MURC/Cavin-4 facilitates recruitment of ERK to caveolae and concentric cardiac hypertrophy induced by α1-adrenergic receptors


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The actions of catecholamines on adrenergic receptors (ARs) induce sympathetic responses, and sustained activation of the sympathetic nervous system results in disrupted circulatory homeostasis. In cardiomyocytes, α1-ARs localize to flask-shaped membrane microdomains known as “caveolae.” Caveolae require both cavin and cavolin proteins for their biogenesis and function. However, the functional roles and molecular interactions of caveolar components in cardiomyocytes are poorly understood. Here, we showed that muscle-restricted coiled-coil protein (MURC)/Cavin-4 regulated α1-AR-induced cardiomyocyte hypertrophy through enhancement of ERK1/2 activation in caveolae. MURC/Cavin-4 was expressed in the caveolae and T tubules of cardiomyocytes. MURC/Cavin-4 overexpression distended the caveolae, whereas MURC/Cavin-4 was not essential for their formation. MURC/Cavin-4 deficiency attenuated cardiac hypertrophy induced by α1-AR stimulation in the presence of caveolae. Interestingly, MURC/Cavin-4 bound to α1A- and α1B-ARs as well as ERK1/2 in caveolae, and spatiotemporally modulated MEK/ERK signaling in response to α1-AR stimulation. Thus, MURC/Cavin-4 facilitates ERK1/2 recruitment to caveolae and efficient α1-AR signaling mediated by caveolae in cardiomyocytes, which provides a unique insight into the molecular mechanisms underlying caveola-mediated signaling in cardiac hypertrophy.

caveola | signal transduction | heart | plasma membrane

Caveolae are plasmalemmal invaginations enriched in cholesterol, glycosphingolipids, and lipid-anchored proteins relative to the bulk of the plasma membrane (1, 2). Owing to their specific lipid composition, caveolae concentrate several signaling molecules involved in cellular processes or trafficking events from the cell surface; therefore, they are recognized as a platform for preassembled complexes of receptors, signal components, and their targets, facilitating efficient and specific cellular responses (3–5). Accumulating evidence has demonstrated that caveola biogenesis and function depend on two distinct caveolar components: caveolins and cavin (6, 7). Caveolin (Cav)-1 and Cav-2 are expressed in most cell types, including adipocytes, endothelial cells, fibroblasts, and smooth myocytes, whereas Cav-3 is expressed exclusively in muscle cells (13), whereas MURC/Cavin-4 is expressed in various cell types except for muscle cells (13), whereas MURC/Cavin-4 is expressed exclusively in myocytes, similar to Cav-3 (11). Recent studies have shown that cavin and cavelins form a complex, called the “cavin–cavin complex,” which modifies caveolar biogenesis and function (14, 15). PTRF/Cavin-1 and SDPR/Cavin-2 are required for caveolar invagination and SRBC/Cavin-3 for caveolar budding to form caveolar vesicles (13, 16, 17). However, the functional role of MURC/Cavin-4 in caveolar morphology is not known.

Alpha-1 adrenergic receptors (α1-ARs) are members of the G protein-coupled receptor (GPCR) family and have been demonstrated to accumulate in caveolar fractions of the myocardium (5). GPCRs are well-known representatives of receptors concentrated in caveolae and transduce several signals from substrates to downstream effectors in caveolae (18). Because disruption of the caveolae affects the response to several GPCRs, caveolae are considered important plasma membrane structures that coordinate GPCRs and their downstream signaling components (5). In our previous study, we showed that MURC/Cavin-4 knockdown suppressed α1-AR agonist-induced atrial natriuretic peptide expression and myofibrillar organization in cardiomyocytes and that transgenic mice overexpressing MURC/Cavin-4 in cardiac tissue

**Significance**

Caveolae are recognized as a platform for preassembled complexes of receptors, signal components, and their targets, facilitating efficient and specific cellular responses at the plasma membrane. ERK is activated at the plasma membrane and an important molecule that has been well studied for its integral role in signal transduction events during physiological adaptation and pathological manifestation. Here we show that although muscle-restricted coiled-coil protein (MURC)/Cavin-4, a muscle-specific caveolar component, is dispensable for caveolar formation in cardiomyocytes, MURC/Cavin-4 serves as an ERK-recruiting protein in the caveolae within cardiomyocytes. The recruiting function of MURC/Cavin-4 is necessary to elicit efficient signaling of the α1-adrenergic receptor–ERK cascade in concentric cardiac hypertrophy. Our findings provide unique insight into the molecular mechanisms underlying caveola-mediated signaling in cardiac hypertrophy.
(MURC-Tg) developed cardiomyocyte hypertrophy at 5 wk of age (11). These results suggest that MURC/Cavin-4 is involved in α1-AR signaling and cardiac hypertrophy.

In the present study, we manipulated MURC/Cavin-4 expression to investigate the role of MURC/Cavin-4 in caveolar morphology and α1-AR–induced cardiac hypertrophy. Overexpression and deletion of MURC/Cavin-4 showed the roles of MURC/Cavin-4 in the caveolar morphology of cardiomyocytes. Furthermore, we found that MURC/Cavin-4 facilitated ERK1/2 recruitment to caveolae and ERK activation in α1-AR–induced concentric cardiomyocyte hypertrophy.

**Results**

**MURC/Cavin-4 Forms Caveolin–Cavin Complexes in the Caveolae and T Tubules and Modulates Caveolar Morphology in Cardiomyocytes.** To reveal the functional significance of MURC/Cavin-4 as a caveolar component in cardiomyocytes, we examined the association of MURC/Cavin-4 with other cavins and caveolins. Expression plasmids encoding MURC/Cavin-4, Cav-3, and PTRF/Cavin-1 were transfected into CV-1 (simian) in origin, and carrying the SV40 genetic material (COS) cells, a fibroblast-like cell line derived from monkey kidney tissue. Immunoblot analysis showed that MURC/Cavin-4 was coimmunoprecipitated with Cav-3 and PTRF/Cavin-1 (Fig. S1 C and B). MURC-HA and Cav-3-T7 expressions were not reduced in supernatants immunoprecipitated with anti-T7 and anti-HA antibodies, respectively (Fig. S1 A), whereas MURC-FLAG expression was reduced in the supernatant immunoprecipitated by the anti-HA antibody, although PTRF/Cavin-1-HA expression was not reduced in the supernatant immunoprecipitated by the anti-FLAG antibody (Fig. S1 B). These results suggest that MURC/Cavin-4 binds to PTRF/Cavin-1 with high affinity, and that MURC/Cavin-3 does not entirely bind to Cav-3.

The bimolecular fluorescence complementation (BiFC) assay confirmed that MURC/Cavin-4, Cav-3, PTRF/Cavin-1, and SDPR/ Cavin-2 interact in living cardiomyocytes (Fig. S1 C and D). Immunoelectron microscopy revealed that MURC/Cavin-4 was expressed in caveolae and T tubules in cardiomyocytes of adult mice (Fig. 1A). These observations are in accordance with our previous finding showing that MURC/Cavin-4 was localized to the Z line in cardiomyocytes (11) because the T-tubule system is in register with the Z lines and the immunostaining pattern of Cav-3 has been shown to coincide with the Z line in the heart (19). Because it was confirmed that MURC/Cavin-4 was expressed by caveolae, we assessed whether MURC/Cavin-4 affected caveolar morphology in cardiomyocytes. In cardiomyocytes of 13-wk-old MURC-Tg mice, the caveolae were significantly distended compared with those of wild-type (WT) mice (Fig. 1B). The effects of MURC/Cavin-4 on caveolae were supported by the results of an in vitro study in which MURC/Cavin-4 overexpression significantly increased the caveolar area and perimeter in cardiomyocytes compared with β-galactosidase (LacZ) overexpression (Fig. 1C). These results indicated that MURC/Cavin-4 modified the morphology of formed caveolae in cardiomyocytes.

**MURC/Cavin-4 Is Associated with α1-ARs at Caveolae in Cardiomyocytes.** We next investigated the localization of α1-ARs in cardiomyocytes. α1-AR exists as three molecular subtypes: α1A, -B, and -D. The α1A and -B subtypes are expressed in the myocardium, whereas the α1D subtype is expressed in vascular muscle (20). Because antibodies for α1-AR subtypes, which are frequently cited, have been shown to be nonspecific (21), we used plasmids encoding red fluorescent protein mCherry-conjugated α1A-AR (ADRA1A) and α1B-AR (ADRA1B). ADRA1A and ADRA1B signals were observed predominantly at the plasma membrane and partly within the cytoplasm (Fig. 2A). ADRA1A and ADRA1B signals were colocalized with endogenous Cav-3 and MURC/Cavin-4 at the plasma membrane and partly within the cytoplasm. Immunoprecipitation and BiFC assays revealed that both ADRA1A and ADRA1B were bound to MURC/Cavin-4 in COS cells and cultured rat cardiomyocytes, respectively (Fig. 2 B and C).

Because Cav-3 has also been demonstrated to bind to α1-ARs (22), we investigated whether Cav-3 could influence the localization of MURC/Cavin-4 and α1-ARs in cardiomyocytes. Cav-3 knockdown impaired the plasma membrane localization of MURC/Cavin-4, resulting in the accumulation of MURC/Cavin-4 in the cytosol of cardiomyocytes (Fig. S2 A–C). However, α1-ARs were retained at the plasma membrane in Cav-3–knocked down cardiomyocytes (Fig. S2 D).

**MURC/Cavin-4 Deficiency Attenuates α1-AR–Induced ERK Activation and Cardiac Hypertrophy.** The above-mentioned observations that α1-ARs bound to MURC/Cavin-4 at caveolae in cardiomyocytes led us to examine whether MURC/Cavin-4 influenced the response to α1-AR stimulation in vivo. To this end, we subjected WT and WT MURC-Tg mice to isoproterenol (ISO) injection. In ISO-treated WT and WT MURC-Tg mice, the area and perimeter of caveolae were measured (Fig. 2A). The effects of MURC/Cavin-4 on caveolar morphology in cardiomyocytes were assessed using electron microscopy. Representative electron microscopic images of mouse heart tissue and adult cultured cardiomyocytes. Representative electron microscopic images of mouse heart tissue and adult cultured cardiomyocytes. Representative electron microscopic images of mouse heart tissue and adult cultured cardiomyocytes. Representative electron microscopic images of mouse heart tissue and adult cultured cardiomyocytes. Representative electron microscopic images of mouse heart tissue and adult cultured cardiomyocytes.
under physiological conditions (Tables S1 and S2). Furthermore, indistinguishable by cardiac mass and function from WT mice demonstrated that, in ADRA1A and ADRA1B double-knockout MURC (23). Therefore, we investigated ERK activation in WT and heart size in could not activate ERK in cardiomyocytes, resulting in a reduced (KO) (23). Phenylephrine (PE) infusion for 7 d caused marked concentric localization of Cav-3 and caveola formation in cardiomyocytes. PE stimulation, both MURC/Cavin-4 and phosphorylated ERK were translocated from the plasma membrane to the perinuclear region (Fig. 4B and C). MURC/Cavin-4 knockdown reduced phosphorylated ERK accumulation at the plasma membrane, where it was colocalized with MURC (24). Upon PE stimulation, both MURC/Cavin-4 and phosphorylated ERK were translocated from the plasma membrane to the perinuclear region (Fig. 4B and C). MURC/Cavin-4 knockdown reduced phosphorylated ERK accumulation at the plasma membrane (Fig. 4D). Immunoblot analysis showed that MURC/Cavin-4 coimmunoprecipitated with phosphorylated and total ERK (Fig. 4E).

The stability of ERK proteins influences the duration of ERK activity (27). Thus, we evaluated the stability of phosphorylated and total ERK using cycloheximide (CHX), a protein biosynthesis inhibitor. The stability of phosphorylated and total ERK proteins was significantly increased following CHX treatment in MURC-expressing cardiomyocytes compared with the level in those expressing LacZ (Fig. S4D).
MURC/Cavin-4 Modulates α1-AR–Induced Cardiomyocyte Hypertrophy Through ERK Activation. We previously demonstrated cardiomyocyte hypertrophy in young MURC-Tg mice (11). In the present study, we found that ERK activity was increased in the cardiomyocytes of 6-wk-old MURC-Tg mice (Fig. 5A) and that MURC/Cavin-4 overexpression promoted cardiomyocyte hypertrophy (Fig. 5B). Furthermore, ERK1/2 siRNA knockdown (Fig. S5) significantly inhibited MURC/Cavin-4– and α1-AR–induced increase in cardiomyocyte size (Fig. 5 C and D). These results indicated that MURC/Cavin-4 was required for ERK activation in cardiomyocyte hypertrophy. ERK activity is regulated by MEK (28); therefore, to determine whether MURC/Cavin-4 modulates ERK activation through MEK1/2, we measured MEK1/2 activity in cardiomyocytes (Fig. S6). Under basal conditions, MURC/Cavin-4 overexpression did not affect MEK1/2 activation, whereas ERK activity was activated by MURC/Cavin-4 overexpression compared with LacZ. MEK1/2 activation in MURC/Cavin-4–overexpressing PE-stimulated cardiomyocytes was significantly enhanced compared with that in LacZ-overexpressing cardiomyocytes. In addition, ERK activation was synergistically enhanced by MURC/Cavin-4 overexpression.
overexpression in response to PE stimulation. Taken together, these findings suggested that MURC/Cavin-4 was capable of regulating ERK activation in a MEK1/2-dependent and independent manner and that MURC/Cavin-4 served as a caveolar platform, thereby allowing activated α1-ARs to elicit MEK1/2 activation efficiently.

Discussion

Similar to caveolins, cavin are caveolar components that modulate caveolar morphology (29). Among the cavin, PTRF/Cavin-1 is required for caveola formation in various cell types including epithelium, smooth muscle, and skeletal muscle (16, 30). A recent study showed that SDPR/Cavin-2 deletion caused a pronounced reduction in caveola abundance in lung endothelium, but not in heart endothelium and that SRBC/Cavin-3 deletion did not affect caveolar formation in these cells (31). We demonstrated in the present study that MURC/Cavin-4 modified the morphology of formed caveolae in cardiomyocytes, whereas MURC/Cavin-4 was not essential to caveolar formation. Thus, all cavin proteins do not necessarily have the ability to form caveolae. Cav-3 KO mice showed a loss of caveolae in cardiomyocytes, leading to ERK activation and cardiac hypertrophy (19). We showed that Cav-3 knockdown reduced MURC/Cavin-4 localization at the plasma membrane, whereas MURC/Cavin-4 deficiency did not impair the membrane localization of Cav-3. Furthermore, unlike Cav-3 KO mice, MURC/Cavin-4-deficient mice exhibit normal caveolar morphology and function under physiological conditions. MURC/Cavin-4 deficiency attenuated ERK activation and cardiac hypertrophy induced by α1-AR stimulation. These results indicate that MURC/Cavin-4 regulates ERK activity and cardiac hypertrophy independently of caveolar morphology.

MURC/Cavin-4 bound to ERK1/2 in cardiomyocytes, and MURC/Cavin-4 and phosphorylated ERK were cotranslocated from the plasma membrane to the perinuclear region in response to PE. MURC/Cavin-4 knockdown attenuated PE-induced phosphorylated ERK accumulation at the plasma membrane. These findings suggest that MURC/Cavin-4 interacts with ERK and promotes ERK recruitment to caveola and subsequent internalization by the caveolae. We also showed that MURC/Cavin-4 increased the stability of phosphorylated and total ERK proteins in cardiomyocytes, which suggests that MURC/Cavin-4 stabilized ERK proteins to prevent their inactivation and/or degradation, thereby sustaining ERK activation. Although scaffold proteins involved in ERK signaling, such as kinase suppressor of Ras (KSR), MAPK kinase (MEK) partner 1, MAPK organizer 1, and β-arrestin, have been identified (32), the relationship between these scaffolds and the caveolae has not been documented. Our results provide the unique documentation that MURC/Cavin-4 is a scaffold protein for ERK in caveolae and contributes to the stabilization of ERK activity in cardiomyocytes. Thus, MURC/Cavin-4 spatiotemporally regulates α1-AR-induced ERK activation in cardiomyocytes.

Several studies have demonstrated that α1-ARs were localized to the plasma membrane (19, 33, 34), whereas Wright et al. (35) showed that α1-ARs were located around or within the nucleus, but not on the plasma membrane, in adult mouse cardiomyocytes. Thus, controversy exists regarding the localization of α1-ARs in cardiomyocytes. Because of a lack of proper antibodies for α1-AR subtypes (21), we expressed fluorescent protein-tagged α1-ARs in cardiomyocytes and showed that α1A- and α1B-ARs were colocalized with MURC/Cavin-4 and Cav-3 at the plasma membrane and partly within the cytoplasm in cardiomyocytes. It has been revealed that many GPCRs form oligomeric complexes, as either homo- or heterooligomers, and that GPCR oligomerization has effects on ligand binding, receptor activation, desensitization, and trafficking, as well as receptor signaling (21). We showed that MURC/Cavin-4 enhanced MEK1/2 activation in response to PE stimulation in cardiomyocytes. Because MURC/Cavin-4 interacts with α1A- and α1B-AR, our observations raise the possibility that MURC/Cavin-4 might contribute to an increase in the accessibility between each of the α1-ARs, which leads to α1-AR oligomerization and promotes α1-AR signaling. A previous
study reported that MEKI was distributed in caveolae (25). On the basis of this evidence, we predicted that MURC/Cavin-4 interacted with MEK1/2 as well as ERK in the caveole, although commu

noprecipitation analysis did not demonstrate an association be-
tween MURC/Cavin-4 and MEK1/2. Considering that MURC/

Cavin-4 is colocized with α-ARs, MURC/Cavin-4 may also
contribute to the functional compartmentation of α-AR signaling
of the caveole, which facilitates the responsiveness of MEK1/2
activation in response to α-AR stimulation, although MURC/

Cavin-4 does not interact with MEK1/2 directly.

In cultured cardiomyocytes, ERK depletion using an antisense
oligonucleotide was shown to down-regulate PE-induced hyper-
trophic responses (36). In the present study, ERK1/2 knockdown
oligonucleotide was shown to down-regulate PE-induced hyper-
trophy. Thus, ERK has a crucial role in

cardiac hypertrophy with ERK1/2 activation in transgenic mice

(37), activated MEK1 induced concentric cardiac hypertrophy


The other materials and methods used for this study are described in SI Materials and Methods.

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ment of Medical Equipment; and the Takeda Science Foundation and the Mitsubishi Pharma Research Foundation.

Materials and Methods

Pearson product-moment correlation coefficient was used to measure the linear correlation between variables. All experiments were performed at least three times and are expressed as the mean ± SEM and were analyzed by one-way ANOVA with post hoc analysis. A P value of <0.05 was considered significant.


12. Rodriguez G, et al. (2011) Molecular genetic and functional characterization implicate MURC/Cavin-4 and PE-induced cardiac hypertrophy is unknown (37), activated MEK1 induced concentric cardiac hypertrophy, which provides unique insight into the molecular mechanisms underlying caveola-
mediated signaling in cardiac hypertrophy.

ERK activation. The discrepancy between these studies may be
explained by a difference in the levels of suppressed ERK ac-
tivity. Eccentric hypertrophy and cardiomyocyte elongation were
observed in ERK1/2-deficient mice, which have complete loss of
ERK activity (38). On the other hand, MURC/Cavin-4 deficiency
maintained baseline ERK activity in the cardiomyocytes compared
with that in control mice under physiological conditions. These
observations suggested that concentric hypertrophy was modu-
lated by ERK activation, which is induced by various stimuli, and that eccentric hypertrophy was modulated by a reduction in basal
ERK activity.

As shown in the present study, although MURC/Cavin-4 is dispensable for caveolar formation in cardiomyocytes, MURC/

Cavin-4 serves as an ERK-recruiting protein in the caveole.

The recruiting function of MURC/Cavin-4 is necessary to elicit efficient signaling of the α-AR/ERK cascade in the caveole in concentric cardiac hypertrophy, which provides unique insight into the molecular mechanisms underlying caveola-
mediated signaling in cardiac hypertrophy.
Supporting Information

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SI Materials and Methods

Transmission Electron Microscopy and Quantitation. Twelve- and 20-wk-old mouse hearts and adult rat cardiomyocytes were fixed with 2% (vol/vol) glutaraldehyde (GA) in 0.1 M cacodylate buffer, postfixed with 2% OsO₄, and stained with uranyl acetate and lead citrate. For preembedding immunogold electron microscopy, tissue sections were washed with PBS and fixed with 4% (wt/vol) paraformaldehyde (PFA)/PBS for 2 h at 4 °C. The sections were then permeabilized in PBS containing 0.5% saponin for 1 h at 4 °C. After blocking, the sections were incubated in 5% (wt/vol) BSA, 5% (wt/vol) normal goat serum, and 0.1% cold fish gelatin in 0.1 M PBS for 30 min at 4 °C and then incubated in rabbit anti-muscle-restricted coiled-coil protein (MURC)/Cavin-4 antibody (1:100 dilution) for 18 h at 4 °C, washed in incubation buffer and incubated with goat anti-rabbit IgG conjugated to 10-nm gold particles (1:100 dilution) for 2 h at room temperature. Tissue sections were washed and finally fixed with 2% GA in 0.1 M cacodylate buffer and postfixed with 2% OsO₄. Microtome sections were examined using an H-7100 (Hitachi) or a JEM-1200EX (JEOL, Ltd.) transmission electron microscope and photographed at a magnification of 40,000× or 20,000×. Caveolae were identified by their characteristic flask shapes and locations at or near the plasma membrane. When the caveolar perimeter or area was measured, two independent fields from each of at least two independent experiments were quantitated for each condition. Similar results were obtained in double-blind experiments.

Neonatal and Adult Cardiomyocyte Culture. Rat and mouse neonatal cardiomyocytes, cultured from 1-d-old Wistar rats and C57BL/6 mice, were prepared as described previously with slight modifications (1–3). Isolated neonatal cardiomyocytes were cultured in serum-containing medium for 24 h and then cultured in serum-free medium. For electron microscopic analysis, adult rat cardiomyocytes were isolated from 8-wk-old male Wistar rats as described previously with minor modifications (4). Adult myocytes were plated on laminin-coated plates and cultured in DMEM–Ham’s F-12 nutrient mixture with 5% (vol/vol) newborn calf serum for 1 h. The media were changed to serum-free media (1% BSA, insulin-transferrin-selenium-ethanolamine) to remove nonmyocytes and the cardiomyocytes were incubated at 37 °C in an atmosphere of 5% CO₂.

Plasmid Construction. The corresponding cDNA fragments for human MURC/Cavin-4, α₁A- and α₁B-adrenergic receptor (AR) (ADRA1A and ADRA1B), were cloned by PCR from a human heart cDNA template as previously described (1). Complementary DNA (cDNA) encoding human MURC/Cavin-4 with a C-terminal anti-DYKDDDDK (FLAG) or HA epitope and human ADRA1A with a C-terminal HA epitope were cloned into pcDNA3.1 (Invitrogen) to generate pcDNA3.1-MURC-FLAG or -HA and pcDNA3.1-ADRA1A-HA, respectively. The cDNA encoding human ADRA1A was cloned into pEGFP-N3 (Clontech), whereas pmCherry-ADRA1A and -ADRA1B were generated by replacing the EGFP gene with the mCherry gene cloned by PCR from pmCherry-C1 (Clontech) as a template. Myc-DDK-tagged human ADRA1B (pCMV6-Myc-DDK-ADRA1B) was purchased from OriGene Technologies, Inc. HA-tagged human polymerase I and transcript release factor (PTRF), pCruz-HA-A-PTRF, T7-tagged human Caveolin (Cav)-3, and pcDNA3.1-Cav-3 were previously described (5). To construct mammalian expression vectors for the bimolecular fluorescence complementation (BiFC) assay, the cDNA fragments of human MURC/Cavin-4, Cav-3, PTRF/Cavin-1, SDPR/Cavin-3, ADRA1A, and ADRA1B were fused to the N- or C-terminals of the divided monomeric Kusabira Green (mKG) fragments of phmKGN-MC, phmKGC-MC, phmKGN-MN, and phmKGC-MN (MBL International) to generate phmKGN-MC-MURC, phmKGC-MC-MURC, phmKGC-MN-MURC, phmKGC-MC-Cav-3, phmKGN-MC-PTRF, phmKGC-MC-PTRF, phmKGN-MN-PTRF, phmKGN-MC-SDPR, phmKGN-MN-SDPR, and phmKGN-MN-ADRA1A, and phmKGN-MN-ADRA1B.

Replication-Defective Recombinant Adenoviruses and Gene Transfer. Recombinant adenoviruses expressing FLAG-tagged mouse MURC/Cavin-4 (Ad-MURC), Ad-β-galactosidase (Ad-LacZ), rMURC/Cavin-4 short hairpin RNA (shRNA) (Ad-rMURC shRNA), and luciferase shRNA (Ad-Luc shRNA) were described previously (1). Twenty-four hours after seeding, the cardiomyocytes were infected with Ad-MURC, Ad-LacZ, Ad-rMURC shRNA, or Ad-Luc shRNA diluted in culture media at a multiplicity of infection of 10 or 20 and incubated at 37 °C for 1 h. The viral suspension was removed and the cardiomyocytes were cultured with serum-depleted culture media. Phenylephrine (PE) was added postinfection.

Generation of MURC−/− Mice and Cardiospecific MURC Transgenic Mice. Genomic MURC DNA was isolated from R1 ES cells and used to create a MURC-targeting construct containing floxP sites and the neomycin resistance gene. The construct was generated in the pBluescript II KS⁺ vector. The 5’ arm of homology consisted of the first floxP site located in the 5’ untranslated region of MURC exon 1. The 3’ arm of homology consisted of a 2.2-kb fragment fused with the FRT-Neo-FRT-floxP cassette located downstream of MURC exon 1. The targeting construct was verified by sequencing and linearized with the NotI endonuclease before electroporation into R1 ES cells. Genomic DNA was extracted from G418-resistant ES cell clones. ES cell DNA was digested with the NdeI and SpeI endonucleases and subjected to Southern blot analysis. For the 5’ probe, a 500-bp fragment was PCR-generated using genomic mouse DNA and specific MURC primers [forward (F), AGTCCGTGCAGCT-TAAATAGGAGT; reverse (R), CTCATTACATCTACTACA-GGACCTT]. For the 3’ probe, a 458-bp fragment was PCR-generated using specific MURC primers (F, CAAGTCAA-GAGGTTTTCTAAGGCATA; R, TCTAACTATAGCAAGGACCCTTC). The PCR products were subsequently radiolabeled with α[³²P] deoxycytidine triphosphate by random priming (In-vitrogen). DNA blots were hybridized with the radiolabeled probes and visualized by autoradiography. The wild-type (WT) allele was represented by a 21.1-kb band, whereas a 9.9- or a 12.9-kb band represented the correctly targeted alleles. Five of 300 G418-resistant ES clones that underwent homologous recombination were identified by Southern blot analysis. Two independent homologous recombinant ES clones were microinjected into blastocysts derived from C57BL/6 mice. Male chimeras were inbred with female C57BL/6 mice to generate germ line-transmitted heterozygous mice (MURC+/− mice). To induce MURC−/− mice, MURC+/− mice were bred with those expressing the CAG promoter-driven cre recombinase gene. Offspring were genotyped by PCR analysis using mouse tail DNA and primers (F, CGGA-TTGCCCATAGGAACCTCTTGCG; R, TTTAAAATGAGG-GTGACCAC). Transgenic mice expressing MURC in the heart (MURC-Tg) were described previously (1). All aspects of animal care and...
experimentation performed in this study were approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of Medicine.

**Immunoprecipitation.** CV-1 (simian) in origin, and carrying the SV40 genetic material (COS) cells were plated in 60-mm dishes and cotransfected with pcDNA3.1–MURC–FLAG and/or pCruz-HA–P-TFRF with pcDNA3.1–MURC–HA, and/or pcDNA3.1–Cav-3–T7 with pcDNA3.1–ADRA1A–HA, and/or pcDNA3.1–MURC–FLAG with pcDNA3.1–MURC–HA, and/or pcCMV6–Mye-DDK–ADRA1B. Cardiomyocytes were also plated in 60-mm dishes and infected with or without Ad-MURC. Cells were cultured for an additional 48 h and lysed with lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF), protease inhibitor mixture (Pierce Biotechnology), 1 mM Na3VO4, 1 mM NaF, and 60 mM octyl glucoside]. Monoclonal antibodies against FLAG M2 (Sigma-Aldrich), HA (Roche Diagnostics), T7 (Novagen Biologies, Inc.), ERK1/2 (Cell Signaling Technology, Inc.), or phospho-ERK1/2 (p-ERK1/2; Cell Signaling Technology, Inc.) were conjugated to magnessphere MS300/carboxyl beads (JSR Micro) according to the manufacturer's protocol. Cell lysates were incubated with antibody-conjugated beads for 3 h at 4°C. After the beads were extensively washed with the lysis buffer, the bound proteins were eluted by boiling the beads in SDS sample buffer and then subjected to SDS/PAGE followed by Western blot analysis.

**Protein–Protein Interaction Assay.** To examine the interaction of target proteins in living cardiomyocytes, we performed the BiFC assay using the CoralHue Flu-Chase kit (MBL International) as described previously (6, 7). In brief, cardiomyocytes were cotransfected with plasmid pairs expressing the target proteins fused to the N-terminal portion of the mKG fragment and a steric structure before dividing, which formed a steric structure before dividing, the chromophore emitted fluorescence.

**Histological Analysis.** Cardiac perfusion-fixation was performed using 4% PFA, the hearts were sectioned and stained with hematoxylin/eosin (H&E).

**RT-Quantitative PCR.** Total RNA was extracted from cells or tissues using the RNeasy Mini Kit (Qiagen) or Trizol reagent (Invitrogen). cDNA synthesis and kinetic real-time PCR were performed as described previously (1, 8). The primer sequences were as follows: mouse BNP (F, GGAAATGGCTCAGACGC; R, CGATCCGCTTATCTTCGCT); rat BNP (F, GGAAATGGCTCAGACGC; R, CGATCCGCTTATCTTCGCT); and rat GAPDH (F, ATGGGAAGCTGTCATACAA; R, GTGATTCCACCCATCCAA).

**Measurement of Cardiomyocyte Size.** Cardiomyocytes were infected with Ad-LacZ or Ad-MURC, and 24 h later the cells were treated with or without U0126 [a highly selective inhibitor of both mitogen-activated protein kinase (MEK) 1 and MEK2] for 48 h. After fixation with 4% PFA, the cells were stained with FITC (Sigma-Aldrich) and DAPI (Invitrogen). Cells were imaged using an immunofluorescence microscope and measured using Image J software (National Institutes of Health, Bethesda). At least 50 cells were measured per sample.

**Western Blot Analysis.** Cell lysates and tissue samples were extracted using a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM EDTA, 1% Triton X-100, 1 mM PMSF, protease inhibitor mixture, 1 mM Na3VO4, and 1 mM NaF. Protein samples were subsequently incubated with primary polyclonal antibody against MURC and monoclonal antibodies against FLAG M2, HA, T7, p-ERK1/2, ERK1/2, phospho-MEK (p-MEK; Cell Signaling Technology, Inc.), MEK (Cell Signaling Technology, Inc.), Na-K ATPase α1 subunit (Santa Cruz Biotechnology, Inc.), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Chemicon International, Inc.). Horseradish peroxidase-conjugated anti-rabbit, -rat, and -mouse IgG (GE Healthcare) were used as secondary antibodies.

**Preparation of Cytosol or Membrane Fraction.** Cell fractionation was performed as previously described (7). In brief, cells were harvested from the 100-mm culture dishes and resuspended in ice-cold homogenization buffer containing 250 mM sucrose, 20 mM phosphate buffer (pH 6.8), and a protease/phosphatase inhibitor mixture (Thermo Fisher Scientific Inc.). Cells were homogenized gently on ice using a Dounce tissue grinder (Sigma-Aldrich) and centrifuged at 1,000 × g for 10 min to remove nuclei and remaining cells. The supernatants were ultracentrifuged at 55,000 rpm for 30 min using an Optima TLX centrifuge (Beckman Coulter, Inc.) with a TLS-100.3 rotor. Supernatants were obtained as the cytosol fraction. Pellets were lysed with the lysis buffer and centrifuged, and the supernatants were obtained as the membrane fraction.

**Protein Stability Assay.** We assessed p-ERK1/2 or ERK1/2 protein stability using cycloheximide (CHX), an inhibitor of protein synthesis, as described previously (11). Cells were exposed to 50 μM CHX for 36 h and protein expression was analyzed by Western blot analysis.

**Immunofluorescence Microscopy.** Specimens were fixed in 4% PFA/PBS and stained with rabbit polyclonal MURC/Cavin-4 antibody, mouse monoclonal anti-Cav-3 antibody (BD Biosciences), mouse monoclonal anti-FLAG M2 antibody, mouse monoclonal anti-p-ERK1/2 antibody, or rabbit polyclonal anti-MURC/Cavin-4 antibody. Secondary antibodies were conjugated with Alexa Fluor 488, 555, or 594, and nuclei were visualized using DAPI.

**Gene Silencing Through RNA Interference.** Rat ERK1- and ERK2-specific and control siRNA duplex oligonucleotides [ Stealth RNAi interface (RNAi)] were purchased from Invitrogen. siRNAs were transiently transfected into cardiomyocytes using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. The medium was changed 4 h posttransfection and cardiomyocytes were harvested or fixed for 72 h after transfection. The siRNA sequences were as follows: ERK1 siRNA-1 [sense (S), 5'-CGACUGAGAUAAGACACCAUGUGCU; antisense (AS), 5'-AGCAGAUGUGGUGUCAUCCGAC]; ERK1
Cav-3 (phmKGN-MN-SDPR and phmKGC-MN-SDPR); MURC
Cav-3 (phmKGN-MC-SDPR and phmKGC-MC-PTRF). Forty-eight hours after transfection, the cardiomyocytes were
fused to divided mKG fragments as follows: MURC
and Cav-3 (phmKGN-MC-MURC and phmKGC-MC-Cav-3); PTRF
SDPR (phmKGN-MC-SDPR and phmKGC-MC-PTRF). MURC

Cav-3 (phmKGN-MC and phmKGC-MC) or NC-2 (phmKGN-MN and phmKGC-MN). NC-1 and -2 were used as negative controls for the BiFC


**Fig. S1.** MURC/Cavin-4 forms caveolin-cavin complexes in cardiomyocytes. (A and B) Immunoprecipitation of MURC and Cav-3 or PTRF/Cavin-1. COS cells were cotransfected with MURC/Cavin-4-HA and Cav-3-T7 (A) or MURC/Cavin-4-FLAG and PTRF/Cavin-1-HA (B). Cell lysates were subjected to immunoprecipitation. Supernatants were collected after immunoprecipitation and immunoblotting was performed. The same volume of supernatant was applied in each lane. (C) Representative images of cardiomyocytes (Left) and lists of plasmid pairs in the BiFC assay (Right). Cardiomyocytes were transfected with either of the following pair of empty plasmids: NC-1 (phmKGN-MC and phmKGC-MC) or NC-2 (phmKGN-MN and phmKGC-MN). NC-1 and -2 were used as negative controls for the BiFC assay. (D) Representative images of cardiomyocytes in the BiFC assay. Cardiomyocytes were cotransfected with pairs of plasmids expressing the indicated proteins fused to divided mKG fragments as follows: MURC + Cav-3 (phmKGN-MC-MURC and phmKGC-MC-Cav-3); PTRF + Cav-3 (phmKGN-MN-PTRF and phmKGC-MN-Cav-3); SDPR + Cav-3 (phmKGN-MN-SDPR and phmKGC-MN-Cav-3); MURC + PTRF (phmKGN-MC-PTRF and phmKGC-MC-MURC); MURC + SDPR (phmKGN-MC-MURC and phmKGC-MC-SDPR); and PTRF + SDPR (phmKGN-MC-SDPR and phmKGC-MC-PTRF). Forty-eight hours after transfection, the cardiomyocytes were observed by fluorescence microscopy. (Scale bars: 20 μm) Cav-3, caveolin-3; IB, immunoblots; IP, immunoprecipitation; NC, negative control; Sup, supernatant.
**Fig. S2.** Cav-3 knockdown induces translocation of MURC/Cavin-4 but not α1-ARs from the plasma membrane to the cytosol in cardiomyocytes. (A) Representative fluorescence images of cardiomyocytes. Cells were transfected with control siRNA or Cav-3 siRNAs. Twenty-four hours after transfection, we performed additional transfection with ADRA1A- or ADRA1B-pmCherry and then, 48 h later, immunostaining was performed with an anti-MURC/Cavin-4 antibody. α1-ARs were observed at the plasma membrane (white arrowheads). Nuclei were stained with DAPI (blue). (Scale bars: 20 μm.) (B) Western blots of Cav-3 in Cav-3 siRNA-transfected cardiomyocytes. (C) Representative Western blots (Upper) and bar graphs of MURC protein levels in cytosol and membrane fractions (Lower). Cav-3, caveolin-3.
Fig. S3. Generation of MURC/Cavin-4–deficient mice and α1-AR–induced MAP kinase activation in the heart. (A) Targeting vector and restriction map of the MURC/Cavin-4 locus (Left) and Southern analysis of wild-type and targeted alleles. Fragments specific to wild-type (21.1 kb) and targeted alleles (9.9 or 12.9 kb) are shown (Right). Boxes represent exons. ES cell DNA was digested with NdeI and SpeI. (B) Western blot analysis of MURC/Cavin-4 expression in heart tissues. (C) MURC−/− mice undergo normal development without any defects. (D) Representative electron microscopic images of caveolae in wild-type and MURC−/− heart tissues from 20-wk-old mice. (E) Representative fluorescence images of mouse heart tissues. Tissue sections were immunostained with anti-MURC/Cavin-4 and anti–Cav-3 antibodies. (Scale bars: 500 nm in D and 20 μm in E.) (F) Western blots of PE-induced ERK activation in mouse neonatal cardiomyocytes. Cardiomyocytes were isolated from 1-d-old WT or MURC−/− mice. **P < 0.01 compared with PBS-treated cells isolated from WT mice. †P < 0.05 compared with PE-treated cells isolated from WT mice. (G) Measurement of p38 and JNK activation in the heart. Wild-type and MURC−/− mice were infused with PE for 2 d with osmotic minipumps. *P < 0.05 compared with vehicle-treated WT mice. WT, wild-type. Data are presented as mean ± SEM.
Fig. S4. MURC/Cavin-4 modulates α1-induced hypertrophic responses and stabilizes ERK1/2 protein in cardiomyocytes. MURC/Cavin-4 mRNA expression (A), quantification of cell surface area (B), and BNP mRNA expression (C). Cardiomyocytes were infected with Ad-Luc- or Ad-rMURC-shRNA at a multiplicity of infection (MOI) of 20. After 72 h of incubation, cells were treated with PE for another 24 h. **P < 0.01 compared with Ad-Luc-shRNA; ††P < 0.01 compared with PE-treated Ad-Luc-shRNA. (D) Representative Western blots (Upper) and quantification of p-ERK1/2, ERK1/2 and GAPDH protein levels (Lower) in cardiomyocytes with CHX (50 μM). Cardiomyocytes were infected with Ad-LacZ or Ad-MURC at an MOI of 10. After 48 h of incubation, the cells were treated with CHX for 36 h. **P < 0.01 or *P < 0.05 compared with time-matched Ad-LacZ. Data are presented as mean ± SEM.

Fig. S5. ERK1/2 siRNA transfection suppresses ERK1/2 expression. Representative Western blots of ERK. Cardiomyocytes were infected with Ad-LacZ at an MOI of 10 and simultaneously transfected with ERK1/2 siRNAs. Cells were harvested 72 h after incubation. ERK1/2 siRNAs1 indicates ERK1 siRNA-1 and ERK2 siRNA-1 cotransfection, and ERK1/2 siRNAs2 indicates ERK1 siRNA-2 and ERK2 siRNA-2 cotransfection.
Fig. 56. MURC/Cavin-4 regulates ERK1/2 activation in MEK1/2-dependent and -independent manners. Representative Western blots of MEK1/2, ERK1/2 and FLAG (Left) and quantification of MEK1/2 or ERK1/2 phosphorylation (Center or Right). Cardiomyocytes were infected with Ad-LacZ or Ad-MURC at an MOI of 10. After 36 h of incubation, cells were treated with PE for 30 min. Data are presented as mean ± SEM. **P < 0.01 compared with untreated Ad-LacZ; ††P < 0.01 compared with PE-treated Ad-LacZ.

Fig. 57. Uncropped illustrations used to prepare Figs. 2B; 3C; 4 A, C, and E; and 5A.
### Fig. S1A

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**IB:** anti-HA

### Fig. S1B

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**IB:** anti-HA

### Fig. S2B

**WB:** Cav3

**WB:** GAPDH

**WB:** MURC

### Fig. S2C

**WB:** GAPDH

**WB:** Na-K ATPase α1

### Fig. S3A

5' probe

3' probe

### Fig. S3B

**WB:** MURC

**WB:** pERK1/2

**WB:** ERK1/2

### Fig. S3E

**WB:** pERK1/2

**WB:** ERK1/2

### Fig. S3F

**WB:** p-p38

**WB:** p38

**WB:** p-JNK

**WB:** JNK

Fig. S8. (Continued)
**Table S1.** Morphometric analysis of WT and MURC<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Data measure</th>
<th>Sham WT</th>
<th>MURC&lt;sup&gt;−/−&lt;/sup&gt; WT</th>
<th>PE, 1 wk WT</th>
<th>MURC&lt;sup&gt;−/−&lt;/sup&gt; WT</th>
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</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>24.8 ± 0.4</td>
<td>25.5 ± 1.7</td>
<td>23.0 ± 1.1</td>
<td>22.1 ± 1.5</td>
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<tr>
<td>VW, mg</td>
<td>96.6 ± 2.2</td>
<td>93.2 ± 4.0</td>
<td>111.4 ± 9.8</td>
<td>99.2 ± 9.5&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>TL, mm</td>
<td>16.8 ± 0.3</td>
<td>16.6 ± 0.2</td>
<td>16.7 ± 0.3</td>
<td>16.6 ± 0.4</td>
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<tr>
<td>VW/BW, mg/g</td>
<td>3.90 ± 0.06</td>
<td>3.66 ± 0.11</td>
<td>4.84 ± 0.37&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>4.48 ± 0.17&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>VW/TL, mg/mm</td>
<td>5.75 ± 0.11</td>
<td>5.63 ± 0.21</td>
<td>6.69 ± 0.62</td>
<td>5.96 ± 0.57&lt;sup&gt;‡&lt;/sup&gt;</td>
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</tbody>
</table>

BW, body weight; TL, tibial length; VW, ventricular weight. Values are expressed as mean ± SEM.

<sup>**</sup>P < 0.01 compared with WT-sham mice.

<sup>†</sup>P < 0.05 compared with WT-PE mice.

**Table S2.** Echocardiographic analysis of WT and MURC<sup>−/−</sup> mice

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<tr>
<th>Data measure</th>
<th>Sham WT</th>
<th>MURC&lt;sup&gt;−/−&lt;/sup&gt; WT</th>
<th>PE, 1 wk WT</th>
<th>MURC&lt;sup&gt;−/−&lt;/sup&gt; WT</th>
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<tbody>
<tr>
<td>LVDd, mm</td>
<td>4.11 ± 0.23</td>
<td>3.76 ± 0.29</td>
<td>3.58 ± 0.30&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>4.01 ± 0.20&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>LVDs, mm</td>
<td>2.99 ± 0.17</td>
<td>2.69 ± 0.24</td>
<td>2.54 ± 0.26</td>
<td>2.86 ± 0.31</td>
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<tr>
<td>IVSTd, mm</td>
<td>0.57 ± 0.09</td>
<td>0.54 ± 0.07</td>
<td>0.84 ± 0.12&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.54 ± 0.09&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>PWtd, mm</td>
<td>0.60 ± 0.07</td>
<td>0.52 ± 0.04</td>
<td>0.86 ± 0.11&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.57 ± 0.13&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>FS, %</td>
<td>27.3 ± 1.3</td>
<td>28.5 ± 1.8</td>
<td>29.0 ± 4.3</td>
<td>28.7 ± 6.1</td>
</tr>
<tr>
<td>EF, %</td>
<td>53.6 ± 1.9</td>
<td>55.9 ± 3.2</td>
<td>56.4 ± 6.5</td>
<td>55.7 ± 7.6</td>
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EF, ejection fraction; FS, fractional shortening; IVSTd, interventricular septum thickness at end-diastole; LVDd, left ventricular dimension at end-diastole; LVDs, left ventricular dimension in systole; PWtd, left ventricular posterior wall thickness at end-diastole. Values are expressed as mean ± SEM.

<sup>‡</sup>P < 0.05 and <sup>†</sup>P < 0.01 compared with WT-sham mice.

<sup>†</sup>P < 0.05 and <sup>‡</sup>P < 0.01 compared with WT-PE mice.