The coiled-coil domain of MURC/Cavin-4 is involved in membrane trafficking of caveolin-3 in cardiomyocytes

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Running title: MURC/Cavin-4 functions as a crucial caveolar component

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ABSTRACT

Muscle-restricted coiled-coil protein (MURC), also referred to as Cavin-4, is a member of the cavin family that works cooperatively with caveolins in caveola formation and function. Cavins are cytoplasmic proteins with coiled-coil domains and form heteromeric complexes, which are recruited to caveolae in cells expressing caveolins. Among caveolins, caveolin-3 (Cav3) is exclusively expressed in muscle cells, similar to MURC/Cavin-4. In the heart, Cav3 overexpression contributes to cardiac protection, while its deficiency leads to progressive cardiomyopathy. Mutations in the MURC/Cavin-4 gene have been identified in patients with dilated cardiomyopathy. Here we show the role of MURC/Cavin-4 as a caveolar component in the heart. In H9c2 cells, MURC/Cavin-4 was localized at the plasma membrane, while a MURC/Cavin-4 mutant lacking the coiled-coil domain (ΔCC) was primarily localized to the cytoplasm. ΔCC bound to Cav3 and impaired membrane localization of Cav3 in cardiomyocytes. Additionally, although ΔCC did not alter Cav3 mRNA expression, ΔCC decreased the Cav3 protein level. MURC/Cavin-4 and ΔCC similarly induced cardiomyocyte hypertrophy; however, ΔCC showed higher hypertrophy-related fetal gene expression than MURC/Cavin-4. ΔCC induced ERK activation in cardiomyocytes. Transgenic mice expressing ΔCC in the heart (ΔCC-Tg mice) showed impaired cardiac function accompanied by cardiomyocyte hypertrophy and marked interstitial fibrosis. ΔCC-Tg hearts showed reduction of the Cav3 protein level and the activation of ERK. These results suggest that MURC/Cavin-4 requires its coiled-coil domain to target the plasma membrane and to stabilize Cav3 at the plasma membrane of cardiomyocytes, and that MURC/Cavin-4 functions as a crucial caveolar component to regulate cardiac function.
NEW & NOTEWORTHY

MURC/Cavin-4 requires its coiled-coil domain (CC) to target the plasma membrane. Deletion of CC in MURC/Cavin-4 (ΔCC) impairs membrane localization of caveolin-3 and induces cardiomyocyte hypertrophy. Transgenic mice expressing ΔCC in the heart show impaired cardiac function. Thus, MURC/Cavin-4 functions as a crucial caveolar component to regulate cardiac function.
INTRODUCTION

Caveolae are plasma membrane invaginations with a flask-shaped morphology that have been implicated in many cellular processes, including signal transduction, endocytosis, transcytosis, and lipid regulation (9, 24). In the heart, caveolae are abundantly present in ventricular, atrial, and nodal cells (1) and have been shown to be important for colocalization of receptors with their signaling partners (26). In cardiomyocytes, caveolin-3 (Cav3), a muscle-specific caveolin family member, is a key determinant of caveolar morphology because genetic deletion of Cav3 shows loss of caveolae in cardiac and skeletal myocytes (5, 35). Cav3-knockout (KO) mice show systolic dysfunction accompanied by cardiac hypertrophy (35). Mutations in the Cav3 gene have been identified in patients with not only muscular dystrophies but also hypertrophic cardiomyopathy and long-QT syndrome (12, 21, 32, 33). Thus, Cav3 regulates caveolar morphology in cardiomyocytes and cardiac function.

Characterizations of polymerase I and transcript release factor (PTRF)/Cavin-1 and subsequently other cavin family members, serum deprivation protein response (SDPR)/Cavin-2 and SDR-related gene product that binds to C kinase (SRBC)/Cavin-3, in caveolar morphology have provided further insights into the complexity of the biogenesis of caveolae (3, 8, 13, 14, 18, 20). PTRF/Cavin-1 and SDPR/Cavin-2 are expressed in a variety of cell types, including myocytes (2, 6, 10, 11, 14, 18). SRBC/Cavin-3 is expressed in many cell types but not muscle cells per se, despite its expression in the heart and skeletal muscle (10, 20). Recent studies revealed that PTRF/Cavin-1 and SDPR/Cavin-2 are required for caveolar formation and SRBC/Cavin-3 for trafficking of caveolar vesicles (8, 10, 14, 18, 20). Homozygous mutations in the PTRF/Cavin-1 gene have been identified in patients who presented with both generalized
lipodystrophy and muscular dystrophy (13). Furthermore, in patients with congenital
generalized lipodystrophy with muscle rippling (CGL4) who have homozygous
PTRF/Cavin-1 mutations, long-QT syndrome and fatal cardiac arrhythmia have been
observed (25, 28). We previously identified MURC, muscle-restricted coiled-coil protein,
which is expressed in cardiomyocytes, smooth muscle cells, and skeletal myocytes (23).
MURC is also referred to as Cavin-4 based on its sequence homology with
PTRF/Cavin-1 and its localization to caveolae (2, 9). We have shown that MURC/Cavin-4
is capable of forming complexes with Cav3, PTRF/Cavin-1, and SDPR/Cavin-2, and that
MURC/Cavin-4 overexpression in cardiomyocytes distends caveolae, although
MURC/Cavin-4 is dispensable for caveola formation (22). MURC/Cavin-4 deficiency
attenuates cardiac hypertrophy induced by α1-adrenergic receptor (AR) stimulation (22).
Furthermore, we identified MURC/Cavin-4 mutations in patients with dilated
cardiomyopathy (27).

The coiled-coil domain has been shown to be responsible for the oligomerization
of proteins, and proteins containing its domain exhibit a large diversity of functions, such
as gene regulation, cell division, membrane fusion, and drug extrusion (4, 19). Although
MURC/Cavin-4 has the coiled-coil domain (human: 44-77 amino acids, mouse: 44-77
amino acids), its functional significance is not well known. In this study, we sought to
identify the roles of the coiled-coil domain in MURC/Cavin-4 in targeting to caveolae of
cardiomyocytes and cardiac function.

MATERIALS AND METHODS

Materials
Rat monoclonal anti-hemagglutinin (HA) antibody (Cat. #: 118674 23001) was from Roche Diagnostics. Mouse monoclonal anti-FLAG (Cat. #: F3166), anti-α-actinin (Cat. #: A7811), and anti-pan-cadherin (Cat. #: C1821) antibodies were from Sigma-Aldrich. Mouse monoclonal anti-T7 (Cat. #: 69522), anti-Cav3 (Cat. #: 610420), anti-\(\text{Na}^+/\text{K}^+\)-ATPase α1 (Cat. #: sc-21712), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cat. #: MAB374) antibodies were from Novagen Biosciences, Inc., BD Transduction Laboratories, Santa Cruz Biotechnology, Inc., and Merck Millipore, respectively. Rabbit polyclonal anti-PTRF/Cavin-1 antibody (Cat. #: ab48824) was from Abcam. Rabbit polyclonal anti-phospho-ERK1/2 (pERK, Cat. #: 9101S) and anti-ERK (Cat. #: 9102S) antibodies were from Cell Signaling Technology, Inc. Horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-glutathione S-transferase (GST) antibody (Cat. #: 011-21891) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Rabbit polyclonal anti-MURC/Cavin-4 antibody was generated as previously described (23, 29). pEGFP-N3 vector was purchased from Clontech. H9c2 cells were purchased from ATCC. The adenovirus expression vector kit (Dual Version) was from Takara Bio Inc. (Otsu, Japan). Human Cav3 expressing vector, pcDNA3.1-hCav3-T7, was kindly provided by Yukiko K. Hayashi (Tokyo Medical University, Tokyo, Japan) (13). Other materials were obtained from commercial sources.

**Plasmid construction**

The corresponding cDNA fragment for mouse *MURC/Cavin-4* (mMURC) with a C-terminal HA epitope was obtained by PCR with pcDNA3-mMURC-FLAG (23) as a template with the following primers: mMURC forward primer (5'-CACCATGGAACACAAACGGATCAGCTT-3') and mMURC-HA reverse primer.
(5'-CTAAGCGTAATCTGGAACATCGTATGGGTATGAGGACTGCTTTAGCTCCAACAGGAG-3'). The HA-tagged mMURC fragment was inserted into pcDNA3 (Invitrogen) to generate pcDNA3-mMURC-HA. Deletion mutants lacking the coiled-coil domain of human *MURC/Cavin-4* (hMURC) and mMURC with a C-terminal FLAG or HA epitope, hΔCC-FLAG, hΔCC-HA, mΔCC-FLAG, and mΔCC-HA, were obtained by using a Quikchange II site-directed mutagenesis kit (Stratagene) with pcDNA3.1-hMURC-FLAG (27), pcDNA3.1-hMURC-HA (22), pcDNA3-mMURC-FLAG, and pcDNA3-mMURC-HA as templates, respectively, with the following primers: hΔCC forward primer (5'-TGGACAAAGTAGCCTCCATCTCGCATAGCAATACAGGGCA-3') and hΔCC reverse primer (5'-TGCCCTGTATTGCTATGCGAGATGGAGGCTACTTTGTCC-3'), and mΔCC forward primer (5'-TGGACAGAGTGGCCAGTGTCTCACACAGCAATACGGGCTA-3') and mΔCC reverse primer (5'-TAGCCCGTATTGCTGTGTGAGACACTGGCCACTCTGTCCA-3'). The cDNA fragments of hΔCC-FLAG, hΔCC-HA, mΔCC-FLAG, and mΔCC-HA were replaced with the hMURC-FLAG, hMURC-HA, and mMURC-FLAG fragments to generate pcDNA3.1-hΔCC-FLAG, pcDNA3.1-hΔCC-HA, pcDNA3-mΔCC-FLAG, and pcDNA3-mΔCC-HA, respectively. The corresponding cDNA fragments for hMURC and hΔCC without a stop codon were cloned by PCR with pcDNA3.1-hMURC-FLAG and pcDNA3.1-hΔCC-FLAG as templates with the following primers: hMURC forward primer-2 (5'-AACCGCTAGCATGGAACATCGTATGGGTATGAGGACTGCTTTAGCTCCAACAGGAG-3') and hMURC reverse primer (5'-GGCCGGATCCGTATGGGTATGAGGACTGCTTTAGCTCCAACAGGAG-3'). The cDNA fragments encoding hMURC and hΔCC without a stop codon were cloned into the Nhel-BamHI site of pEGFP-N3 to generate pEGFP-hMURC and pEGFP-hΔCC, respectively. The Cav3 fragment from pcDNA3.1-hCav3-T7 was inserted into pGEX-6P-2 (GE Healthcare) to
generate pGEX-hCav3. All of the PCR fragments were confirmed by DNA sequencing.

**Transfection**

H9c2 cells were plated in two-well culture plates. The following day, the cells were transfected with 1.0 μg of an expression vector containing hMURC-EGFP or hΔCC-EGFP using Lipofectamine LTX (Invitrogen). Cells were cultured for another 72 hours and then fixed for fluorescence microscopy.

**Confocal immunofluorescence microscopy**

Specimens were fixed with 4% paraformaldehyde (PFA) and stained with anti-Cav3, anti-α-actinin, and MURC/Cavin-4 antibodies. Secondary antibodies were conjugated with Alexa Fluor 488 or 555 (Invitrogen), and nuclei were visualized using 4',6-diamino-2-phenylindole (DAPI; Invitrogen).

**Preparation of cytosol or membrane fraction**

Cell fractionation was performed as previously described (16, 22). 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 cocktail (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 ultra-centrifuged at 55,000 rpm for 30 min using an Optima TLX centrifuge (Beckman Coulter, Inc.) with a TLA-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. Because pan-cadherin and Na^+/K^+-ATPase are markers for the membrane fraction, and GAPDH is a marker for the cytosol fraction (36, 37), anti-pan-cadherin, anti-Na^+/K^+-ATPase α1, and anti-GAPDH antibodies were used to verify the purity of membrane and cytosol fractions.

**Immunoprecipitation**

COS cells were plated in 60-mm dishes. The following day, the cells were transfected with pcDNA3.1-hCav3-T7 and either pcDNA3-mMURC-HA or pcDNA3-mΔCC-HA. The total plasmid amount was adjusted with an empty vector plasmid. Cells were cultured for another 48 hours and then lysed with a lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 50 mM EDTA, 1% Triton X-100, 60 mM octyl glucoside (DOJINDO, Kumamoto, Japan), 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM Na_3VO_4, and 1 × protease inhibitor (Pierce). Cell lysates were incubated with an anti-HA or an anti-T7 antibody and subsequently with protein A-Sepharose beads (GE Healthcare) at 4°C overnight. After the beads had been extensively washed with the lysis buffer, the bound proteins were eluted by boiling the beads in SDS sample buffer and subjected to SDS-PAGE, followed by Western blot analysis.

**Western blot analysis**

Total cell lysates or fractionated lysates were electrophoresed in sodium dodecyl sulfate (SDS)-polyacrylamide gels (PAGE) and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were subsequently incubated with primary antibodies against HA, GAPDH, pan-cadherin, FLAG, Cav3, PTRF/Cavin-1, Na^+/K^+-ATPase α1, phospho-ERK1/2 (pERK1/2), ERK1/2, and MURC/Cavin-4.
Horseradish peroxidase-conjugated anti-rat IgG, anti-mouse IgG, and anti-rabbit IgG antibodies (GE Healthcare) were used as secondary antibodies. For immunoblotting of GST, peroxidase-conjugated anti-GST antibody was used. Signal intensities were measured using Image J software (National Institutes of Health).

**GST pulldown assay**

Two micrograms of GST-Cav3 was bound to glutathione-Sepharose beads. The beads were incubated with lysates from COS cells transfected with pcDNA3-mMURC-FLAG or pcDNA3-mΔCC-FLAG for 2 hours at room temperature. Sepharose beads alone were used as a control. Beads were washed five times and proteins were eluted for Western blotting.

**Primary cardiomyocyte culture**

Rat neonatal cardiomyocytes, cultured from 1-day-old Wistar rats, were prepared as described previously with slight modifications (23, 31). Briefly, ventricles were digested enzymatically, and cardiomyocytes were purified over a Percoll gradient. The culture medium was changed to serum-free medium after 24 hours.

**Replication-defective recombinant adenoviruses and gene transfer**

The cDNA encoding hΔCC-FLAG was inserted into a pAxCAwtit2 cosmid vector in an adenovirus expression vector kit (Dual Version). A recombinant adenovirus expressing hΔCC1-FLAG (Ad-ΔCC) was generated as described previously (23, 27). A recombinant adenovirus expressing hMURC-FLAG, Ad-MURC, was as described previously (27). Ad-LacZ was used as a control as described previously (23, 27). Twenty-four hours after
seeding, the cardiomyocytes were infected with Ad-LacZ, Ad-MURC, or Ad-ΔCC and incubated at 37°C for 1 hour. The viral suspension was removed and the cardiomyocytes were cultured with serum-depleted culture media. Expression of mRNA and protein was examined in cardiomyocytes infected with recombinant adenoviruses for 48 and 72 hours, respectively.

**Measurement of cardiomyocyte surface**

Cardiomyocytes infected with Ad-LacZ, Ad-MURC, or Ad-ΔCC were fixed with 4% PFA, and the cells were stained with a mouse monoclonal anti-α-actinin antibody, Alexa Fluor 488, and DAPI. Cells were imaged using an immunofluorescence microscope and measured using ImageJ software (National Institutes of Health). At least 100 cells were measured per sample.

**RNA extraction and quantitative reverse transcriptase (RT)-PCR**

Total RNA was extracted from cells or tissues using an RNeasy Mini kit (Qiagen) or Trizol reagent (Invitrogen) and then treated with DNase I (Qiagen) to remove any residual DNA. Total RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Synthesized cDNA was analyzed by kinetic real-time PCR using TAKARA PCR Thermal Cycler Dice (TAKARA BIO INC., Japan) with Platinum SYBR Green qPCR Super Mix (Invitrogen) as described previously (23, 29). The primers used were as follows: mouse *atrial natriuretic peptide* (*ANP, Nppa*) forward primer (5’-AACCTGCTAGACCACCTGGA-3’) and mouse *ANP* reverse primer (5’-TGCTTTTCAAGAGGCAGAT-3’); mouse *brain natriuretic peptide* (*BNP, Nppb*) forward primer (5’-CTGAAGGTGCTGTCCAGAT-3’) and mouse *BNP* reverse primer
Animals

Transgenic mice expressing mΔCC-FLAG in the heart (ΔCC-Tg) were generated as described previously (23). Briefly, the cDNA encoding mΔCC-FLAG was cloned into the third 5'-untranslated exon of αMHC promoter plasmid clone 26 (a generous gift from...
Experiments were performed on ΔCC-Tg mice and gender-, strain-, and age-matched NTg controls (C57BL/6 background). All of the 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.

Echocardiography and morphometric analysis
Echocardiographic analysis of mice was performed as described previously (23). Briefly, after the mice had been anesthetized with 2,2,2-tribromoethanol (0.20 mg/g, Sigma-Aldrich), echocardiography was performed using SONOS 5500 (Hewlett-Packard) equipped with a 15-MHz microprobe. After echocardiography, the mice were sacrificed by cervical dislocation and then weighed. Hearts were excised, rinsed in PBS, and weighed. The body weight or the left tibial length was measured to normalize the heart weight.

Histological analysis
Cardiac perfusion-fixation was performed using 4% PFA/PBS. Hearts were cut into and paraffin sections, 3 μm thick, were stained with hematoxylin and eosin (H&E) or Masson's trichrome. Frozen specimens for immunostaining were cut into 10 μm thick sections. Cross-sectional areas of cardiomyocytes were measured in heart sections stained with H&E using ImageJ software (National Institutes of Health).

Transmission electron microscopy and quantitation
Transmission electron microscopy was performed as described previously (22). Briefly,
12-week-old mouse hearts were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed with 2% OsO₄, and stained with uranyl acetate and lead citrate. The thickness of the sections for transmission electron microscopy was about 80 nm. Microtome sections were examined under an H-7100 transmission electron microscope (HITACHI, Japan) and photographed at magnifications of ×5,000 and ×20,000. Caveolae were identified by their characteristic omega-shaped membrane profiles open at the cell surface. The depths of caveolae were measured from the caveolar orifice to their bottom in electron micrographs.

**Statistical analysis**

All experiments were performed at least three times. Data are expressed as means ± standard errors (SEM) and were analyzed by Student’s *t*-test for comparisons between two groups or one-way analysis of variance followed by the Tukey-Kramer test for multiple comparisons. A probability (*p*) value of <0.05 was considered significant.

**RESULTS**

**Requirement of the coiled-coil domain in MURC/Cavin-4 for plasma membrane targeting**

To investigate the function of the coiled-coil domain in MURC/Cavin-4 in cardiomyocytes, we generated ΔCC, a human MURC/Cavin-4 mutant lacking the coiled-coil domain. An expression vector expressing EGFP-tagged full-length human MURC/Cavin-4 or human ΔCC was transduced into H9c2 cells, a permanent cell line derived from rat cardiac tissue (22). Fluorescence microscopy revealed that the full-length human
MURC/Cavin-4-EGFP was colocalized with Cav3 at the plasma membrane of H9c2 cells, while human ΔCC-EGFP was not localized to the plasma membrane but distributed to the cytoplasm (Fig. 1A). Additionally, Cav3 staining was not observed at the plasma membrane of H9c2 cells expressing human ΔCC-EGFP. However, in H9c2 cells that were not transduced with human ΔCC-EGFP, Cav3 staining was observed at the plasma membrane. To further examine localization of human ΔCC, cell fractionation studies were performed using HEK 293 cells. The full-length human MURC/Cavin-4 was mainly detected in the membrane fraction, but almost all human ΔCC was detected in the cytosol fraction (Fig. 1B). Thus, the coiled-coil domain in MURC/Cavin-4 is critical for targeting MURC/Cavin-4 to the plasma membrane in cardiomyocytes.

To assess whether ΔCC is capable of associating with Cav3, we performed immunoprecipitation using expression vectors expressing Cav3-T7, ΔCC-HA, and full-length MURC/Cavin-4-HA. ΔCC was associated with Cav3, although its association was less than that of full-length MURC/Cavin-4 with Cav3 (Fig. 1C). Additionally, we performed GST pulldown assay to assess the association of Cav3 with full-length MURC/Cavin-4 and ΔCC. The GST pulldown assay showed that full-length MURC/Cavin-4 as well as ΔCC was associated with Cav3 (Fig. 1D).

Reduced Cav3 protein level induced by ΔCC in cardiomyocytes

We then examined whether full-length MURC/Cavin-4 and ΔCC affect Cav3 and PTRF/Cavin-1 expression in cardiomyocytes. Full-length MURC/Cavin-4 did not affect only Cav3 and Ptrf/Cavin-1 mRNA expression but also Cav3 and PTRF/Cavin-1 protein levels in cardiomyocytes (Fig. 2, A and B). ΔCC also did not affect Cav3 and Ptrf/Cavin-1 mRNA expression in cardiomyocytes; however, it decreased the Cav3 protein level but
not the PTRF/Cavin-1 protein level in cardiomyocytes compared with LacZ and full-length MURC/Cavin-4 (Fig. 2, A and B). Cell fractionation studies revealed that ΔCC reduced the Cav3 protein level in the membrane fraction of cardiomyocytes compared with LacZ, and that PTRF/Cavin-1 protein expression did not differ among the membrane fractions of cardiomyocytes expressing LacZ, full-length MURC/Cavin-4, and ΔCC (Fig. 2C).

**Hypertrophic responses induced by ΔCC in cardiomyocytes**

We previously showed that full-length MURC/Cavin-4 induces cardiomyocyte hypertrophy (22). To assess and compare physiological properties of full-length MURC/Cavin-4 and ΔCC, cardiomyocytes were transduced with LacZ as a control, full-length MURC/Cavin-4, or ΔCC by using recombinant adenoviruses, Ad-LacZ, Ad-MURC, and Ad-ΔCC. Forty-eight hours after infection with Ad-LacZ, Ad-MURC, and Ad-ΔCC, cardiomyocytes expressing full-length MURC/Cavin-4 and ΔCC showed increases in surface area compared with cardiomyocytes expressing LacZ (Fig. 3A). Cardiomyocytes expressing ΔCC showed higher hypertrophy-related fetal gene expression, such as ANP, BNP, and SkA mRNA expression, and higher βMHC/αMHC ratio, than cardiomyocytes expressing full-length MURC/Cavin-4 (Fig. 3B). Additionally, ΔCC enhanced ERK activation in cardiomyocytes compared with full-length MURC/Cavin-4 (Fig. 3C). Thus, ΔCC induced hypertrophic responses in cardiomyocytes.

**Impaired cardiac function in ΔCC-Tg mice**

We next generated transgenic mice expressing ΔCC-FLAG in the heart, ΔCC-Tg mice, to examine the role of the coiled-coil domain in MURC/Cavin-4 in vivo (Fig. 4A). We made 2 lines of ΔCC-Tg mice (Fig. 4B). The cardiac expression level of the transgenic
protein ΔCC-FLAG was higher in line 2 of the ΔCC-Tg mice than in line 1. Therefore, we used line 2 of the ΔCC-Tg mice and compared them with gender-, strain-, and age-matched non-transgenic (NTg) controls in following experiments. We measured heart weights at 12-13 weeks of age in NTg and ΔCC-Tg mice (Table 1). The heart-weight-to-tibial-length (HW/TL) ratio in ΔCC-Tg mice was significantly higher than that in NTg mice. In addition, the lung weights, which were evaluated by the lung-weight-to-body-weight (LW/BW) and lung-weight-to-tibial-length (LW/TL) ratios, in ΔCC-Tg mice were also significantly higher than those in NTg mice. Cardiac sections of ΔCC-Tg mice showed chamber dilatation compared with those of NTg mice (Fig. 4C). Cross-sectional areas of cardiomyocytes and interstitial fibrosis increased in ΔCC-Tg hearts compared with those in NTg hearts (Fig. 4, D and E).

To address the functional consequences of ΔCC expression in vivo, we performed echocardiography on NTg and ΔCC-Tg mice at 12-13 weeks of age. ΔCC-Tg mice exhibited chamber dilatation and systolic dysfunction compared with NTg mice (Table 2). Consistent with these observations, ANP and BNP mRNA expression was elevated in ΔCC-Tg hearts compared with that in NTg hearts (Fig. 4F). Thus, ΔCC-Tg mice showed severe cardiac dysfunction compared with NTg mice. These results suggest that the non-membrane-targeting MURC/Cavin-4 affects cardiac function.

Reduced Cav3 protein expression in ΔCC-Tg hearts
Because we previously showed that full-length MURC/Cavin-4 overexpression induces dilatation of caveolae in cardiomyocytes (22), we then examined whether ΔCC could influence the morphology of caveolae in cardiomyocytes. Transmission electron microscopy revealed that ΔCC overexpression had no effect on the size or shape of
caveolae in cardiomyocytes (Fig. 5A). Immunostaining showed that anti-MURC/Cavin-4 antibody-recognizing protein, which contains exogenous ΔCC and endogenous MURC/Cavin-4, was distributed to both the cytosol and the plasma membrane of cardiomyocytes in ΔCC-Tg mice (Fig. 5B). Although immunostained Cav3 was detected at the plasma membrane of cardiomyocytes in ΔCC-Tg mice, western blotting revealed that the Cav3 protein level was decreased in ΔCC-Tg hearts compared with that in NTg hearts (Fig. 5C). PTRF/Cavin-1 protein level was similar between NTg and ΔCC-Tg hearts. ERK activation was induced in the heart of ΔCC-Tg mice compared with that of NTg mice (Fig. 5D). These results suggest that impaired localization of MURC/Cavin-4 reduces the number of caveolae in cardiomyocytes and the Cav3 protein level accompanying ERK activation in the heart.

**DISCUSSION**

Here, we demonstrated that deletion of the coiled-coil domain in MURC/Cavin-4 impairs the proper localization of MURC/Cavin-4 and reduces the Cav3 protein level at the plasma membrane of cardiomyocytes. ΔCC induces ERK activation in cardiomyocytes. Furthermore, ΔCC overexpression in the heart induces cardiac dysfunction accompanied by cardiomyocyte hypertrophy and interstitial fibrosis.

We have shown that MURC/Cavin-4 is localized to caveolae of cardiomyocytes in the heart and is associated with other caveola-associated proteins, such as Cav3, PTRF/Cavin-1, and SDPR/Cavin-2, in cardiomyocytes (22, 23). In the present study, we demonstrated that ΔCC is distributed throughout the cytoplasm but is not localized at the plasma membrane of cardiomyocytes. Previous studies have shown that deletions of the coiled-coil domain in SDPR/Cavin-2 and the leucine zipper domains in PTRF/Cavin-1
and SRBC/Cavin-3 impair their targeting to caveolae (8, 20, 34). Furthermore, a minimal N-terminal domain, termed HR1, of zebrafish Cavin4a has been shown to be required for association with lipid membrane (17). Our findings also indicate that the coiled-coil domain in MURC/Cavin-4 is essential for its membrane targeting in cardiomyocytes. We showed that ΔCC is capable of binding to Cav3 in immunoprecipitation and GST studies, which indicates that the function of the Cav-3 binding domain is retained in ΔCC. ΔCC decreased in Cav3 protein expression at the plasma membrane of cardiomyocytes, as shown in Figs. 1A and 2C, which also indicates that the coiled-coil domain in MURC/Cavin-4 is required for Cav3 localization at the plasma membrane of cardiomyocytes. ΔCC did not alter Cav3 mRNA expression in cardiomyocytes, while it decreased Cav3 protein levels in cardiomyocytes and ΔCC-Tg hearts. These findings suggest that deletion of the coiled-coil domain in MURC/Cavin-4 impairs Cav3 trafficking to the plasma membrane of cardiomyocytes, and that stability of the Cav3 protein is a prerequisite for its proper localization at the plasma membrane of cardiomyocytes. On the other hand, PTRF/Cavin-1 protein levels were not altered by ΔCC in cardiomyocytes, as shown in Figs. 2C, suggesting that the coiled-coil domain in MURC/Cavin-4 has different effects on the regulation of Cav3 and PTRF/Cavin-1 in cardiomyocytes.

Cav3 regulates caveolar formation in cardiac and skeletal muscle cells as shown in previous studies indicating that Cav3 deficiency leads to decreased numbers or the absence of caveolar structures in striated muscle, and that Cav3 overexpression in cardiomyocytes increases the number of caveolae (5, 7, 15, 30, 35). Although Cav3 protein levels were reduced in ΔCC-Tg hearts and the membrane fraction of cardiomyocytes expressing ΔCC, the size or shape of caveolae in cardiomyocytes was not affected in ΔCC-Tg hearts, which implies that because endogenous MURC/Cavin-4
protein is expressed in cardiomyocytes overexpressing ΔCC, endogenous MURC/Cavin-4 protein contributes to proper membrane localization and stabilization of Cav3 protein against ΔCC actions, and that the decreased Cav3 protein levels in ΔCC-Tg hearts are insufficient to alter caveolar morphology. In Cav3-KO mice, Cav3 deficiency has been shown to induce ERK activation in the heart and cardiac hypertrophy, which results in impaired cardiac function (35). We showed that ΔCC overexpression in cardiomyocytes induces ERK activation and hypertrophic responses, and that ΔCC-Tg hearts showed ERK activation and cardiac dysfunction accompanying cardiomyocyte hypertrophy and interstitial fibrosis. Therefore, ΔCC-induced reduction of Cav3 protein likely contributes to ERK activation and the development of cardiac dysfunction in ΔCC-Tg mice.

In MURC/Cavin-4-deficient cardiomyocytes, Cav3 is retained at the plasma membrane (22). Moreover, MURC/Cavin-4-deficient mice exhibit normal caveolar morphology and cardiac function under physiological conditions, while upon α1-AR stimulation, MURC/Cavin-4-deficient mice show attenuation of cardiac hypertrophy accompanied by suppressed ERK activation (22). On the other hand, ΔCC leads to Cav3 reduction and ERK activation in cardiomyocytes despite the absence of ΔCC at the plasma membrane. As a consequence, ΔCC-Tg mice exhibit cardiac dysfunction. These findings suggest that MURC/Cavin-4 at the caveolae is necessary to elicit efficient signaling of the α1-AR/ERK cascade, while cytoplasmic MURC/Cavin-4 mislocalization causes alterations of caveola-associated proteins and signaling in cardiomyocytes, which result in cardiac dysfunction. Thus, MURC/Cavin-4 works as a critical caveolar component in cardiomyocytes.

As shown in the present study, deletion of the coiled-coil domain in
MURC/Cavin-4 impairs the localization of MURC/Cavin-4 in cardiomyocytes and causes decreases in Cav3 protein level at the plasma membrane accompanied by ERK activation, which results in cardiac dysfunction. These findings indicate the critical roles of MURC/Cavin-4 as a caveolar component in cardiomyocytes and provide insights into molecular mechanisms underlying caveola-mediated signaling in cardiac function.

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FIGURE LEGENDS

Figure 1. The coiled-coil domain in MURC/Cavin-4 is required for membrane targeting.

(A) Top, the schema of the full-length human MURC/Cavin-4 and human MURC/Cavin-4 mutant lacking the coiled-coil domain (ΔCC). Bottom, representative fluorescence microscopic images of H9c2 cells transfected with pEGFP-hMURC or pEGFP-hΔCC. The experiments were performed at least three times. Seventy-two hours after transfection, H9c2 cells were fixed, immunostained for Cav3, and then examined by fluorescence microscopy. Of note, in the right panels, Cav3 was localized at the plasma membrane in the adjacent H9c2 cell that had not been transduced with human ΔCC-EGFP, while it was not localized at the plasma membrane in the H9c2 cell expressing human ΔCC-EGFP. Arrows highlight the localization of Cav3 at the plasma membrane. The exposure time to take the images of ΔCC was longer than that of MURC/Cavin-4. Bar, 20 μm. (B) Immunoblotting of MURC-HA and ΔCC-HA. pcDNA3.1-hMURC-HA or pcDNA3.1-hΔCC-HA was transfected into HEK293 cells, and lysates were subjected to sucrose gradient analysis. Thirty μg protein of the membrane fraction and twenty μg protein of the cytosol fraction were loaded for each well. Samples were assessed by immunoblotting using anti-HA, anti-GAPDH, and anti-pan-cadherin antibodies. The fractionation experiments were performed at least three times. (C) Immunoprecipitation of Cav3 with full-length MURC/Cavin-4 or ΔCC. pcDNA3-mMURC-HA or pcDNA3-mΔCC-HA was transfected with or without pcDNA3.1-hCav3-T7 into COS cells. Cell lysates were subjected to immunoprecipitation with the anti-HA or anti-T7 antibody. IP: immunoprecipitation, IB: immunoblotting. (D)
Pulldown assay of Cav3 with MURC/Cavin-4 or ΔCC. pcDNA3-mMURC-FLAG or pcDNA3.1-mΔCC-FLAG was transfected into COS cells. Cell lysates were incubated with GST-Cav3 fusion protein or GST alone. The pulldown samples were analyzed by probing with anti-FLAG and anti-GST antibodies.

**Figure 2. ΔCC induces Cav3 protein reduction in cardiomyocytes.**

(A) The mRNA expression of Cav3 and Ptrl/Cavin-1 in cardiomyocytes infected with Ad-LacZ, Ad-MURC, or Ad-ΔCC. (B) Left, representative immunoblotting of FLAG-tagged protein, PTRF/Cavin-1, and Cav3 in cardiomyocytes infected with Ad-LacZ, Ad-MURC, or Ad-ΔCC. Right, bar graphs showing quantification of protein expression of PTRF/Cavin-1 and Cav3 in cardiomyocytes. (C) Left, representative immunoblotting of Cav3 and PTRF/Cavin-1 in the membrane (upper) and cytosol (lower) fractions of cardiomyocytes infected with Ad-LacZ, Ad-MURC, or Ad-ΔCC. Right, bar graphs showing protein expression of Cav3 and PTRF/Cavin-1 in the membrane (upper) and cytosol (lower) fractions of cardiomyocytes. The experiments were performed at least three times. Data are presented as mean ± SEM. *p<0.05 compared with Ad-LacZ, **p<0.01 compared with Ad-LacZ, ††p<0.01 compared with Ad-MURC. NS, not significant.

**Figure 3. ΔCC induces hypertrophic responses in cardiomyocytes.**

(A) Left, representative staining of cardiomyocytes with an anti-α-actinin antibody. Bar, 20 μm. Right, quantitation of cell surface areas of cardiomyocytes. Cultured neonatal rat cardiomyocytes were infected with Ad-LacZ, Ad-MURC, or Ad-ΔCC. (B) The mRNA expression of ANP (Nppa), BNP (Nppb), SkA (Acta1), αMHC (Myh6), and βMHC (Myh7) in cardiomyocytes infected with Ad-LacZ, Ad-MURC, or Ad-ΔCC. (C) Left,
representative immunoblotting of ERK in cardiomyocytes infected with Ad-LacZ, Ad-MURC, or Ad-ΔCC. Right, bar graphs showing phosphorylation of ERK in cardiomyocytes. Data are presented as mean ± SEM. *p<0.05 compared with Ad-LacZ, **p<0.01 compared with Ad-LacZ, ††p<0.01 compared with Ad-MURC.

**Figure 4. ΔCC-Tg mice show cardiac dilatation accompanied by cardiomyocyte hypertrophy and interstitial fibrosis.**

(A) Schematic of transgenic vectors showing inserted FLAG-tagged mouse ΔCC under the αMHC promoter and three exons prior to the first coding ATG site. (B) Expression of ΔCC-FLAG protein in the heart. Heart lysates from NTg and ΔCC-Tg mice (lines 1 and 2) were immunoblotted with antibodies against FLAG, MURC/Cavin-4, and GAPDH which is an internal control. The experiments were performed at least three times. (C) Representative H&E staining sections of hearts from NTg and ΔCC-Tg mice at 12-13 weeks of age. Bar, 1 mm. (D) Left, representative H&E staining sections of hearts from NTg and ΔCC-Tg mice at 12-13 weeks of age. Bar, 20 μm. Right, cross-sectional areas of cardiomyocytes of NTg and ΔCC-Tg mice at 12-13 weeks of age. (E) Left, representative Masson’s trichrome staining sections of hearts from NTg and ΔCC-Tg mice at 12-13 weeks of age. Bar, 100 μm. Right, fibrotic area in hearts from NTg and ΔCC-Tg mice at 12-13 weeks of age. (F) The mRNA expression of ANP (Nppa) and BNP (Nppb) in the heart of NTg, MURC-Tg, and ΔCC-Tg mice. Data are presented as mean ± SEM. **p<0.01 compared with NTg, ††p<0.01 compared with MURC-Tg.

**Figure 5. ΔCC-Tg mice show morphological abnormalities of the plasma membrane and mislocalization of Cav3 in the heart.**
(A) Upper, representative electron microscopic images of NTg and ΔCC-Tg hearts. Bar, 1 μm. Lower left, manually superimposed caveolar membrane profiles from electron microscopic images of NTg and ΔCC-Tg cardiomyocytes. Bar, 200 nm. Lower right, quantification of the perimeters and depths of caveolae in cardiomyocytes of NTg and ΔCC-Tg hearts. Multiple electron micrographs were obtained for each heart (n=3 each), and depths of caveolae were measured in each image. Caveolae were counted as omega-shaped membrane profiles open at the cell surface. (B) Left, immunostaining of heart sections from NTg and ΔCC-Tg mice with anti-MURC/Cavin-4 and anti-Cav3 antibodies. Bar, 100 μm. (C) Expression of MURC/Cavin-4, Cav3, and PTRF/Cavin-1 in NTg and ΔCC-Tg hearts. Right, bar graphs showing protein expression of PTRF/Cavin-1 and Cav3 in NTg and ΔCC-Tg hearts. (D) Activation of ERK in NTg and ΔCC-Tg hearts. Right, bar graphs showing phosphorylation of ERK in NTg and ΔCC-Tg hearts. Data are presented as mean ± SEM. *p<0.05 compared with NTg. NS, not significant.
<table>
<thead>
<tr>
<th></th>
<th>NTg (n=8)</th>
<th>ΔCC-Tg (n=3)</th>
</tr>
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<tbody>
<tr>
<td>BW (g)</td>
<td>23.4 ± 1.23</td>
<td>29.5 ± 0.42*</td>
</tr>
<tr>
<td>TL (mm)</td>
<td>16.9 ± 0.11</td>
<td>17.3 ± 0.09</td>
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<tr>
<td>HW/BW (mg/g)</td>
<td>4.32 ± 0.10</td>
<td>5.48 ± 0.21**</td>
</tr>
<tr>
<td>HW/TL (mg/mm)</td>
<td>5.97 ± 0.35</td>
<td>9.33 ± 0.49**</td>
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<tr>
<td>LW/BW (mg/g)</td>
<td>5.51 ± 0.20</td>
<td>10.58 ± 1.35**</td>
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<tr>
<td>LW/TL (mg/mm)</td>
<td>7.51 ± 0.18</td>
<td>18.04 ± 2.41**</td>
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BW: body weight, TL: tibial length, HW: heart weight, LW: lung weight. Values are expressed as mean ± SEM. *P<0.05 compared with NTg, **P<0.01 compared with NTg.
Table 2. Echocardiographic analysis of NTg and ΔCC-Tg mice

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<th>NTg (n=7)</th>
<th>ΔCC-Tg (n=8)</th>
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<tbody>
<tr>
<td>LVDd (mm)</td>
<td>3.15 ± 0.10</td>
<td>3.61 ± 0.16*</td>
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<tr>
<td>LVDs (mm)</td>
<td>1.60 ± 0.08</td>
<td>2.73 ± 0.15**</td>
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<tr>
<td>IVSd (mm)</td>
<td>0.77 ± 0.01</td>
<td>0.70 ± 0.03</td>
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<tr>
<td>LVPWd (mm)</td>
<td>0.73 ± 0.01</td>
<td>0.69 ± 0.04</td>
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<tr>
<td>LVFS (%)</td>
<td>48.8 ± 2.5</td>
<td>24.6 ± 2.4**</td>
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</table>

LVDd: left ventricular dimension at end-diastole, LVDs: left ventricular dimension in systole, IVSd: interventricular septum thickness at end-diastole, LVPWd: left ventricular posterior wall thickness at end-diastole, LVFS: left ventricular fractional shortening. Values are expressed as mean ± SEM. *P<0.05 compared with NTg, **P<0.01 compared with NTg.
**A**

<table>
<thead>
<tr>
<th>MURC/Cavin-4</th>
<th>Cav3</th>
<th>Merged</th>
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<tbody>
<tr>
<td>Red</td>
<td>Green</td>
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<tr>
<td>Red</td>
<td>Green</td>
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<tr>
<td>Red</td>
<td>Green</td>
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**B**

<table>
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<th>HEK293 cells</th>
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<th>Cytosol fraction</th>
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<td>MURC-HA</td>
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<td>−</td>
</tr>
<tr>
<td>ΔCC-HA</td>
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<tr>
<td>GAPDH</td>
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<tr>
<td>pan-cadherin</td>
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**C**

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<tr>
<td>ΔCC-HA</td>
<td>− +</td>
</tr>
<tr>
<td>T7-Cav3</td>
<td>+ −</td>
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**D**

<table>
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<th>Input</th>
<th>GST-Cav3</th>
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<tbody>
<tr>
<td>MURC-FLAG</td>
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<tr>
<td>IB: anti-FLAG</td>
<td>IB: anti-FLAG</td>
<td>IB: anti-GST</td>
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**Fig. 2**

### Cardiomyocytes

<table>
<thead>
<tr>
<th>Infection</th>
<th>Ad-LacZ</th>
<th>Ad-MURC</th>
<th>Ad-ΔCC</th>
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<td>PTRF/Cavin-1</td>
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<tr>
<td>GAPDH</td>
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### Membrane

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<th>Ad-ΔCC</th>
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### Cytosol

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<td>Cav3</td>
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<td>GAPDH</td>
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Fig. 3

A. Image showing cell morphology with Ad-LacZ, Ad-MURC, and Ad-ΔCC treatments.

B. Bar graphs showing gene expression fold changes for Nppa/Gapdh, Nppb/Gapdh, Acta1/Gapdh, and Myh7/Myh6 in Ad-LacZ, Ad-MURC, and Ad-ΔCC groups. Significant differences are indicated by ** and ††.

C. Western blot analysis showing pERK/ERK fold changes in Cardiomyocytes treated with Ad-LacZ, Ad-MURC, and Ad-ΔCC. Significant differences are indicated by * and †††.
Fold increase
Nppb/Gapdh
10
5
0

NTg
ΔCC
ΔTg

Fig. 4

A B C

MURC/Cavin-4
FLAG
GAPDH

D
NTg
ΔCC-Tg

Nppa/Gapdh

E
NTg
ΔCC-Tg

Nppb/Gapdh

F
NTg
ΔCC-Tg

**

0
2
4
6
8

Fold change
Fibrotic area (%)

αMHC
promoter
ex1
ex2
ex3
hGH-polyA

ΔCC

**

NTg
ΔCC
ΔTg

**

NTg
ΔCC
ΔTg

**

NTg
ΔCC
ΔTg

**

Fig. 4