible that the role of endogenous MuRF1 is to inhibit pathological hypertrophy and cardiac dysfunction during PO, and that downregulation of endogenous MuRF1 during PO promotes hypertrophy and cardiac dysfunction. On the contrary, based on the results of Willis et al., excessive upregulation might also promote hypertrophy and dysfunction by some unknown mechanism. We speculate that excessive upregulation of the E3 ligase may affect additional targets that may not be targeted at lower levels of MuRF1.

Although the PO-induced downregulation of MuRF1 was significant after 2 weeks of TAC, downregulation develops gradually. Thus, the immediate early phase of PO-induced hypertrophy may be induced through MuRF1 downregulation-independent mechanisms, such as direct post-translational modification of class II histone deacetylases (HDACs). On the contrary, PO-induced downregulation of MuRF1 may contribute to the development of later phase cardiac hypertrophy and heart failure.

Transcription of MuRF1 is upregulated during cardiac unloading in the heart, and MuRF1 is upregulated in the rat model of chronic heart failure caused by permanent coronary ligation. Upregulation of MuRF1 transcription is also observed during denervation-induced atrophy in skeletal muscle, where it is induced preferentially in fast fibers rather than slow fibers. However, MuRF1 is downregulated in the mouse model of PO (and in this study), with estrogen attenuating TAC-induced downregulation of MuRF1. MuRF1 is upregulated by NF-κB-, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α)-, and FoxO-dependent mechanisms. However, the molecular mechanism mediating downregulation of MuRF1 during PO remains to be elucidated.

Previous studies have suggested that the CnA–NFAT pathway plays an important role in mediating pathological hypertrophy. Activation of CnA in response to hypertrophic stimuli is mediated by many mechanisms. We show that the TAC-induced increases in the activity of CnA are significantly enhanced in Murf1−/− mice, suggesting that MuRF1 is a negative regulator of CnA in the heart. We think that the enhancement of CnA is functionally significant because the enhancement of TAC-induced cardiac hypertrophy and LV dysfunction in Murf1−/− mice was

Figure 6. Effects of inhibition of CnA on the enhancement of transverse aortic constriction (TAC)-induced fibrosis and apoptosis in Murf1−/− mice. Tg-ZAKI, Murf1−/−, Murf1−/−-Tg-ZAKI, or nontransgenic (NTg) mice were subjected to TAC for 4 weeks. A, Apoptosis was evaluated with TUNEL staining. Right image shows the results of the quantitative analysis. *P<0.05 (N=10–15). B, Interstitial fibrosis was evaluated with Masson Trichrome staining. Right image shows the results of the quantitative analysis. ∗P<0.05 (N=9).
significantly attenuated when TAC-induced activation of CnA was inhibited by ZAKI-4β, an endogenous inhibitor of CnA. 24

Several lines of evidence suggest that endogenous MuRF1 directly regulates the protein stability of CnA through ubiquitination. First, downregulation of MuRF1 significantly attenuates degradation of CnA in cardiomyocytes in vitro, indicating that endogenous MuRF1 negatively regulates the stability of CnA in a cell-autonomous manner. Second, endogenous MuRF1 and CnA physically interact with one another. The fact that their interaction decreased during TAC correlates well with the increased protein level of CnA during TAC. Third, the extent of polyubiquitination of CnA after TAC is significantly attenuated in Murf1−/− mice. Finally, MuRF1 is able to directly polyubiquitinate CnA in test tubes, indicating that CnA can be a direct substrate of MuRF1.

The activity of CnA is regulated by many endogenous molecules, including MCIP1 5 and CIB1. 6 However, to our knowledge, the functional involvement of MuRF1 in the regulation of CnA has not been demonstrated in the heart. It has previously been shown that muscle atrophy F-box (MAFbx) causes degradation of CnA in cardiomyocytes. However, this was demonstrated using gain-of-function experiments. 25 Taking into account our recent observation using loss-of-function experiments that MAFbx mediates, rather than inhibits, cardiac hypertrophy, 26 we propose that, under physiological conditions, MuRF1, rather than MAFbx, controls the level of CnA. Importantly, despite modest increases in NFAT activity in Murf1−/− mice subjected to sham operation, these mice do not show obvious cardiac hypertrophy. Thus, it is possible that, although NFAT enhances TAC-induced hypertrophy, modest elevation of NFAT alone may not be sufficient for inducing hypertrophy at baseline.

MuRF1 is localized in the M-line region and colocalized with α-actinin, one of the major components of the Z-disc in heart tissue. 7 CnA is tethered to α-actinin at the Z-disc in cardiomyocytes. 27 Thus, MuRF1 may regulate CnA signaling in the microdomain near the Z-disc. It has been shown that both MuRF1 and MuRF2 are required for maintenance of type-II muscle fibers, possibly through upregulation of myozenin-1, a negative regulator of calcineurin. 28 Although the involvement of myozenin-1 in the regulation of CnA by MuRF1 cannot be excluded, downregulation of MuRF1 was sufficient for upregulation of calcineurin during PO in the heart.

Several other molecules act downstream of MuRF1. MuRF1 regulates protein kinase Cε (PKCε) through interaction with the receptor for activated C-kinase 1 (RACK1) 16 and affects transcription through interaction with serum response factor 10 and glucocorticoid modulatory element binding factor-1. 29 MuRF1 also regulates muscle contraction through degradation of myosin heavy chain, 30 troponin I, 31

Figure 7. Hypothetical model of muscle-specific RING finger 1 (MuRF1) and calcineurin A (CnA) interactions in response to pressure overload (PO) in the heart. Expression of CnA is increased but that of MuRF1 is decreased in response to PO, and MuRF1 negatively regulates pathological hypertrophy in part through proteasomal degradation of CnA.
and myosin-binding protein C. In addition, MuRF1 regulates microtubule dynamics, myofilament structure, and muscle metabolism, including carbohydrate metabolism and branched chain amino acid metabolism. Whether these molecules located downstream of MuRF1 are also involved in the progression of pathological hypertrophy and LV dysfunction initiated by downregulation of MuRF1 remains to be determined.

In conclusion, we have identified MuRF1 as an important negative regulator of cardiac hypertrophy (Figure 7). Because endogenous MuRF1 is downregulated during PO, a pharmacological intervention that maintains, but does not increase, the level of MuRF1 may prevent the progression of pathological hypertrophy and heart failure.

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Disclosures

None.

References

CLINICAL PERSPECTIVE

Cardiac hypertrophy caused by hemodynamic overload is initially adaptive, but the prolonged presence of hypertrophy leads to the development of heart failure. Calcineurin A (CnA), a calcium/calmodulin-dependent serine/threonine phosphatase, is upregulated in response to hypertrophic stimuli and induces cardiac hypertrophy by dephosphorylating the nuclear factor of activated T cells (NFAT) family. We show here that muscle-specific RING finger 1 (MuRF1), an E3 ubiquitin ligase, polyubiquitinates CnA and degrades it through the ubiquitin proteasome system (UPS). Hypertrophic stimuli downregulate MuRF1, which in turn induces accumulation of CnA and the consequent development of cardiac hypertrophy and dysfunction. Thus, MuRF1 is an important negative regulator of cardiac hypertrophy and heart failure. Mutations of TRIM63, the gene encoding MuRF1, are found in patients with hypertrophic cardiomyopathy. These results suggest that intervention to stimulate MuRF1 may inhibit cardiac hypertrophy and heart failure by stimulating degradation of CnA through the UPS.
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Supplement Material

Methods

Primary cultures of neonatal rat ventricular myocytes
Primary cultures of ventricular cardiomyocytes were prepared from 1-day-old Crl:(WI) BR-Wistar rats (Charles River Laboratories). A cardiomyocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient as described 1.

Construction of adenoviruses
Recombinant adenovirus vectors were constructed, propagated, and titrated as previously described 2. Briefly, pBHGloxΔE1,3 Cre (Microbix), including the ΔE1 adenoviral genome, was co-transfected with the pDC316 shuttle vector containing the gene of interest into HEK293 cells using LipofectAMINE 2000 (Life Technologies). Through homologous recombination, the test genes were integrated into the E1-deleted adenoviral genome. The viruses were propagated in HEK293 cells. We made replication-defective human adenovirus type 5 (devoid of E1) harboring myc-MurF1. Adenovirus harboring β-galactosidase (Ad-LacZ) was used as a control.

Construction of short hairpin RNA (shRNA) adenoviral expression vector
The pSilencer 1.0-U6 expression vector was purchased from Ambion. The U6 RNA polymerase III promoter and the polylinker region were subcloned into the adenoviral shuttle vector pDC311 (Microbix). A hairpin-forming oligo corresponding to bases 616-638 (5′-GGAGGAAGCTGAGCCACAAGTTCAAGAGAAGCTTGTGGCTCAGT TTACTCCTTTTTGGAAA-3′) of the rat MuRF1 cDNA and its antisense were synthesized with Apal and HindIII overhangs, annealed, and subcloned distal to the U6 promoter. The loop sequence is underlined. A recombinant adenovirus was made by homologous recombination in HEK293 cells as described above.
**Immunoblot analysis**

For immunoblot analyses, heart homogenates were prepared in RIPA lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% IGEPAL CA-630, 0.1% SDS, 0.5% deoxycholic acid, 10 mM Na4P2O7, 5 mM EDTA, 0.1 mM Na3VO4, 1 mM NaF, 0.5 mM 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 0.5 mg/ml aprotinin, and 0.5 mg/ml leupeptin. Immunoblots were performed as previously described. The antibodies used include anti-MuRF1 (ECM Biosciences), anti-CnA (BD Biosciences), anti-NFAT1 (Abcam), anti-Histone H3 (Cell Signaling Technologies), anti-creatine kinase (CK)-MB, anti-Troponin-I and anti-ubiquitin (Santa Cruz), anti-β-myosin heavy chain (MYH) and anti-GAPDH (SIGMA) antibodies.

**Nuclear and cytosol extract preparation**

Cells were washed in PBS and resuspended in hypotonic buffer, containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 100 μM Na3VO4, 1 mM DTT, and 500 μM PMSF. The cells were then lysed by adding 10% IGEPAL CA-630 and vortexing vigorously. Nuclei were pelleted by centrifugation at 800 X g for 30 min. The supernatant (cytosol) was saved for analysis. The nuclei were then resuspended in hypertonic buffer, containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 100 μM Na3VO4, 1 mM DTT, and 500 μM PMSF, and rocked for 30 min on a shaking platform at 4ºC. The samples were centrifuged at 16,000 X g for 5 min, and the supernatant (nuclear extracts) was saved.

**Immunoprecipitation**

Cardiac myocytes were lysed with IGEPAL CA-630 buffer (50 mM Tris-HCl (pH 7.4), 1% IGEPAL CA-630, 10 mM EDTA, 150 mM NaCl, 50 mM NaF, 1 μM leupeptin, and 0.1 μM aprotinin). Primary antibody was covalently immobilized on protein A/G agarose using the
Pierce® Crosslink Immunoprecipitation Kit according to the manufacturer's instructions (Thermo Scientific). Samples were incubated with immobilized antibody beads for at least 2 h at 4°C. After immunoprecipitation, the samples were washed with TBS five times. They were then eluted with glycine-HCl (0.1 M, pH 3.5), and the immunoprecipitates were subjected to immunoblotting using specific primary antibodies and a conformation-specific secondary antibody that recognizes only the native IgG (Cell Signaling).

**Luciferase assay**

Hearts were harvested and weighed, and equal amounts of the heart homogenates were subjected to luciferase assays using a luciferase assay system (Promega)\(^4\).

**Calcineurin activity assay**

Phosphatase activity was measured by using a calcineurin assay kit (Enzo Life Sciences) according to the manufacturer’s instructions. Heart homogenates from Tg-ZAKI, Murf1\(^{-/-}\), Murf1\(^{-/-}\)-Tg-ZAKI and NTg mice were prepared, and calcineurin activity was measured as the dephosphorylation rate of a synthetic phosphopeptide substrate (RII peptide) in the presence or absence of EGTA. The amount of PO\(_4\) release was determined photometrically using the Biomol Green reagent (Enzo Life Sciences). The activity of calcineurin in each sample was normalized to the amount of total protein in the sample.

**Calcineurin turnover assay**

Cycloheximide (CHX) chase for evaluating calcineurin turnover was performed as described previously with modification \(^5\). Briefly, cultured cardiomyocytes were transduced with either Ad-sh-MuRF1 or Ad-sh-scramble. Ninety-six hours following transduction, cells were incubated with 50 ng/ml CHX for the indicated periods of time, after which lysates were immediately collected into lysis buffer.
**Transverse aortic constriction (TAC)**

The method used to impose PO on mice has been described. As controls, sham operations were performed without constricting the aorta. After 2 or 4 weeks, echocardiography and hemodynamic measurements were performed as described.

**Echocardiography**

Mice were anesthetized with 12 µl/g of body weight of 2.5% Avertin (Sigma), and echocardiography was performed with ultrasonography (Acuson Sequoia C256, Siemens Medical Solutions) as previously described.

**Histological analyses**

Heart specimens were fixed with formalin, embedded in paraffin, and sectioned into 6 mm-thick slices. Interstitial fibrosis was evaluated by Masson’s Trichrome staining. Myocyte cross-sectional area was measured from images taken of wheat germ agglutinin (WGA)-stained 1 mm-thick sections. The outline of 100-200 myocytes was traced in each section, using the MetaMorph image system. DNA fragmentation was detected in situ using TUNEL staining.

**Immunofluorescence microscopy**

Cardiomyocytes were fixed with 3.7% paraformaldehyde in PBS for 10 min and incubated with PBS containing 0.5% Triton X-100 for 15 min. After being washed with PBS, the cells were first incubated with primary antibodies in PBS, and then incubated with secondary antibodies in PBS.

**Analysis of messenger RNA (mRNA) expression**
Total RNA from the heart was extracted using TRIzol (Life Technologies) and first-strand cDNA was generated using a first-strand cDNA synthesis kit (GE Healthcare Biosciences)\textsuperscript{11}. Real-time PCR was carried out using specific primers as described\textsuperscript{11}.

References


Cardiac-specific overexpression of \( \alpha \)1 receptor mutant lacking \( \alpha \)q/\( \alpha \)i coupling causes hypertrophy and bradycardia in transgenic mice. *J Clin Invest.* 2005;115:3045-3056


Online Table 1

Postmortem pathologic measurements in control and Murf1−/− mice 2 weeks after TAC

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Murf1−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>TAC</td>
</tr>
<tr>
<td>N</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>BW (g)</td>
<td>25.6±1.1</td>
<td>24.8±0.7</td>
</tr>
<tr>
<td>TL (mm)</td>
<td>18.0±0.2</td>
<td>17.9±0.3</td>
</tr>
<tr>
<td>LV (mg)</td>
<td>88.3±2.7</td>
<td>126.7±8.7^A</td>
</tr>
<tr>
<td>RV (mg)</td>
<td>18.6±1.4</td>
<td>22.3±1.7</td>
</tr>
<tr>
<td>LV weight/BW (mg/g)</td>
<td>3.47±0.11</td>
<td>5.08±0.27^A</td>
</tr>
<tr>
<td>Lung weight/BW (mg/g)</td>
<td>5.24±0.15</td>
<td>6.30±0.87</td>
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<tr>
<td>Liver weight/BW (mg/g)</td>
<td>45.0±4.5</td>
<td>40.8±1.9</td>
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<tr>
<td>LV weight/TL (mg/mm)</td>
<td>4.99±0.15</td>
<td>7.05±0.41^A</td>
</tr>
<tr>
<td>Lung weight/TL (mg/mm)</td>
<td>7.31±0.09</td>
<td>8.70±1.17</td>
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<tr>
<td>Liver weight/TL (mg/mm)</td>
<td>62.7±5.8</td>
<td>56.4±2.8</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. ^A^P < 0.01 compared with same genotype sham mice. ^B^P < 0.05 compared with WT 2 weeks after TAC.
**Online Table 2**

Postmortem pathologic measurements in control and *Murf1*/*−−* mice 4 weeks after TAC

<table>
<thead>
<tr>
<th></th>
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<th>Murf1*−−*</th>
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<tbody>
<tr>
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<td>Sham</td>
<td>TAC</td>
</tr>
<tr>
<td>N</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>BW (g)</td>
<td>26.4±1.1</td>
<td>28.1±1.1</td>
</tr>
<tr>
<td>TL (mm)</td>
<td>18.0±0.2</td>
<td>17.6±0.2</td>
</tr>
<tr>
<td>LV (mg)</td>
<td>88.3±2.7</td>
<td>155.0±12.7&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>RV (mg)</td>
<td>18.6±1.4</td>
<td>21.4±1.7&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>LV weight/BW (mg/g)</td>
<td>3.58±0.15</td>
<td>5.50±0.30&lt;sup&gt;A&lt;/sup&gt;</td>
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<tr>
<td>Lung weight/BW (mg/g)</td>
<td>5.24±0.15</td>
<td>6.48±0.49&lt;sup&gt;A&lt;/sup&gt;</td>
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<tr>
<td>Liver weight/BW (mg/g)</td>
<td>45.0±4.5</td>
<td>39.3±1.8</td>
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<tr>
<td>LV weight/TL (mg/mm)</td>
<td>4.99±0.15</td>
<td>8.88±0.64&lt;sup&gt;A&lt;/sup&gt;</td>
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<tr>
<td>Lung weight/TL (mg/mm)</td>
<td>7.31±0.09</td>
<td>10.4±0.51&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver weight/TL (mg/mm)</td>
<td>62.7±5.8</td>
<td>63.5±4.2</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. <sup>A</sup>*P* < 0.01 compared with same genotype sham mice. <sup>C</sup>*P* < 0.01 compared with WT 4 weeks after TAC.
### Online Table 3

Echocardiographic analyses in control and Murf1\(^{-/-}\) mice 2 weeks after TAC

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Murf1(^{-/-})</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>TAC</td>
</tr>
<tr>
<td>N</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>DSEPWT (mm)</td>
<td>0.69±0.02</td>
<td>0.97±0.04(^B)</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>4.13±0.13</td>
<td>3.99±0.12</td>
</tr>
<tr>
<td>DPWT (mm)</td>
<td>0.64±0.03</td>
<td>0.92±0.04(^B)</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.97±0.11</td>
<td>2.88±0.11</td>
</tr>
<tr>
<td>EF (%)</td>
<td>62.9±1.8</td>
<td>57±3</td>
</tr>
<tr>
<td>FS (%)</td>
<td>28.2±1.1</td>
<td>27.8±1.0</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>412±19</td>
<td>411±13</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. \(^A\)\(P < 0.05\), \(^B\)\(P < 0.01\) compared with same genotype sham mice. \(^C\)\(P < 0.01\) compared with WT 2 weeks after TAC.

DSEPWT: diastolic septal wall thickness; LVEDD: left ventricular end-diastolic dimension; DPWT: diastolic posterior wall thickness; LVESD: left ventricular end-systolic dimension; EF: ejection fraction; FS: fractional shortening; HR: heart rate.
### Online Table 4

Echocardiographic analyses in control and *MurfI*/* mouse 4 weeks after TAC

<table>
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<th>MurfI/*</th>
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<td>TAC</td>
<td>Sham</td>
<td>TAC</td>
</tr>
<tr>
<td>N</td>
<td>7</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td>DSEPWT (mm)</td>
<td>0.69±0.02</td>
<td>1.23±0.06&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.74±0.04</td>
<td>1.25±0.05&lt;sup&gt;A&lt;/sup&gt;</td>
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<tr>
<td>LVEDD (mm)</td>
<td>4.13±0.13</td>
<td>3.95±0.12</td>
<td>4.02±0.16</td>
<td>4.16±0.15</td>
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<tr>
<td>DPWT (mm)</td>
<td>0.64±0.03</td>
<td>1.15±0.08&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.72±0.04</td>
<td>1.20±0.07&lt;sup&gt;A&lt;/sup&gt;</td>
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<tr>
<td>LVESD (mm)</td>
<td>2.97±0.11</td>
<td>2.89±0.11</td>
<td>2.81±0.14</td>
<td>3.21±0.07</td>
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<tr>
<td>EF (%)</td>
<td>62.87±1.76</td>
<td>50.51±2.66</td>
<td>65.70±1.49</td>
<td>40.25±2.36&lt;sup&gt;AB&lt;/sup&gt;</td>
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<tr>
<td>FS (%)</td>
<td>28.23±1.12</td>
<td>26.83±1.47</td>
<td>30.04±1.00</td>
<td>16.67±1.68&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>412±19</td>
<td>405±14</td>
<td>413±15</td>
<td>432±18</td>
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</table>

Data are mean ± SEM. <sup>A</sup><sub>P</sub> < 0.01 compared with sham mice of the same genotype. <sup>B</sup><sub>P</sub> < 0.05 compared with WT 4 weeks after TAC.

DSEPWT: diastolic septal wall thickness; LVEDD: left ventricular end-diastolic dimension; DPWT: diastolic posterior wall thickness; LVESD: left ventricular end-systolic dimension; EF: ejection fraction; FS: fractional shortening; HR: heart rate.
Online Figure I. A, Upper: Representative images of neonatal rat cultured cardiomyocytes treated with PE or IGF-I and the indicated adenoviruses. The cells were observed under a fluorescent microscope. Lower: Relative cell size of myocytes treated with the indicated adenoviruses in the presence or absence of PE or IGF-I for 48 hours was examined * \( P<0.05 \) (\( N=9 \)). B, Cultured cardiomyocytes were treated with CK59, a CaMKII inhibitor, and then harvested after 48 hr. Immunoblot analysis was performed using anti-MuRF1 antibody.
Online Figure II. A, Expression of NFAT in mouse hearts in response to TAC (4 weeks) was determined by immunoblotting. B, Left: Immunohistochemistry of NFAT. Arrows indicate nuclear localization of NFAT. Right: NFAT-positive myocytes were counted. * P<0.05 (N=3). C, Left: The extent of nuclear expression of NFAT was determined by triple immunostaining with anti-NFAT antibody (green), anti-troponin I antibody (red) and DAPI (blue). Right: Nuclear NFAT-positive myocytes were counted. * P<0.05 (N=3). D, Cytosolic and nuclear fractions were prepared from Ad-shScramble- and Ad-shMuRF1-transduced cardiomyocytes. Immunoblot analyses were conducted with NFAT antibody. The purity of the nuclear fraction was confirmed by the lack of Troponin I. Representative immunoblot images are shown.