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 caveolin and cavin 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 cavins (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 smooth, skeletal, and cardiac myocytes. Caveolin deficiency leads to caveolar loss, which is accompanied by alterations in signaling responses (8, 9). Cavins are also structural components of caveolae and assume four isoforms, namely, polymerase I and transcript release factor (PTRF)/Cavin-1, serum deprivation protein response (SDPR)/Cavin-2, SDR-related gene product that binds to C kinase (SRBC)/Cavin-3, and muscle-related coiled-coil protein (MURC) (6), which is also known as "Cavin-4" because of its sequence homology with other cavins and localization to the caveolae (10). Previously, we reported the association of MURC/Cavin-4 with SDPR/Cavin-2 and identified MURC/Cavin-4 mutations in dilated cardiomyopathy patients (11, 12). PTRF/Cavin-1 and SDPR/Cavin-2 are expressed in various cell types, including myocytes (10, 11), and SRBC/Cavin-3 is expressed in many 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 cavins and caveolins form a complex, called the "caveolin–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 caveola 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.

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(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 *A* and *B*). MURC-HA and Cav-3-T7 expressions were not reduced in supernatants immunoprecipitated by 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. 1*A*). 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. 1*B*). 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. 1*C*). 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. 2*A*). 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*A*).

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

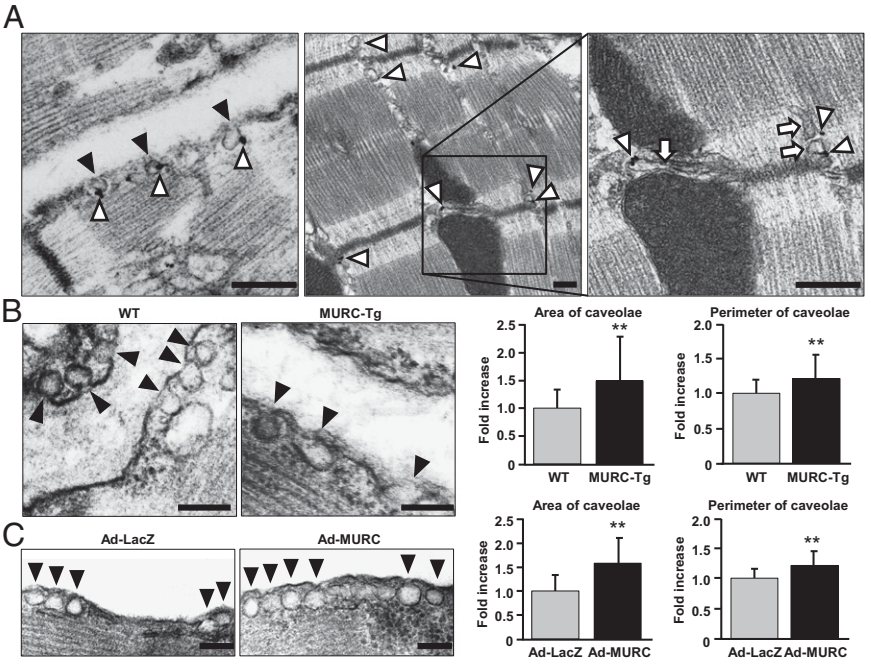

Fig. 1. MURC/Cavin-4 exists in the caveolae and T tubules and modulates caveolar morphology in cardiomyocytes. Representative electron microscopic images of mouse heart tissue and adult cultured cardiomyocytes. (*A*) Immunogold staining of MURC/Cavin-4 (white arrowheads) was observed at caveolae (black arrowheads) and T tubules (white arrows) of cardiomyocytes. (*B* and *C*) Representative shapes of caveolae in mouse heart tissue (*B*, *Left*) and rat cultured cardiomyocytes with or without MURC/Cavin-4 overexpression (*C*, *Left*). Caveolae were identified by their characteristic flask shapes and locations at or near the plasma membrane. Relative caveolar areas and perimeters were measured in cardiomyocytes from heart tissue (*B*, *Right*) and cultured cardiomyocytes (*C*, *Right*). Data are presented as mean ± SEM. $^{**}P < 0.01$ compared with controls. (Scale bars: 500 nm in *A*; and 200 nm in *B* and *C*.)

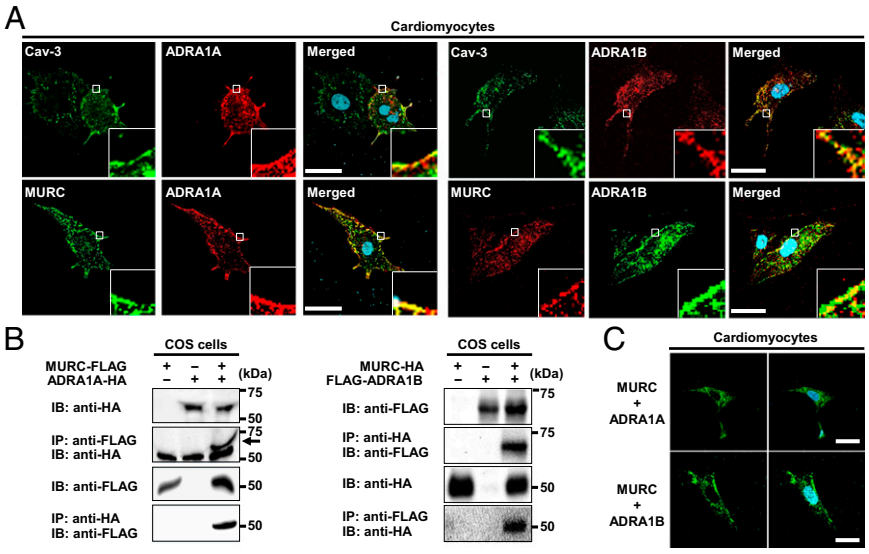

Fig. 2. MURC/Cavin-4 is associated with α1-ARs at caveolae in cardiomyocytes. (*A*) Representative fluorescence images of rat neonatal cardiomyocytes. Cardiomyocytes were cotransfected with ADRA1A- or ADRA1B-pmCherry. MURC/Cavin-4 and Cav-3 were costained with ADRA1A and ADRA1B (white boxes). (*B*) COS cells were transfected with MURC/Cavin-4-FLAG and/or ADRA1A-HA (*Left*), or with MURC/Cavin-4-HA and/or FLAG-ADRA1B (*Right*). The black arrow indicates ADRA1A-HA. (*C*) In situ association of MURC/Cavin-4 with α1-ARs in living cardiomyocytes was confirmed using the BiFC assay. Cardiomyocytes were cotransfected with phmKGC-MN-MURC and phmKGN-MN-ADRA1A or phmKGN-MN-ADRA1B. Nuclei were stained with DAPI (blue). (Scale bars: 20 μm in *A* and *C*.) IB, immunoblots; IP, immunoprecipitation. mKGC, monomeric Kusabira Green.

MURC/Cavin-4–null ($MURC^{-/-}$) mice (Fig. S3 *A* and *B*) to α1-AR–induced cardiac hypertrophy. $MURC^{-/-}$ mice are fertile, exhibit normal growth under normal conditions (Fig. S3*C*), and are indistinguishable by cardiac mass and function from WT mice under physiological conditions (Tables S1 and S2). Furthermore, caveolae and Cav-3 localization at the plasma membrane are retained in cardiomyocytes of $MURC^{-/-}$ mice (Fig. S3 *D* and *E*), indicating that MURC/Cavin-4 is not essential to the membrane localization of Cav-3 and caveola formation in cardiomyocytes. Phenylephrine (PE) infusion for 7 d caused marked concentric cardiac hypertrophy in WT mice, whereas MURC/Cavin-4 depletion suppressed cardiac hypertrophy, as evaluated using a cross-sectional area (Fig. 3*A*). Morphological and echocardiographic analyses also showed attenuated cardiac hypertrophy in $MURC^{-/-}$ mice (Tables S1 and S2). In the hearts of $MURC^{-/-}$ mice, BNP and βMHC mRNA expression levels were reduced in response to PE, compared with those of WT mice (Fig. 3*B*). A previous study demonstrated that, in ADRA1A and ADRA1B double-knockout (KO) (α1AB-KO) mice, ERK activation was reduced and PE could not activate ERK in cardiomyocytes, resulting in a reduced heart size in α1AB-KO mice compared with that in male WT mice (23). Therefore, we investigated ERK activation in WT and $MURC^{-/-}$ hearts. After PE infusion for 2 d, ERK activation was significantly suppressed in the hearts of $MURC^{-/-}$ mice compared with WT mice (Fig. 3*C*). In neonatal mouse cardiomyocytes isolated from WT and $MURC^{-/-}$ mice, PE-induced ERK activation was also significantly suppressed in $MURC^{-/-}$ cardiomyocytes compared with the level in WT cardiomyocytes (Fig. S3*F*). Mitogen-activated protein kinases (MAPKs), including ERK1/2, modulate cardiac hypertrophy through G proteins (24). Therefore, we assessed p38 and JNK activities in the hearts of WT and $MURC^{-/-}$ mice (Fig. S3*G*). The basal activity of p38 did not differ between WT and $MURC^{-/-}$ hearts, whereas JNK activation was significantly increased in $MURC^{-/-}$ hearts compared with the level in WT hearts. However, both p38 and JNK activities were not enhanced by PE infusion in the hearts of WT and $MURC^{-/-}$ mice.

MURC/Cavin-4 Serves as an ERK-Recruiting Protein in the Caveolae and Modulates α1-AR–Induced Hypertrophic Responses in Cardiomyocytes. To clarify the molecular mechanisms responsible for MURC/Cavin-4–mediated cardiac hypertrophy, we knocked down MURC expression in neonatal rat cardiomyocytes. PE increased MURC/Cavin-4 mRNA expression in cardiomyocytes, whereas MURC/Cavin-4 RNA interference (RNAi) using Ad-rMURC short hairpin RNA (shRNA) attenuated PE-induced MURC/Cavin-4 mRNA expression (Fig. S4*A*). MURC/Cavin-4 RNAi reduced PE-induced increases in cell size and BNP mRNA expression (Fig. S4 *B* and *C*), which is consistent with the in vivo study described above. PE-induced ERK activation was also suppressed in neonatal rat cardiomyocytes infected with Ad-MURC-shRNA as well as mouse cardiomyocytes isolated from $MURC^{-/-}$ mice (Fig. 4*A*). In cardiomyocytes infected with Ad-Luc-shRNA, ERK activation was detected 5 min after PE stimulation, which peaked at 30 min and then declined, whereas in cardiomyocytes infected with Ad-MURC-shRNA, ERK activation peaked at 5 min and then declined, which was significantly suppressed compared with that in Ad-Luc-shRNA–infected cardiomyocytes, suggesting that MURC/Cavin-4 plays a determinant role in the modulation of signaling amplitude and the duration of ERK activation in response to PE stimulation.

A previous study using immunoelectron microscopy showed that ERK was concentrated in caveolae (25). ERK is activated at the plasma membrane by several GPCRs and subsequently translocates to the nucleus and cytoplasm (26). Therefore, we investigated the functional role of MURC/Cavin-4 in ERK localization. In unstimulated cardiomyocytes, total and phosphorylated ERK was distributed throughout the cytoplasm and partially at the plasma membrane, where it was colocalized with MURC (Fig. 4*B*). Upon PE stimulation, both MURC/Cavin-4 and phosphorylated ERK were translocated from the plasma membrane to the perinuclear region (Fig. 4 *B* and *C*). MURC/Cavin-4 knockdown reduced phosphorylated ERK accumulation at the plasma membrane (Fig. 4*D*). Immunoblot analysis showed that MURC/Cavin-4 coimmunoprecipitated with phosphorylated and total ERK (Fig. 4*E*).

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. S4*D*).

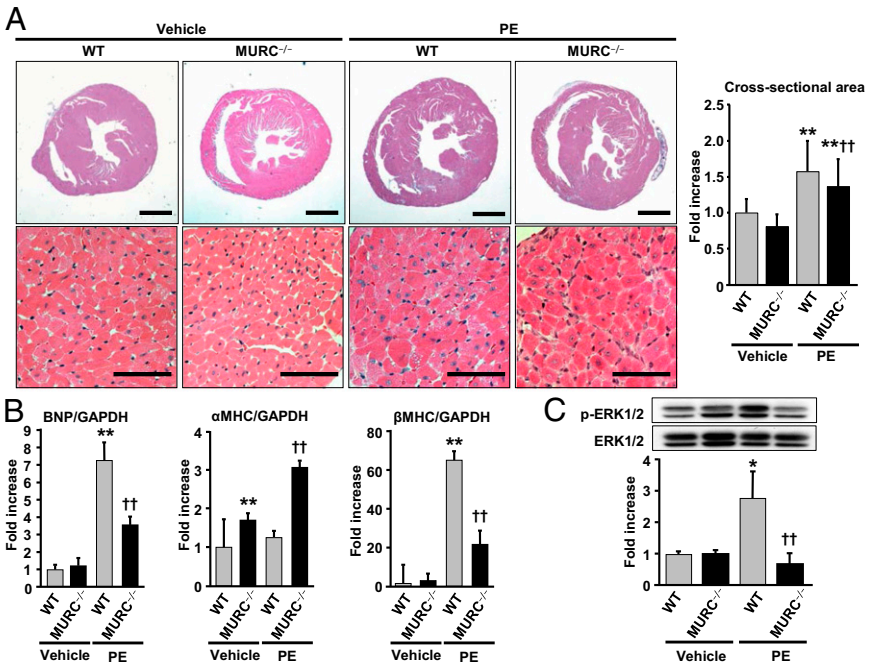

Fig. 3. MURC/Cavin-4 deletion attenuates α1-AR–induced ERK1/2 activation and cardiac hypertrophy. (*A*) Representative H&E staining of WT and *MURC*$^{-/-}$ heart paraffin-embedded sections (*A*, *Upper*, *Left*) and measurement of relative myocyte cross-sectional area (*A*, *Upper*, *Right*). WT and *MURC*$^{-/-}$ mice were infused with PE for 7 d with osmotic minipumps. Higher magnification images are shown (*Lower*). (Scale bars: 1 mm in *Upper*; and 50 μm in *Lower*.) A bar graph showing cross-sectional areas relative to vehicle-treated WT mice. (*B*) Measurements of BNP, αMHC, and βMHC mRNA levels in the heart. (*C*) Measurement of ERK1/2 activation in the heart. WT and *MURC*$^{-/-}$ mice were infused with PE for 2 d with osmotic minipumps. Data are presented as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with vehicle-treated WT mice; $^{\dagger\dagger}P < 0.01$ compared with PE-treated WT mice.

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. 5*A*) and that MURC/Cavin-4 overexpression promoted cardiomyocyte hypertrophy (Fig. 5*B*). 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 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

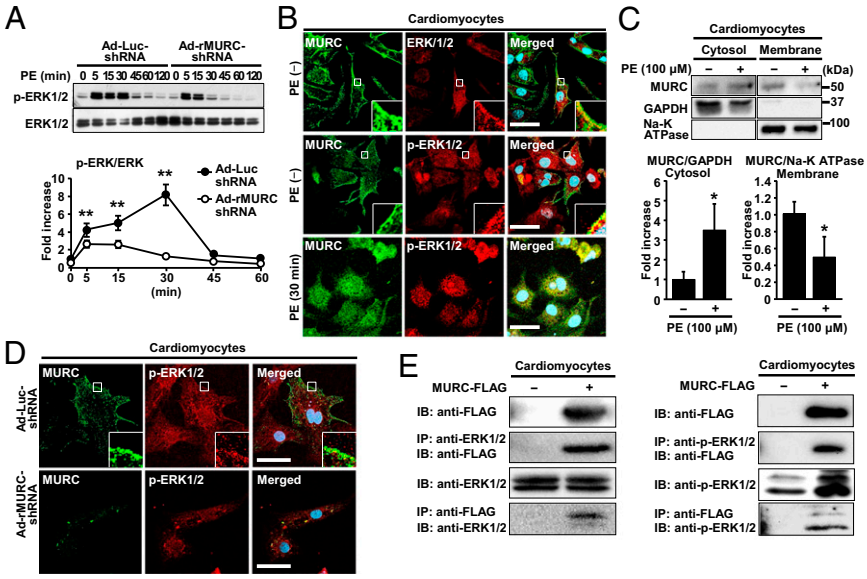

Fig. 4. MURC/Cavin-4 serves as an ERK1/2-recruiting protein in the caveolae and modulates α1-induced hypertrophic responses in cardiomyocytes. (*A*) Representative Western blots (*Upper*) and quantification of ERK phosphorylation (*Lower*) in cardiomyocytes with 100 μM PE. Data are presented as mean ± SEM. $^{**}P < 0.01$ compared with Ad-LucshRNA; $^{\dagger\dagger}P < 0.01$ compared with PE-treated Ad-Luc shRNA. (*B*) Representative immunostaining of ERK1/2 and MURC/Cavin-4 in cardiomyocytes. MURC/Cavin-4 was costained with ERK1/2 and p-ERK1/2 (white boxes). (*C*) Representative Western blots (*Upper*) and quantification of MURC protein levels in the cytosol and membrane fractions (*Lower*). Cells were treated with 100 μM PE for 30 min. (*D*) Representative immunostaining of p-ERK1/2 and MURC in MURC knocked-down cardiomyocytes. (Scale bars: 20 μm in *B* and *D*.) (*E*) Cell lysates were subjected to immunoprecipitation with anti-FLAG, anti-ERK1/2 (*Left*) and anti–p-ERK1/2 (*Right*) antibodies. Nuclei were stained with DAPI (blue).

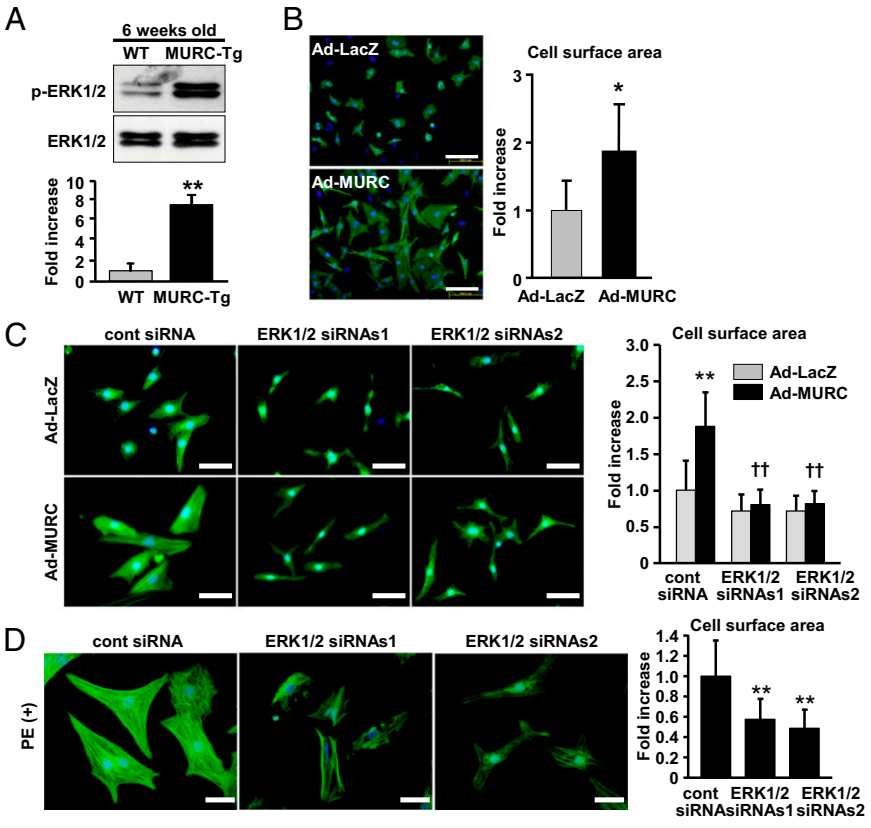

Fig. 5. MURC/Cavin-4 modulates α1-AR–induced cardiomyocyte hypertrophy through ERK1/2 activation. (*A*) Representative Western blots (*Upper*) and quantification of ERK1/2 phosphorylation (*Lower*) in 6-wk-old WT and MURC-Tg mice. $^{**}P < 0.01$ compared with WT mice. (*B*) Representative phalloidin-FITC–stained cardiomyocytes (*Left*) and quantification of cell surface area (*Right*). $^{*}P < 0.05$ compared with Ad-LacZ. (*C*) Representative fluorescence images of cardiomyocytes (*Left*) and quantification of cell surface area (*Right*). $^{**}P < 0.01$ compared with control siRNA-transfected Ad-LacZ; $^{\dagger\dagger}P < 0.01$ compared with control siRNA-transfected Ad-MURC. (*D*) Representative phalloidin-FITC–stained cardiomyocytes stimulated by PE. Cardiomyocytes were transfected with ERK1/2 siRNA and then stimulated by 100 μM PE for 48 h (*Left*), and cell surface area was quantified (*Right*). $^{**}P < 0.01$ compared with control siRNA. (Scale bars: 100 μm in *B*; and 50 μm in *C* and *D*.) All cells were stained by phalloidin-FITC. Nuclei were stained by DAPI (blue). 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. Data are presented as mean ± SEM.

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, cavins are caveolar components that modulate caveolar morphology (29). Among the cavins, 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 caveolae 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 MEK1 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 caveolae, although coimmunoprecipitation analysis did not demonstrate an association between MURC/Cavin-4 and MEK1/2. Considering that MURC/Cavin-4 is colocalized with α1-ARs, MURC/Cavin-4 may also contribute to the functional compartmentation of α1-AR signaling of the caveolae, which facilitates the responsiveness of MEK1/2 activation in response to α1-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 hypertrophic responses (36). In the present study, ERK1/2 knockdown inhibited MURC/Cavin-4– and PE-induced cardiomyocyte hypertrophy. Thus, ERK has a crucial role in α1-AR– and MURC/Cavin-4–induced cardiac hypertrophy. Furthermore, MURC/Cavin-4 knockdown inhibited PE-induced cardiomyocyte hypertrophy associated with ERK inactivation. These findings suggested that MURC/Cavin-4 mediated PE-induced ERK signaling in cardiomyocytes.

Although the role of ERK signaling in cardiomyocyte hypertrophy is unknown (37), activated MEK1 induced concentric cardiac hypertrophy with ERK1/2 activation in transgenic mice (28). ERK1/2-deficient mice showed increases in the heart:body weight ratio and reductions in cardiac contractility under physiological conditions, which resulted in eccentric hypertrophy and cardiomyocyte elongation (38). Furthermore, angiotensin II and PE stimulation worsened eccentric cardiac growth in ERK1/2-deficient mice. However, in our present study, 1 wk of PE infusion inhibited concentric hypertrophy in *MURC*$^{-/-}$ mice, but did not promote eccentric hypertrophy, despite the inhibition of ERK activation. The discrepancy between these studies may be explained by a difference in the levels of suppressed ERK activity. 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 modulated 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 caveolae within cardiomyocytes. The recruiting function of MURC/Cavin-4 is necessary to elicit efficient signaling of the α1-AR/ERK cascade in the caveolae in concentric cardiac hypertrophy, which provides unique insight into the molecular mechanisms underlying caveola-mediated signaling in cardiac hypertrophy.

Materials and Methods

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

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

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1. Shaul PW, Anderson RG (1998) Role of plasmalemmal caveolae in signal transduction. *Am J Physiol* 275(5 Pt 1):L843–L851.
2. Parton RG, Simons K (2007) The multiple faces of caveolae. *Nat Rev Mol Cell Biol* 8(3):185–194.
3. Razani B, Woodman SE, Lisanti MP (2002) Caveolae: From cell biology to animal physiology. *Pharmacol Rev* 54(3):431–467.
4. Cohen AW, Hnasko R, Schubert W, Lisanti MP (2004) Role of caveolae and caveolins in health and disease. *Physiol Rev* 84(4):1341–1379.
5. Harvey RD, Calaghan SC (2012) Caveolae create local signalling domains through their distinct protein content, lipid profile and morphology. *J Mol Cell Cardiol* 52(2):366–375.
6. Hansen CG, Nichols BJ (2010) Exploring the caves: Cavins, caveolins and caveolae. *Trends Cell Biol* 20(4):177–186.
7. Briand N, Dugail I, Le Lay S (2011) Cavin proteins: New players in the caveolae field. *Biochimie* 93(1):71–77.
8. Williams TM, Lisanti MP (2004) The Caveolin genes: From cell biology to medicine. *Ann Med* 36(8):584–595.
9. Gratton JP, Bernatchez P, Sessa WC (2004) Caveolae and caveolins in the cardiovascular system. *Circ Res* 94(11):1408–1417.
10. Bastiani M, et al. (2009) MURC/Cavin-4 and cavin family members form tissue-specific caveolar complexes. *J Cell Biol* 185(7):1259–1273.
11. Ogata T, et al. (2008) MURC, a muscle-restricted coiled-coil protein that modulates the Rho/ROCK pathway, induces cardiac dysfunction and conduction disturbance. *Mol Cell Biol* 28(10):3424–3436.
12. Rodriguez G, et al. (2011) Molecular genetic and functional characterization implicate muscle-restricted coiled-coil gene (MURC) as a causal gene for familial dilated cardiomyopathy. *Circ Cardiovasc Genet* 4(4):349–358.
13. McMahon KA, et al. (2009) SRBC/cavin-3 is a caveolin adapter protein that regulates caveolae function. *EMBO J* 28(8):1001–1015.
14. Hayashi YK, et al. (2009) Human PTRF mutations cause secondary deficiency of caveolins resulting in muscular dystrophy with generalized lipodystrophy. *J Clin Invest* 119(9):2623–2633.
15. Bastiani M, Parton RG (2010) Caveolae at a glance. *J Cell Sci* 123(Pt 22):3831–3836.
16. Hill MM, et al. (2008) PTRF-Cavin, a conserved cytoplasmic protein required for caveola formation and function. *Cell* 132(1):113–124.
17. Hansen CG, Bright NA, Howard G, Nichols BJ (2009) SDPR induces membrane curvature and functions in the formation of caveolae. *Nat Cell Biol* 11(7):807–814.
18. Allen JA, Halverson-Tamboli RA, Rasenick MM (2007) Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci* 8(2):128–140.
19. Woodman SE, et al. (2002) Caveolin-3 knock-out mice develop a progressive cardiomyopathy and show hyperactivation of the p42/44 MAPK cascade. *J Biol Chem* 277(41):38988–38997.
20. Jensen BC, Swigart PM, De Marco T, Hoopes C, Simpson PC (2009) α1-Adrenergic receptor subtypes in nonfailing and failing human myocardium. *Circ Heart Fail* 2(6):654–663.
21. Breitwieser GE (2004) G protein-coupled receptor oligomerization: Implications for G protein activation and cell signaling. *Circ Res* 94(1):17–27.
22. Fujita T, et al. (2001) Accumulation of molecules involved in alpha1-adrenergic signal within caveolae: Caveolin expression and the development of cardiac hypertrophy. *Cardiovasc Res* 51(4):709–716.
23. O'Connell TD, et al. (2003) The $\alpha_{(1A/C)}$- and $\alpha_{(1B)}$-adrenergic receptors are required for physiological cardiac hypertrophy in the double-knockout mouse. *J Clin Invest* 111(11):1783–1791.
24. Muslin AJ (2008) MAPK signalling in cardiovascular health and disease: Molecular mechanisms and therapeutic targets. *Clin Sci (Lond)* 115(7):203–218.
25. Liu P, Ying Y, Anderson RG (1997) Platelet-derived growth factor activates mitogen-activated protein kinase in isolated caveolae. *Proc Natl Acad Sci USA* 94(25):13666–13670.
26. Kholodenko BN, Hancock JF, Kolch W (2010) Signalling ballet in space and time. *Nat Rev Mol Cell Biol* 11(6):414–426.
27. Lu Z, Xu S, Joazeiro C, Cobb MH, Hunter T (2002) The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. *Mol Cell* 9(5):945–956.
28. Bueno OF, et al. (2000) The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. *EMBO J* 19(23):6341–6350.
29. Nabi IR (2009) Cavin fever: Regulating caveolae. *Nat Cell Biol* 11(7):789–791.
30. Liu L, et al. (2008) Deletion of Cavin/PTRF causes global loss of caveolae, dyslipidemia, and glucose intolerance. *Cell Metab* 8(4):310–317.
31. Hansen CG, Shvets E, Howard G, Riento K, Nichols BJ (2013) Deletion of cavin genes reveals tissue-specific mechanisms for morphogenesis of endothelial caveolae. *Nat Commun* 4:1831.
32. Dhanasekaran DN, Kashef K, Lee CM, Xu H, Reddy EP (2007) Scaffold proteins of MAP-kinase modules. *Oncogene* 26(22):3185–3202.
33. Petrashevskaya NN, Bodi I, Koch SE, Akhter SA, Schwartz A (2004) Effects of α1-adrenergic stimulation on normal and hypertrophied mouse hearts. Relation to caveolin-3 expression. *Cardiovasc Res* 63(3):561–572.
34. Koga A, et al. (2003) Adenovirus-mediated overexpression of caveolin-3 inhibits rat cardiomyocyte hypertrophy. *Hypertension* 42(2):213–219.
35. Wright CD, et al. (2008) Nuclear α1-adrenergic receptors signal activated ERK localization to caveolae in adult cardiac myocytes. *Circ Res* 103(9):992–1000.
36. Glennon PE, et al. (1996) Depletion of mitogen-activated protein kinase using an antisense oligodeoxynucleotide approach downregulates the phenylephrine-induced hypertrophic response in rat cardiac myocytes. *Circ Res* 78(6):954–961.
37. Kehat I, Molkentin JD (2010) Extracellular signal-regulated kinase 1/2 (ERK1/2) signaling in cardiac hypertrophy. *Ann N Y Acad Sci* 1188:96–102.
38. Kehat I, et al. (2011) Extracellular signal-regulated kinases 1 and 2 regulate the balance between eccentric and concentric cardiac growth. *Circ Res* 108(2):176–183.