Research Article

The CR9 element is a novel mechanical load-responsive enhancer that regulates natriuretic peptide genes expression

Yohei Miyashita1,2,3 | Osamu Tsukamoto1 | Ken Matsuoka1 | Kenta Kamikubo1 | Yuki Kuramoto3 | Hai Ying Fu4 | Tomoya Tsubota1 | Hirona Hasuie1 | Toshiro Takayama5 | Hiroaki Ito6 | Tatsuro Hitsumoto1 | Chisato Okamoto1 | Hidetaka Kioka3 | Ryohei Oya1 | Haruki Shinomiya3 | Hideyuki Hakui3 | Yasunori Shintani7 | Hisakazu Kato1 | Masafumi Kitakaze8 | Yasushi Sakata3 | Yoshihiro Asano3 | Seiji Takashima1

1Department of Medical Biochemistry, Graduate School of Medicine/Frontier Biosciences, Osaka University, Suita, Japan
2Department of Legal Medicine, Graduate School of Medicine, Osaka University, Suita, Japan
3Department of Cardiovascular Medicine, Graduate School of Medicine, Osaka University, Suita, Japan
4Department of Cardiorenal and Cerebrovascular Medicine, Faculty of Medicine, Kagawa University, Takamatsu, Japan
5School of Engineering, Department of Mechanical Engineering, Tokyo Institute of Technology, Tokyo, Japan
6Department of Physics, Graduate School of Science, Chiba University, Chiba, Japan
7Department of Molecular Pharmacology, National Cerebral and Cardiovascular Center, Suita, Japan
8Hanwa Daini Senboku Hospital, Osaka, Japan

Correspondence
Osamu Tsukamoto, Department of Medical Biochemistry, Osaka University, Suita, Japan.
Email: tsuka@medbio.med.osaka-u.ac.jp
Yoshihiro Asano, Department of Cardiovascular Medicine, Graduate School of Medicine/Frontier Biosciences, Osaka University, Suita, Japan.
Email: asano@cardiology.med.osaka-u.ac.jp

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Abstract
Enhancers regulate gene expressions in a tissue- and pathology-specific manner by altering its activities. Plasma levels of atrial and brain natriuretic peptides, encoded by the Nppa and Nppb, respectively, and synthesized predominantly in cardiomyocytes, vary depending on the severity of heart failure. We previously identified the noncoding conserved region 9 (CR9) element as a putative Nppb enhancer at 22-kb upstream from the Nppb gene. However, its regulatory mechanism remains unknown. Here, we therefore investigated the mechanism of CR9 activation in cardiomyocytes using different kinds of drugs that induce either cardiac hypertrophy or cardiac failure accompanied by natriuretic peptides upregulation. Chronic treatment of mice with either catecholamines or doxorubicin increased CR9 activity during the progression of cardiac hypertrophy to failure, which is accompanied by proportional increases in Nppb expression. Conversely, for cultured cardiomyocytes, doxorubicin decreased

Abbreviations: Adr, adrenaline; BDM, 2.3-butanedione monoxime; BNP, brain natriuretic peptide; BW, body weight; CDD, contraction deformation distance; CMV, cytomegalovirus; CR, conserved region; Dox, doxorubicin; FBS, fetal bovine serum; FS, fractional shortening; HR, heart rate; HW, heart weight; Luc, luciferase; LVIDd, left ventricular internal dimension in diastole; LVPWd, left ventricular posterior wall thickness in diastole; miniP, minimal promoter; MLP, muscle LIM protein; PE, phenylephrine; Tg, transgenic.

Yohei Miyashita and Ken Matsuoka contributed equally to this manuscript.

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CR9 activity and \textit{Nppb} expression, while catecholamines increased both. However, exposing cultured cardiomyocytes to mechanical loads, such as mechanical stretch or hydrostatic pressure, upregulate CR9 activity and \textit{Nppb} expression even in the presence of doxorubicin. Furthermore, the enhancement of CR9 activity and \textit{Nppa} and \textit{Nppb} expressions by either catecholamines or mechanical loads can be blunted by suppressing mechanosensing and mechanotransduction pathways, such as muscle LIM protein (MLP) or myosin tension. Finally, the CR9 element showed a more robust and cell-specific response to mechanical loads than the \(-520\)-bp BNP promoter. We concluded that the CR9 element is a novel enhancer that responds to mechanical loads by upregulating natriuretic peptides expression in cardiomyocytes.

**Keywords**

cardiomyocyte, CR9, enhancer, mechanical loads, natriuretic peptides

## 1 | INTRODUCTION

Cells can increase or decrease the expression levels of specific genes in response to changes in either the intracellular or the extracellular environment, changing the type and number of genes to be expressed and thereby enabling short- or long-term adaptation.\textsuperscript{1} For instance, stem cells need to switch the expression patterns of genes in response to external stimuli in order to differentiate into cells with various characteristics.\textsuperscript{1,2} Gene expression is regulated by the integrated action of \textit{cis}-regulatory elements such as promoters and distant-acting enhancers.\textsuperscript{3} Promoters are proximal-acting \textit{cis}-regulatory DNA elements to which RNA polymerase and transcription factors bind in order to initiate transcription of a specific gene into mRNA. Enhancers are distal-acting \textit{cis}-elements that reside millions of base pairs away from the transcription start sites of genes that they regulate. Enhancers selectively activate genes in a tissue-specific and temporal-specific manner at particular developmental stages or under various physiological and pathological conditions.\textsuperscript{3,5} Recently, enhancer activation was proposed to occur prior to a series of changes in specific gene expression in response to changing cellular environments.\textsuperscript{2,6} In other words, the alteration of specific gene expression in response to a pathological situation is initiated by altering the activities of a pathology-specific enhancer or enhancers in cells.

Natriuretic peptides are hormones with pleiotropic effects such as the decrease in vascular tone, the excretion of electrolyte and water from kidney, anti-fibrotic and anti-hypertrophic effects in heart. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), encoded by \textit{Nppa} and \textit{Nppb} genes, respectively, are synthesized predominantly in the cardiac myocytes\textsuperscript{7,8} and released into the blood, although many other cell types have been reported to express BNP.\textsuperscript{9,10} BNP is routinely used in clinical practice for diagnosing heart failure,\textsuperscript{1,12} because BNP expression levels are low in healthy individuals but markedly altered in patients suffering from heart failure depending on the pathological condition.\textsuperscript{13} This spatiotemporal expression profile of BNP led us to attempt to identify a cardiomyocyte-specific and stress-responsive enhancer that activates \textit{Nppb} transcription in response to pathogenic heart conditions. By analyzing the 50-kb \textit{Nppa–Nppb} locus flanked by two insulators to which CCCCTC binding factors (CTCFs) bind, we previously identified a possible stress-responsive CR9 enhancer that is a highly conserved 650-bp region located 22-kb upstream from the \textit{Nppb} gene.\textsuperscript{14} The CR9 region coincides with RNA polymerase II and transcriptional coactivator protein p300 binding sites, overlaps with the gene area modified by monomethylation of histone H3 on Lys 4 (H3K4me1), and interacts with \textit{Nppa} and \textit{Nppb} according to the 3C assay, thereby fulfilling the criteria for an enhancer element.\textsuperscript{14} By using a noninvasive and quantitative live imaging system of transgenic mouse lines (Tg(CR9-luc) mice), in which the CR9 enhancer element and a minimal CMV promoter drive expression of a luciferase reporter gene, we have demonstrated cardiac-specific activation of CR9 during the progression of heart failure in both pressure overload-induced and phenylephrine-induced cardiac failure models.\textsuperscript{14} Importantly, CR9 activity reportedly shows strong positive correlation with \textit{Nppa} and \textit{Nppb} genes expression levels,\textsuperscript{14} which suggested that CR9 regulates the expression of both natriuretic peptides in response to hemodynamic cardiac overload.

Clinically, serum BNP levels increase under pathophysiological conditions associated with cardiac hypertrophy and failure, which correlates strongly with left ventricular wall stress.\textsuperscript{13} Ventricular wall stress, in turn, is directly proportional to left ventricular radius and pressure. The provocative stimuli associated with cardiac hypertrophy and cardiac failure, such as mechanical stretching\textsuperscript{15,16} and catecholamines,\textsuperscript{7} are known to upregulate \textit{Nppb} gene expression in cultured cardiomyocytes.
Doxorubicin, an anthracycline antibiotic used for treating cancers, can also cause cardiac failure and elevated plasma BNP concentration in patients due to its dose-dependent cardiotoxicity. However, the mechanism by which Nppb gene expression is upregulated by various kinds of stimulations associated with cardiac hypertrophy or failure remains largely unknown.

As far as we know, the CR9 is the only enhancer susceptible to cardiac pathological conditions, which was identified in the whole genome. Analyzing the CR9 activation can provide insight into the mechanism underlying the upregulation of natriuretic peptides expression by stimulations associated with cardiac hypertrophy and failure, such as mechanical loads, neurohumoral stimulations, and drugs with cardiotoxicity. Because the pathology-specific enhancer can alter gene expression in response to pathological stimuli, we hypothesized that the CR9 enhancer can respond to the specific conditions associated with cardiac hypertrophy and failure, thereby leading to increased natriuretic peptides expression. Therefore, we aimed to investigate the pathophysiological factors and pathways that trigger CR9 enhancer activity in parallel with Nppa and Nppb genes expression and report our findings here.

2 MATERIALS AND METHODS

2.1 Reagents and antibodies

2.3-Butanedione monoxime (BDM), phenylephrine (PE), doxorubicin (Dox), and adrenaline (Adr) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MYK-461 was purchased from APExBIO (Houston, TX, USA). IL-1b was purchased from R&D systems. Bright-Glo Luciferase Assay System and VivoGlo Luciferin, In Vivo Grade were purchased from Promega (Madison, WI, USA). MTT assay kit for cell viability (Cell Titer 96®AQueous One Solution Cell Proliferation Assay) was also purchased from Promega. The following antibodies were purchased from the indicated suppliers: anti-MLP (1:500; abcam, #42504); anti-Gapdh antibody (1:2000; Millipore, MAB374); anti-p44/42 MAPK (Erk1/2) (1:3000; Cell Signaling, #4695); anti-Phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) (1:3000; Cell Signaling, #4377); HRP-coupled goat anti-rabbit (1:8000; Cappel, #55696); and anti-mouse IgG (1:8000; Cappel, #55550).

2.2 Tg-CR9-Luc mice

We used the Tg-CR9-Luc mice previously reported in which the CR9 enhancer element (650-bp fragment) and a minimal CMV promoter driving the luciferase reporter gene were introduced in the germline. The Tg-CR9-Luc mice allow us the live imaging for real-time monitoring of the CR9 enhancer activity noninvasively and quantitatively.

2.3 Treatments of Tg-CR9-Luc mice with doxorubicin (Dox), phenylephrine (PE), or adrenaline (Adr)

Transgenic mice aged 10 weeks and weighing 25-30 g were treated with Dox (24 mg/kg/2 weeks) intraperitoneally or with PE (2.1 g/kg/4 weeks) or Adr (33.6 mg/kg/4 weeks) using an osmotic minipump (Alzet, Cupertino, CA, USA) to induce cardiac hypertrophy or failure, as previously reported.

2.4 Echocardiographic measurements

At 2 and 4 weeks after the treatment, the echocardiographic measurements were performed in surviving mice under light anesthesia with 1.0%–1.5% isoflurane. A two-dimensional parasternal short-axis views were obtained at the levels of the papillary muscles. After it had been ensured that the imaging was on axis, two-dimensional targeted M-mode tracings were recorded.

2.5 Body weight and organ weights

After an addition of avertin, mice were euthanized by cervical dislocation under deep anesthesia with avertin (2.5%wt/vol, total 10 μL/g of intraperitoneal injection). Hearts and lungs were then excised and weighed.

2.6 In vivo bioluminescence imaging

In vivo bioluminescence imaging was performed as the previously reported. The mice were anesthetized and shaved from the neck to the lower torso to allow the optimal visualization of fluorescence. Five minutes after the injection of β-luciferin solution (VivoGlo Luciferin) intraperitoneally (150 mg/kg i.p.), mice were imaged using an in vivo live imaging system (IVIS Lumina II; Caliper Life Sciences, Waltham, MA, USA). The bioluminescence intensity of firefly luciferase was measured at the region of interest and expressed in relative light units (RLU/min) using Living Image 4.0 (Caliper Life Sciences). To calculate the enhancer activity in the heart, we defined the ratio of heart to brain luciferase intensities as the cardiac-specific enhancer activity.

2.7 Primary culture of neonatal rat cardiomyocytes and cardiac fibroblasts

Ventricular myocytes or cardiac fibroblasts obtained from 1- or 2-day-old Wistar rats were prepared and cultured...
overnight in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 1% (w/v) streptomycin, 1% (w/v) penicillin and 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere at constant humidity, as described previously.²⁴ Twenty-four hours before the stimulations, the medium was replaced with DMEM with 1% FBS.

2.8 | CR9 enhancer assay in cultured rat neonatal cardiomyocytes

The lentiviral particles were produced as previously reported.¹⁴ In short, the CR9 fragment (650-bp) was subcloned into a lentiviral vector encoding the firefly luciferase reporter (pGreenFire Transcriptional Reporter Lentivector: System Biosciences, Mountain View, CA, USA). 293T cells were cotransfected with the lentiviral vector and the three lentiviral packaging plasmids (ie, pMDLg/pRRE, pRSV-Rev, and pMD2.VSV.G) using Lipofectamine 2000 (Invitrogen). The supernatant from 293T cells containing the lentiviral particles was collected 48 hours after transfection, sterilized using a 0.45-m cellulose acetate filter, and concentrated by centrifugation (Peg-it Virus Precipitation Solution, System Biosciences).

Rat neonatal cardiomyocytes were plated in 96-well plates. Twenty-four hours after the plating, the medium was replaced with a serum-free medium containing the lentiviral vector carrying the CR9-reporter gene. Twenty-four hours after the transduction, the cells were exposed to 100-µM PE, 1-µM Dox, or 10-nM Adr for 24 hours prior to the luciferase assay. Luciferase assay was performed using Bright-Glo Luciferase Assay System (Promega) according to the manufacturer’s instructions. In brief, Bright-Glo Reagent equal to the volume of culture medium was added in each plate/well and mix. Two minutes later, luminescence was measured by a luminometer.

2.9 | siRNA transfection

To knock down endogenous MLP, rat neonatal cardiomyocytes were transfected with siRNAs (30 nM) targeting MLP (siMLP-1, sense:ggccgucguucaacauuTT; antisense: auuguug-gaaugcagggccAA; siMLP-2, sense: caaguucucgcaauuTT; antisense: aacuuccgcagagaucugGA) using Lipofectamine RNAiMAX (Invitrogen). siControl was used as a negative control. Efficiency of siRNA-mediated knockdown was confirmed at 48 and 72 hours after incubation with siRNAs.

2.10 | RNA extraction and quantitative PCR

The total RNA extracted from rat cardiomyocytes or murine hearts using the RNA-Bee RNA isolation reagent (Tel-Test, Friendswood, TX, USA) was converted to cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. The quantitative PCR was performed using the Thunderbird SYBR qPCR Mix (Toyobo) and the StepOnePlus real-time PCR System (Applied Biosystems). All samples were processed in duplicate. The level of each transcript was quantified according to the threshold cycle (Ct) method using Actb as an internal control. The following primers sequences were used for real-time PCR for each gene: Nppb mouse Fw gtcagctgttggttgtg, Rv cgccccgaagggaaagaaga; Nppb rat Fw gtcagctgtggctgtg, Rv ccagctggggaagaag; Actb mouse Fw ctaggccaccgctgaaag, Rv aacccacagccagaca; Actb rat Fw cccgcaagtaacctctt, Rv egctatccatggaagct.

2.11 | Measurement of BNP concentration

BNP concentrations in culture media of cardiomyocytes or plasma from mice were measured by ELISA (BNP-45 Rat ELISA Kit, AssayMax) according to the manufacturer’s instructions.

2.12 | Cell motion imaging of cultured cardiomyocytes

Twenty-four hours after the plating of rat neonatal cardiomyocytes, culture media were replaced with serum-free medium containing the lentivector carrying the CR9-reporter gene. Eight hours after the medium replacement, cardiomyocytes were treated with PE, Dox, or Adr in the presence or absence of MYK461 or 2.3-BDM. Five minutes after drug treatment, video images of cultured rat neonatal cardiomyocytes were recorded under the electrical stimulation (1 Hz, 15 V) as sequential phase-contrast images with a 4 × objective at a frame rate of 150 frames/s and a resolution of 2048 × 2048 pixels using the SI8000 cell motion imaging system (Sony Corporation, Tokyo, Japan), and then parameters of cell contraction and relaxation were calculated.

2.13 | Periodic mechanical stretch stimulation of cultured cardiomyocytes

Neonatal rat cardiomyocytes were seeded into a silicone culture plates (STB-CH-10, Strex Inc, Osaka, Japan) at a density of 1 × 10⁵ cells/chamber. Twenty-four hours after the plating, the medium was replaced with a serum-free medium containing the lentiviral vector carrying the CR9-reporter gene. Twenty-four hours later, the medium was exchanged with serum-free medium again, and then the chamber was
attached to a stretching apparatus (STB-140, Strex), and a cyclic uniaxial stretch (120% of resting length, 0.5 Hz) was applied for 12 hours (at 37°C, 5% CO₂).

### 2.14 Periodic hydrostatic pressure stimulation of cultured cardiomyocytes

Confluent cultures of rat neonatal cardiomyocytes were exposed to a hydrostatic pressure, as previously described with a slight modification. Twenty-four hours after the plating, the medium was replaced with serum-free DMEM containing 1% FBS, 25-mM HEPES-NaOH, pH 7.4 (H4034, Sigma-Aldrich), 2-mM l-glutamine (25300-081, Thermo Fisher Scientific), and 1% P/S (ie, the experimental medium), and then cells were exposed to the periodic hydrostatic pressure (HP) of repeated cycles (0.01 Hz) between 101 and 135 kPa (absolute) for 12 hours using our original HP system as described previously. Pressure was applied to cells by compressing the volume of the medium. The system was maintained at 37°C in a CO₂ incubator (5% CO₂).

### 2.15 Effects of MYK-461 on the a1-adrenergic signaling pathway by phenylephrine

MYK461 is a specific inhibitor of cardiac myosin ATPase with an IC₉₀ value of 490 nM in the bovine system and 711 nM in the human system. Rat neonatal cardiomyocytes were plated in 35-mm dishes. Twenty-four hours after the plating, the medium was replaced with a serum-free medium. Twenty-four hours later, cells were treated with 0.5-µM MYK-461 for 30 minutes and then were subsequently exposed to 50-µM phenylephrine for 10 minutes prior to the immunoblot analysis for phosphorylated ERK and ERK.

### 2.16 Effects of hypoxia on the CR9 activity and Nppb gene expression in C2C12 cells

C2C12 cells, a mouse myoblast cell line, were plated in 35-mm dishes and 96-well plates for the quantitative real-time RT-PCR and luciferase assay, respectively. Twenty-four hours after the plating, the medium was replaced with medium containing the lentiviral vector carrying the CR9-reporter gene. Twenty-four hours after the transduction, C2C12 cells were cultured in 95% N₂ and 5% CO₂ atmosphere for 8 hours to produce hypoxic condition and then measured the Nppb gene expression level by quantitative real-time RT-PCR and luciferase activities.

### 2.17 Effects of IL-1β on the CR9 activity and Nppb gene expression in neonatal rat cardiomyocytes

Rat neonatal cardiomyocytes were plated in 96-well plates or 35-mm dishes. Twenty-four hours after the plating, the medium was replaced with serum-free medium containing the lentiviral vector carrying the CR9-reporter gene. Twenty-four hours after the transduction, the cells were exposed to IL-1β (1 or 10 ng/mL) for 24 hours prior to the luciferase assay or quantitative real-time RT-PCR for Nppb gene.

### 2.18 Statistical analysis

All data are expressed as means ± SD. Repeated measures, two-way ANOVA was used for multiple group comparisons over multiple time points. One-way ANOVA was performed for multiple-group comparisons at single time point. Tukey post hoc analysis was used for all ANOVAs. In cases of experiment in a small sample size, nonparametric analysis as a Kruskal–Wallis test was performed, and Dunn’s multiple comparisons test was also performed as a post hoc analysis. Values of P < .05 in each analysis were considered to represent a significant difference.

### 3 RESULTS

#### 3.1 CR9 enhancer activity is upregulated in pharmacologically induced cardiac hypertrophy and cardiac failure

We previously demonstrated that CR9 enhancer activity, measured by CR9-driven luciferase activity, can be activated in hypertrophic and failing hearts of mice exposed to pressure overload. In the present study, to determine whether the CR9 element can respond to pharmacologically induced cardiac hypertrophy or cardiac failure, we treated Tg(CR9-luc) mice with either phenylephrine (PE), adrenaline (Adr), or doxorubicin (Dox), all of which are known to induce cardiac hypertrophy or cardiac failure (Figure 1A). Both PE and Adr groups showed significant increases in heart weight/body weight (HW/BW) ratio (Table 1), left ventricular posterior wall thickness in diastole (LVPWd), and %LV fractional shortening (%LVFS) (Figure 1B and Table 2) on Day 28, which indicate the development of cardiac hypertrophy. The Dox group showed significant LV dilatation with reduced %LVFS on Day 28 after initiating Dox treatment (Figure 1B and Table 2), which indicates the development of cardiac failure. Importantly, the ratio of heart to brain luciferase intensity, a measure of cardiac-specific CR9 enhancer activity, increased over time from Days 14 to 28 in all groups.
FIGURE 1 CR9 enhancer-driven transcription is activated in pharmacologically induced cardiac failure and hypertrophy. A, Transgenic mouse expressing the luciferase reporter construct containing the CR9 element followed by the minimal CMV promoter (Tg(CR9-luc) mice) and the experimental design. B, Sequential M-mode echocardiograms (upper) and in vivo live imaging (IVIS) (lower) of representative Tg(CR9-luc) mice before treatment as well as 14 and 28 days after treatment. Open bars in echocardiograms indicate maximal left ventricular internal dimension in diastole (LVIDd) and maximal left ventricular internal dimension in systole (LVIDs). Insets in in vivo live images show magnified images of the heart. Color scale depends on the ratio relative to brain intensity. C, Time course changes in cardiac-specific CR9 enhancer activities determined by IVIS (B) were expressed by the heart to brain ratio of luciferase intensity. n = 10 in Dox, PE, and Adr groups; n = 10 in control group. Two-way repeated measures analysis of variance (ANOVA). D and E, Relative Nppb mRNA levels in the (D) ventricular myocardium and (E) plasma BNP concentrations of Tg(CR9-luc) mice at 28 days after the treatments were measured by quantitative RT-PCR or ELISA, respectively. Nppb mRNA levels were normalized to those of the housekeeping gene Actb. The average of the Nppb/Actb mRNA ratio in the saline group was defined as 1. n = 10 in Dox, PE, and Adr groups; n = 10 in control group. One-way ANOVA, Tukey’s post hoc test. Values are means ± SD. *P < .05. **P < .01. Adr, adrenaline; Dox, doxorubicin; PE, phenylephrine
Cultured rat neonatal cardiomyocytes were treated with either 1-μM Dox, 100-μM PE, or 10-nM Adr before luminescence intensities were measured. Cardiomyocytes expressing CR9-miniP<sub>Nppb</sub>-Luc showed increased luciferase activity following treatment with PE or Adr and decreased activity following Dox treatment (Figure 2D). In contrast, cardiomyocytes expressing miniP<sub>Nppb</sub>-Luc did not show significant changes in luciferase activity except following PE treatment (Figure 2E). Thus, changes in Nppb mRNA levels and BNP concentration were proportional to CR9-miniP<sub>Nppb</sub>-Luc but not miniP<sub>Nppb</sub> activity. These results suggest that the minimal Nppb promoter does not respond to pharmacological stimuli that cause cardiac hypertrophy or cardiac failure; instead, the CR9 enhancer element is required for the Nppb promoter to respond to such stimuli.

Another point to be emphasized was that the treatment of cultured cardiomyocytes with Dox decreased CR9 activity (Figure 2D), Nppb mRNA expression level (Figure 2A), and the BNP concentration (Figure 2B), which conflict with the results in vivo experiments (Figure 1). Thus, the change in CR9 activity and Nppb expression following Dox treatment appears to differ between in vivo and in vitro settings.

### 3.2 CR9 enhancer activity and endogenous BNP expression are upregulated in cultured cardiomyocytes by treatment with either phenylephrine or adrenaline but suppressed by doxorubicin

In order to elucidate the mechanism by which CR9 activity and Nppb gene expression are upregulated in the hearts of pharmacologically induced cardiac hypertrophy or cardiac failure, we investigated the effects of PE, Adr, or Dox in cultured rat neonatal cardiomyocytes. The treatment with PE or Adr upregulated the mRNA expression level of the Nppb gene (Figure 2A) and the BNP concentration (Figure 2B), while treatment with Dox downregulated Nppb expression. Next, we prepared the two distinct lentiviral vectors with the luciferase reporter gene (Figure 2C): one is driven by the minimal −122-bp Nppb promoter alone (miniP<sub>Nppb</sub>-Luc), which includes GATA and MCAT elements, while the other is driven by the minimal −122-bp Nppb promoter fused to a 650-bp CR9 enhancer fragment (CR9-miniP<sub>Nppb</sub>-Luc).<sup>6</sup> Lentivirus-transduced cardiomyocytes were treated with

### 3.3 Mechanical stimuli increase CR9 activity and Nppb mRNA levels in cultured cardiomyocytes even in the presence of doxorubicin

To explain the discrepancy between in vivo and in vitro changes in the change in CR9 activity and Nppb expression in response to Dox treatment, we hypothesized that the observed increase in CR9 activity and Nppb expression in vivo hearts may be attributed to increased wall stress resulting from cardiac failure induced by the cardiotoxicity of Dox but not from the cardiotoxicity itself. To test this hypothesis, we subjected cultured cardiomyocytes to mechanical stimuli in the presence of Dox. First, we subjected cultured rat neonatal cardiomyocytes to cyclic mechanical stretching (20% of cell length, 0.5 Hz for 12 hours, Figure 3A), which mimics the volume overload during diastole.<sup>23</sup> Mechanical stretching increased Nppb mRNA levels (Figure 3B) and BNP concentration in the...
Importantly, mechanical stretching increased reporter gene expression only in cardiomyocytes expressing CR9-miniP<sup>Nppb</sup>-Luc (Figure 3D), which was proportional to Nppb mRNA levels (Figure 3B), but not in those expressing miniP<sup>Nppb</sup>-Luc (Figure 3E).

Next, we exposed cultured rat neonatal cardiomyocytes to mechanical periodic hydrostatic pressure (0.01 Hz between 101 and 135 kPa absolute for 12 hours, Figure 3F), which mimics the compressive force that these cells experience during systole.<sup>23</sup> In addition to mechanical stretching, hydrostatic pressure increased Nppb mRNA levels (Figure 3G) and BNP concentration in the culture medium (Figure 3H) even in the presence of Dox. Again, hydrostatic pressure increased reporter gene expression only in cardiomyocytes expressing CR9-miniP<sup>Nppb</sup>-Luc (Figure 3I), and the increase was proportional to the increase in Nppb mRNA levels (Figure 3G), but not in cells expressing miniP<sup>Nppb</sup>-Luc (Figure 3J).

Together, the above results indicate that mechanical stimuli play important roles in CR9 enhancer activation and Nppb expression in cardiomyocytes. In addition, increased CR9 activity and Nppb expression in the in vivo failing heart following Dox treatment may be attributable to increased mechanical stresses associated with cardiac failure.

### 3.4 The CR9 enhancer element activates transcription in a manner specific to pathological condition and cell type

Because the proximal −520 bp of the Nppb promoter, which includes GATA (GATA4) and Nkx2.5 elements, was previously proposed to be essential for mechanical stretch-induced cardiomyocyte hypertrophy,<sup>24</sup> we went on to compare the CR9 enhancer element with the proximal −520 bp of the Nppb promoter regarding the specificity of the pathological condition (Figure 4A). We prepared three distinct lentiviral vectors with the luciferase reporter gene (Figure 4A): the first is driven by the minimal −122-bp Nppb promoter alone which contains the GATA site (miniP<sup>Nppb</sup>-Luc), the second is driven by −520 bp of the Nppb promoter (−520 P<sup>Nppb</sup>-Luc), which contains the minimal 122-bp Nppb promoter, and the third is driven by the minimal −122-bp Nppb promoter fused to a 650-bp CR9 enhancer fragment (CR9-miniP<sup>Nppb</sup>-Luc). Consistent with a previous report,<sup>24</sup> the proximal −520 bp of the Nppb promoter responded to mechanical stretching (Figure 4B). However, the CR9 element showed a more potent ability to respond to mechanical stretching (Figure 4B). Similarly, the CR9 element also showed a more robust response to mechanical pressure (Figure 4C) and to phenylephrine treatment (Figure 4D) than the proximal −520 bp of the Nppb promoter. Importantly, the responses of the CR9 element against mechanical stresses and phenylephrine treatment could not be observed when the CR9-miniP<sup>Nppb</sup>-Luc
was transduced in cardiac fibroblasts (Figure 4E-G), indicating that the CR9 element behaves in a cell-type specific manner.

Consistent with these results, Nkx2.5 sites within the CR9 element are well conserved among mammalian species (Figure S2). In contrast, the −387 Nkx2.5 site within the proximal −520 bp of the Nppb promoter, which was previously reported to be a Nkx2.5 binding element critical for stretch-activated Nppb transcription,[24] showed four nucleotide differences out of seven between human and mouse DNA sequences (Figure S1) and was not conserved in dog, horse, and opossum (Figure S1). These results suggest that CR9 is a critical enhancer element for sensing mechanical stress and modulating gene expression in cardiomyocytes in response to such stress.

3.5 Knocking down MLP expression decreases CR9 enhancer activity and endogenous BNP expression induced by mechanical and adrenergic stimulation

Next, we focused on the signal transduction pathways that convert mechanical and adrenergic stimuli into biochemical signals that trigger CR9 activity and Nppb mRNA expression.
Cardiomyocytes possess an intrinsic system for responding to mechanical loads: mechanosensing and mechanotransduction pathways, which upregulate fetal gene programs that include Nppb. Cardiomyocytes sense extracellular and intracellular mechanical stimuli using costameres located at the Z-disc, and the integrin adhesion complex in the costamere is connected to cardiac myosin in the sarcomere. MLP, encoded by Csrp3, at the Z-disc is one of the mechanosensor
proteins expressed in cardiomyocytes, because the increase in Nppb mRNA expression upon mechanical stretching was ablated in cardiomyocytes isolated from MLP knockout (Csrp3−/−) mice. Hence, we knocked down MLP expression using siRNA for 48 hours in cultured cardiomyocytes (Figure 5A,B) before exposing them to either mechanical stresses or adrenergic stimuli. The induction of Nppb mRNA expression (Figure 5C) as well as the activation of the CR9 enhancer (Figure 5D) was suppressed by MLP knockdown following not only the mechanical stretching but also application of hydrostatic pressure. Notably, PE-induced CR9 activation and Nppb expression were also suppressed by MLP knockdown. These results suggest that the mechanosensory pathway at the Z-disc contributes to signal transduction of mechanical loads and adrenergic stimuli in order to induce CR9 activation and Nppb expression partially through MLP.

**Figure 4** CR9 enhancer element enables pathology-specific Nppb transcription. A. The luciferase reporter constructs containing either the minimal −122-bp Nppb promoter alone (miniP−122-Luc), −520 bp of the Nppb promoter which contains the minimal −122-bp Nppb promoter (−520P−122-Luc), or the −122-bp Nppb promoter fused to the 650-bp CR9 enhancer fragment (CR9-miniP−122-Luc). B-D, Cultured cardiomyocytes transduced with the respective luciferase reporter constructs were exposed to either (B) mechanical stretching (0.5 Hz, 20% elongation), (C) periodic hydrostatic pressure (0.01 Hz between 101 and 135 kPa absolute for 12 h), or (D) adrenergic stimulation (100-µM phenylephrine). E-G, Cultured cardiac fibroblasts transduced with the respective luciferase reporter constructs were exposed to either (E) mechanical stretching, (F) hydrostatic pressure, (G) or adrenergic stimulation in the same ways as in (B)–(D). Twelve hours after the stimulations, the luciferase reporter activities were measured. The luciferase activities were normalized to that of the respective controls that were not exposed to the stimulations, and the values in the controls were defined as 1 in (b)–(G). n = 4 in each group. Kruskal–Wallis test, Dunn’s post hoc test. Values are means ± SD. *P < .05

**Figure 5** MLP knockdown suppresses Nppb expression and CR9 enhancer activation. **A** and **B**, Western blot analyses of MLP expression in cardiomyocytes isolated from wild-type and MLP knockout (Csrp3−/−) mice. **C**, Real-time PCR analyses of Nppb mRNA expression in cardiomyocytes isolated from wild-type and MLP knockout (Csrp3−/−) mice. **D**, Real-time PCR analyses of CR9 mRNA expression in cardiomyocytes isolated from wild-type and MLP knockout (Csrp3−/−) mice.
3.6 | Cardiac myosin contributes to transduction of signals from mechanical and adrenergic stimuli to the CR9 enhancer in cardiomyocytes

Next, we inferred that cardiac myosin tension in the sarcomere may affect mechanosensing and mechanotransduction, because cardiac myosin is connected to the Z-disc and the costamere. Accordingly, we exposed cultured cardiomyocytes to either mechanical or adrenergic stimuli in the presence of MYK-461, a specific inhibitor of cardiac myosin II ATPase. MYK-461 suppressed the sarcomere tension expressed by average contraction deformation distance (CDD) (Figure 6A,B), which is used as a surrogate for the traction force developed by cultured cardiomyocytes, without affecting cell viability (Figure 6C) and other cell response (Figure S3).

In fact, the upregulation of Nppb expression accompanied by CR9 activation upon mechanical stretching (Figure 6D,G) or hydrostatic pressure (Figure 6E,H) were suppressed by MYK-461 treatment. We obtained similar results when we used 1 mM of 2,3-butanedione 2-monoxime (BDM), another
cardiac myosin ATPase inhibitor, as a substitute for MYK-461 (Figure S4). These results indicate that the activation pathway for the CR9 enhancer and Nppb mRNA expression upon mechanical stimulation involves the mechanosensing and mechanotransduction pathway from the costamere to the sarcomere. In addition, adrenergic stimuli (PE)-induced up-regulation of CR9's activity and Nppb expression were also suppressed by MYK-461 cotreatment (Figure 6F,I), indicating that the observed effects by adrenergic stimuli can also be explained in part by activation of the mechanosensing and mechanotransduction pathway leading from the costamere to the sarcomere. Furthermore, Nppa gene showed a similar expression pattern with Nppb gene (Figure 6J-L), suggesting the coregulation of BNP and ANP expression by the CR9 enhancer.

3.7 | The effects of other stimuli on the CR9 activity

Finally, we investigated the effects of other stimuli on the CR9 activity. IL-1β is an inflammatory cytokine that up-regulates Nppb gene expression. Thus, we treated culture cardiomyocytes with IL-1β and demonstrated that IL-1β upregulated Nppb expression in parallel with the enhanced CR9 activity in cardiomyocytes (Figure S5). Hypoxia is known to upregulate Nppb expression in C2C12 cells. Indeed, the mRNA expression level of Nppb was upregulated by hypoxia for 8 hours in C2C12 cells (Figure S6), although its expression level was substantially lower than that of cardiomyocytes. Interestingly, the parallel increase of the CR9 activity was not observed in the hypoxia-induced upregulation in Nppb expression, which suggested that the CR9 was not involved in BNP expression in response to the hypoxia in C2C12 cells.

4 | DISCUSSION

We have investigated the regulatory mechanism of CR9 enhancer activity under pathogenic heart conditions. Our major findings in the present study were as follows: (a) the CR9 enhancer can be activated by mechanical cues and adrenergic stimulations, which are proportional to Nppb expression in cardiomyocytes; (b) the increase in CR9 activity and Nppa and Nppb expression were suppressed by either suppressing myosin tension or by knocking down MLP, both of which are components of the mechanosensation and mechanotransduction pathway (Figure 7); and (c) the CR9 element can respond to mechanical stimuli and to catecholamine more robustly than the −520-bp Nppb promoters and confer pathological condition-specific induction of downstream genes.

Using the Tg(CR9-luc) mice, we previously demonstrated that pressure overloading by TAC surgery induced continuous upregulation of CR9 enhancer-driven transcription in mice hearts. In the present study, on the other hand, we used cardiac hypertrophy or cardiac failure models induced by pharmacological agents such as PE, adrenaline, or DOX. All three drugs successfully induced cardiac hypertrophy or cardiac failure, and CR9 enhancer activity continued to be upregulated during the progression of cardiac hypertrophy or cardiac failure, as well as cardiac hypertrophy induced by TAC surgery. Importantly, the levels of CR9 activity in live imaging analysis were proportional to Nppb mRNA levels and plasma BNP concentration on Day 28. Our results indicate that CR9 activity in this live imaging system accurately reflects the level of Nppb expression in various mouse pathological heart models. Correspondingly, treatment with either PE or adrenaline upregulated CR9 activity associated with the upregulation of Nppb transcription in cultured cardiomyocytes. However, treatment with Dox unexpectedly showed opposite responses between in vivo hearts and cultured cardiomyocytes; CR9 activity and Nppb transcription were upregulated in vivo hearts but suppressed in cultured cardiomyocytes. These findings are consistent with those of a previous report which found that Dox suppresses Nppb expression in cultured cardiomyocytes by inhibiting RNA synthesis due to intercalation of the drug in genomic DNA.

Accordingly, we inferred that the upregulation of CR9 activity observed in vivo hearts was triggered by increased wall stress resulting from cardiac damage followed by contractile failure with LV dilation induced by the cardiotoxicity of Dox but not from the cardiotoxicity itself. Indeed, mechanical loads such as mechanical stretching or hydrostatic pressuring upregulated CR9 activity and Nppb expression in cultured cardiomyocytes even in the presence of Dox. Thus,
it seems likely that the plasma concentration of BNP starts to rise in doxorubicin-induced cardiomyopathy when the degree of hemodynamic mechanical loading following LV pump failure and LV dilatation overwhelm the suppressive effect of doxorubicin on Nppb expression. We believe that it is worth emphasizing that we only observed the marked upregulation of CR9 activity by mechanical loads when the CR9 element was present upstream of the promoter and the reporter gene. These results strongly support our hypothesis that the CR9 element is critical for upregulating Nppb gene expression in cardiomyocytes in response to mechanical loads. Furthermore, we found that the CR9 enhancer can be activated only in cardiomyocytes but not in cardiac fibroblasts. Accordingly, we conclude that the CR9 selectively regulates cardiomyocyte Nppb expression in pathological condition of cardiac hypertrophy and failure. Indeed, we could not observe any CR9 signals that correlated with the development of cardiac hypertrophy or failure in other parts of body except heart in Tg(CR9-Luc) mice. Furthermore, the hypoxia-induced upregulation of Nppb expression was not accompanied with the upregulation of the CR9 activity in C2C12 cells. Considering that the enhancer can act as the temporal and/or spatial cis-regulatory element to control certain gene expression, our results suggest that CR9 acts as an enhancer by sensing the various types of mechanical stresses in cardiomyocytes and regulating Nppb expression. However, we do not deny the possibility that the CR9 can respond to the other stimuli to regulate Nppb expression. Indeed, IL-β increased Nppb gene expression accompanied with the upregulation of the CR9 activity in cardiomyocytes. Considering that circulating natriuretic peptide levels are higher in patients with rheumatic diseases than healthy controls even in the absence of clinically apparent cardiovascular diseases, cytokines, such as IL-1β, IL-6, and TNF-α, could also modulate the CR9 activity to regulate Nppb expression. However, it is unable to give a definite answer about the mechanism how cytokines modulate the CR9 activity at this time. It is also interesting to examine whether the CR9 regulates Nppb expression in other cell types, because BNP was reported to be induced in other than cardiomyocytes such as satellite cells in response to inflammation and hypoxia.

In the current study, Nppa gene showed similar expression patterns with Nppb gene against the mechanical stimulations and phenylephrine treatment. Previous study demonstrated the physical contact between Nppa, Nppb genes, and their regulatory elements for the coordinated induction of these
genes. We also previously demonstrated the long-range physical interaction between the CR9 element and the proximal promoters of both the Nppa and Nppb genes by a 3C assay. Although we mostly used Nppb expression as parameter in the current study, Nppa expression is usually coregulated with Nppb expression except few disease conditions such as inflammation. Thus, we suggest that the CR9 element drives the expression of both natriuretic peptides in cardiomyocytes by physically interacting with the proximal promoters of both the Nppa and Nppb genes.

Mechanical stimulation activates hypertrophic pathways that are mediated by the mechanical stress-induced release of hormonal peptides such as adrenergic agonists, angiotensin II, and endothelin from neighboring cardiac fibroblasts. Aside from these paracrine pathways, cardiomyocytes possess intrinsic response systems for mechanical stimuli, namely, mechanosensing and mechanotransduction pathways. Cardiomyocytes are always exposed to a variety of mechanical stimuli, both static and dynamic, including compressive and tensile stresses and strains, and fluid shear stresses and alterations in substrate stiffness. These mechanical stimuli are detected by mechanosensors and transduced into transcriptional responses, including the re-expression of fetal gene programs that include Nppb. In cardiomyocytes, these mechanical stimuli are sensed through the costameres through which cardiac myosin attach to the extracellular matrix using the integrin adhesion complex, the dystrophin–glycoprotein complex, and desmin intermediate filaments (Figure 7). Among these, the stretching dynamics of talin molecules in the integrin adhesion complex are expected to alter mechanosensing and mechanotransduction depending on the mechanical environment. Mechanical environmental changes are also sensed by measuring the combined forces from nonmuscle and muscle myosin in cardiomyocytes. Because cardiac myosin in the sarcomere is connected to the integrin adhesion complex in costameres through titin and cytoplasmic actin filaments, which are in turn connected through nonmuscle myosin, the tension developed by cardiac myosin in sarcomeres may affect the stretching dynamics of talin in costameres (Figure 7). Indeed, the transcriptional response of the CR9-driven reporter gene and Nppb towards treatment with either PE or Ad was blunted by suppressing the tension from sarcomeres with MYK-461, a specific cardiac myosin ATPase inhibitor. Hence, we suggest that increased Nppb expression following by adrenergic stimulation could be caused in part by elevated tension in sarcomeres in cardiomyocytes treated with adrenergic agonists.

The Z-disc or sarcomere also acts as a mechanical sensor and is involved in both mechanosignaling and mechanotransduction (Figure 7). MLP is thought to be involved in mechanosignaling processes at the Z-disc because cardiomyocytes isolated from MLP knockout (MLP−/−) mice were defective in inducing Nppb expression upon mechanical stretching. Consistent with this, our data demonstrated that siRNA-mediated MLP knockdown blunted the induction of Nppb expression upon mechanical stretch stimulation, which is accompanied by reduced CR9 activity. Furthermore, the induction of CR9 activity and Nppb expression was also suppressed upon mechanical pressure stimulation. Taken together, these findings led us to propose that CR9 can respond to various mechanical loads that are sensed by the Z-discs and costameres in cardiomyocytes and that mechanosensory signals can be transduced to CR9 through cardiac myosin-generated tension.

In this study, we exposed cultured cardiomyocytes to two distinct mechanical loads: mechanical stretching and mechanical pressure. There are two major hemodynamic mechanical loads for the heart: volume overload and pressure overload, both of which have been critically implicated in the pathogenesis of cardiac hypertrophy and cardiac failure. Most studies of the mechanical stress-induced cardiomyocyte hypertrophy have been performed with primary cardiomyocyte cultures seeded on an elastic substrate subjected to either static or cyclic stretching, which mimics volume overload in the in vivo heart. Consistent with previous reports, we observed upregulation of Nppb expression by stretch stimulation in cultured cardiomyocytes. In contrast, few studies have examined the effect of mechanical pressure, which mimics pressure overload in the in vivo heart, at the level of cultured cardiomyocytes because of the absence of an experimental device. In the current study, we used the pressuring device for cultured cells, which we previously developed. However, it could not be a true “afterload” for cultured cardiomyocytes, because it does not work to oppose myocyte shortening. Instead, our device imparts cyclical hydrostatic pressure. Stimulation with periodic hydrostatic pressure caused the upregulation of Nppb expression in cultured cardiomyocytes, as did the mechanical stretch model. Thus, ours is the first study to demonstrate that mechanical periodic hydrostatic pressure upregulates Nppb mRNA levels in cultured cells. The molecular mechanism how the periodic hydrostatic pressure is sensed by the cells remains to be elucidated, because the hydrostatic pressure external and internal to the cell was equal in our system, which results in negligible deformation of the cells. However, despite the lack of conspicuous deformation, the biological responses to the hydrostatic pressure can be induced in a variety of cell types. In bone marrow stem cells, physiological periodic hydrostatic pressure induced osteogenic lineage commitment. In innate immune cells, an inflammatory response was initiated by periodic hydrostatic pressure via the mechanically activated ion channel PIEZO1. Accordingly, we speculated that the hydrostatic stress may share similar signaling with tensile stress through the ECM-integrin cytoskeleton linkages to transduce mechanical signals. This pressure stimulation device will provide new insights into the mechanism of hydrostatic pressure-induced cardiac hypertrophy and failure and into the differences in pathological mechanisms depending on the type of mechanical load.
Our live imaging system for measuring CR9 enhancer activity in the hearts of Tg(CR9-luc) mice is advantageous in that there is no need to draw blood repeatedly or to remove the heart from a mouse to determine BNP level in pathological hearts. Therefore, it permits repetitive observations of BNP levels in hearts of the same individual after a certain intervention. Accordingly, we suggest that the in vivo live imaging system used here is widely applicable to studies of cardiac hypertrophy and cardiac failure or to in vivo screening of cardiotoxicity of drugs such as anti-cancer drugs.

How mechanical signals are translated into transcriptional responses remains to be determined. When CR9 acts as an enhancer, transcription factors induced by mechanical loads may bind to the CR9 element. A previous study demonstrated that the combination of tandem GATA consensus sites and an Nkx2.5 binding element in the BNP promoter are critical for stretch-activated BNP transcription. Although the CR9 element also contains both GATA and Nkx2.5 consensus sites, the CR9 element exerted a more potent response against mechanical cues and catecholamine than the proximal 520 bp of the Nppb promoter. One reason for this is a difference in conserved Nkx2.5 consensus sites between CR9 and the proximal 520 bp of the Nppb promoter. The Nkx2.5 site within CR9 is highly conserved among species compared with the Nkx2.5 site in the proximal 520 bp of the BNP promoter. Another reason is that the response of CR9 can be caused by something other than GATA and Nkx2.5 elements. Thus, future studies are necessary to identify transcription factors that are upregulated and bind to the CR9 enhancer element in response to mechanical stresses.

There are two limitations in the present study. First, there is a possibility that “mispositioning” of CR9 sequence immediately upstream of the BNP promoter may upregulate the downstream gene expression nonspecifically in cultured cardiomyocytes. However, we suggest it unlikely because CR9 sequence positioned immediately upstream of the promoter did not upregulate the reporter gene in cardiac fibroblasts and other CR sequences positioned immediately upstream of the promoter did not upregulate the downstream gene in cardiomyocytes. The next is that in vivo experiments did not show a causal link between CR9 and Nppb expression but just show a parallel increase of both. Obtaining direct evidence of CR9 as the enhancer for Nppb gene transcription in response to mechanical stresses requires a CR9 knockout mouse. Therefore, we have been working to develop one. In future studies, we will aim to gather direct evidence of the causal link that CR9 drives natriuretic peptides transcription in response to mechanical stresses in cardiomyocytes.

In conclusion, CR9-driven transcription is activated by mechanical loads in cardiomyocytes through the mechanosensing and mechanotransduction pathway. In the pathological conditions of hearts with volume overloading or pressure overloading, CR9 may be activated to upregulate natriuretic peptides expression.

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CONFLICT OF INTEREST
The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS
O. Tsukamoto and K. Matsuoka designed research; O. Tsukamoto, K. Matsuoka, and Y. Miyashita analyzed data; K. Matsuoka, Y. Miyashita, and O. Tsukamoto performed research; O. Tsukamoto, K. Matsuoka, and Y. Miyashita wrote the paper; Y. Shintani, H. Kioka, M.H. Kato, M. Kitakaze, Y. Sakata, Y. Asano, and S. Takashima offered valuable advices; and O. Tsukamoto, K. Matsuoka K, Kamikubo, Y. Kuramoto, T. Tsubota, H. Fu, T. Takayama, H. Ito, T. Hitsumoto, C. Okamoto, R. Oya, H. Hakui, and H. Shinomiya contributed new reagents or analytic tools.

ETHICS STATEMENT
All procedures were performed according to the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) and were approved by the Animal Experiments Committee, Osaka University (approval no. 29-0071).

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**SUPPORTING INFORMATION**
Additional Supporting Information may be found online in the Supporting Information section.

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