Endothelial Restoration of Receptor Activity–Modifying Protein 2 Is Sufficient to Rescue Lethality, but Survivors Develop Dilated Cardiomyopathy

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Abstract—RAMPs (receptor activity–modifying proteins) serve as oligomeric modulators for numerous G-protein–coupled receptors, yet elucidating the physiological relevance of these interactions remains complex. Ramp2 null mice are embryonic lethal, with cardiovascular developmental defects similar to those observed in mice null for canonical adrenomedullin/calcitonin receptor-like receptor signaling. We aimed to genetically rescue the Ramp2−/− lethality in order to further delineate the spatiotemporal requirements for RAMP2 function during development and thereby enable the elucidation of an expanded repertoire of RAMP2 functions with family B G-protein–coupled receptors in adult homeostasis. Endothelial-specific expression of Ramp2 under the VE-cadherin promoter resulted in the partial rescue of Ramp2−/− mice, demonstrating that endothelial expression of Ramp2 is necessary and sufficient for survival. The surviving Ramp2+/− Tg animals lived to adulthood and developed spontaneous hypotension and dilated cardiomyopathy, which was not observed in adult mice lacking calcitonin receptor-like receptor. Yet, the hearts of Ramp2+/− Tg animals displayed dysregulation of family B G-protein–coupled receptors, including parathyroid hormone and glucagon receptors, as well as their downstream signaling pathways. These data suggest a functional requirement for RAMP2 in the modulation of additional G-protein–coupled receptor pathways in vivo, which is critical for sustained cardiovascular homeostasis. The cardiovascular importance of RAMP2 extends beyond the endothelium and canonical adrenomedullin/calcitonin receptor-like receptor signaling, in which future studies could elucidate novel and pharmacologically tractable pathways for treating cardiovascular diseases. (Hypertension. 2016;68:667-677. DOI: 10.1161/HYPERTENSIONAHA.116.07191.)

Online Data Supplement

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RAMPs (receptor activity–modifying proteins) are single-pass transmembrane proteins that physically interact with numerous G-protein–coupled receptors (GPCRs) to regulate receptor trafficking, ligand-binding specificity, and downstream G-protein coupling and signaling. Biochemical and pharmacological studies have revealed functional RAMP interactions with many GPCRs, including calcitonin receptor-like receptor (CLR=protein; Calcrl=gene), calcitonin receptor, parathyroid hormone receptor 1 and 2 (PTHR1 and PTHR2), glucagon receptor (GCGR), secretin receptor, vasointestinal peptide receptor 1 and 2 (VIPR1 and VIPR2), calcium-sensing receptor (CaSR), an estrogen receptor (GPR30), and likely additional RAMP-interacting GPCRs will be identified in the future.1–4 These phenotypes are essentially identical to those observed in mice lacking the genes that encode for CLR and other GPCRs.5–7,9–11 As expected, based on their broad tissue distribution, the phenotypes of Ramp null mice are extensive and largely reflect their interactions with multiple GPCRs.8 Ramp1 and Ramp3 null mice survive to adulthood, whereas Ramp2−/− mice exhibit embryonic lethality at midgestation caused by cardiovascular defects in the heart, blood, and lymphatic vasculatures.5–7,9,11 These phenotypes are essentially identical to those observed in mice lacking the genes that encode for CLR and its Ramp2-associated ligand, adrenomedullin (AM=protein;
Adm−gene), indicating that RAMP2 is essential for canonical CLR/AM signaling during embryonic development. Yet, mice that are haploinsufficient for Ramp2 also exhibit an expanded constellation of endocrine-related phenotypes that are not observed in Calcrl and Adm haploinsufficient mouse models. Therefore, these data imply that RAMP2 must exert functional modulation of other GPCRs, which is supported by its in vitro biochemical interaction with calcitonin receptor, PTHR1/2, GCGR, and VIPR1/2. To date, ascribing a functional relevance for these other putative RAMP2-interacting GPCR pathways in adult physiological systems has been precluded by the embryonic lethality of Ramp2−/− mice.

Here, we have developed a novel Ramp2 transgenic mouse model which overexpresses Ramp2 specifically in the endothelium, with the hypothesis that restoration of RAMP2 in the endothelium will rescue the lethality-causing vascular defects associated with loss of CLR/AM signaling during embryogenesis. Indeed, endothelial RAMP2 is sufficient to rescue the embryonic lethality of many Ramp2−/− mice. Thus, the surviving mice, which express Ramp2 in the endothelium but lack Ramp2 in every other cell type, provided us with the opportunity to further elucidate the functional implications of the loss of Ramp2 on adult cardiovascular physiology and respective changes in other Ramp2-associated GPCRs.

Methods

Mouse Studies

Previously published SvEv−Ramp2−/− and Calcrl−/− mice were both fully backcrossed (>10 generations) onto a C57BL/6J genetic background for these studies. A novel endothelial-specific Ramp2 transgenic mouse was generated and crossed to the Ramp2−/− mice. All Ramp2 mice used in this study were maintained on an isogenic C57BL/6J genetic background. The Calcrl−/− mice were crossed with either the SilTg(Thg−cre)1Her (SM22-Cre) or the inducible CAGG-CreERT mouse to conditionally delete Calcrl specifically within developmental vascular smooth muscle cells (VSMCs) or ubiquitously in adult mice, respectively. Adult CAGG-CreERT females were administered with tamoxifen as previously published. Genotyping and reverse transcription polymerase chain reaction (RT-PCR) primers and probes are listed in Table S1 in the online-only Data Supplement. Females between 4 and 8 months of age were used in all Ramp2-associated studies, males 3 to 4 months of age for VSMC-specific Calcrl studies, and females 16-month-old for ubiquitous Calcrl studies. Mice were acclimated and conscious for both hearts and thin VSMC walls.9,19,20 The aortic endothelium of Ramp2−/− mice bred normally, with expected Mendelian and sex ratios at birth, and no obvious phenotypic defects.

Transgenic Endothelial Ramp2 Partially Rescues Embryonic Lethality of Ramp2−/− Mice

We sought to determine whether endothelial Ramp2 expression could rescue the previously reported Ramp2−/− embryonic lethality by interbreeding the hemizygous Tg(Cdh5-Ramp2) animals with Ramp2−/− nontransgenic (ntg) animals, on an isogenic C57BL/6J genetic background. Genotyping and reverse transcription polymerase chain reaction (RT-PCR) primers and probes are listed in Table S1 in the online-only Data Supplement. Females between 4 and 8 months of age were used in all Ramp2-associated studies, males 3 to 4 months of age for VSMC-specific Calcrl studies, and females 16-month-old for ubiquitous Calcrl studies. Mice were acclimated and conscious for both noninvasive tail cuff blood pressure and echocardiography analysis and anesthetized with isoflurane for intra-arterial blood pressure measurements. Organs weights and heart chamber dissections were normalized to either body weight or tibia length (TL) as previously described. Biological numbers of 3 to 10 mice per genotype was used for each experiment. Endothelial cells from adult mice were isolated with magnetic-associated cell sorting using CD31-specific antibodies as previously described. Cardiomyocytes from adult mice were isolated using collagenase digestion as previously described. All animal experiments were approved by the Institutional Animal Care and Use Committee of The University of North Carolina at Chapel Hill.

Statistical Analysis

Statistical analysis was determined with GraphPad 5.0, and data are represented as mean±SEM. The unpaired Student t test was used to compare 2 groups, whereas 1-way ANOVA with Tukey multiple comparison test was used to compare ≥3 groups. Survival data were compared by Mantel–Cox and Gehan–Breslow–Wilcoxon tests. Significant differences are represented as *P<0.05, **P<0.01, ***P<0.001. Additional detailed Methods are provided in the online-only Data Supplement.

Results

Generation and Characterization of Endothelial-Specific Ramp2 Transgenic Animals

A diagram of the Cdh5−Ramp2 transgene (Tg) depicts the murine vascular cadherin 5 (Cdh5) promoter driving the expression of a flagged-tagged murine Ramp2 cDNA (Figure S1A in the online-only Data Supplement), which was successfully integrated into the genome of 2 independent founder lines on the C57BL/6J genetic background. Expression of FLAG−Ramp2 protein was confirmed using both transgenic founder lines in a variety of adult tissues including the heart, kidney, lung, and intestine (Figure S1B). Semiquantitative RT-PCR revealed the presence of FLAG transcript within whole-adult lung tissue and CD31+ endothelial-enriched cells in the Tg animals, but not in the wild-type endothelium (Figure S1C), which resulted in a modest increase in overall Ramp2 gene expression levels within lung CD31+ endothelial cells of Tg animals compared with wild-type animals (Figure S1D). Ramp2 expression was significantly higher in the left ventricles (LV) of Tg animals than in those of wild-type—a finding that we attribute to the endothelial-driven transgene expression, as compared with the low levels of endogenous Ramp2 expression in isolated cardiomyocytes (Figure S1E) and previously reported expression in VSMCs.17,18 Tg(Cdh5−Ramp2) mice bred normally, with expected Mendelian and sex ratios at birth, and no obvious phenotypic defects.
controls, despite detectable expression of Ramp2 in Ramp2−/− Tg hearts (Figure 1G through 1I). Consistent with previous reports, these data reflect that Ramp2 is required for normal VSMC and cardiac development and that endothelial Ramp2 restoration is not sufficient to rescue these defects, thus confirming nonendothelial roles of Ramp2 during cardiovascular development.

Unlike the Ramp2−/− ntg embryos, which uniformly die by e15.5, Ramp2−/− Tg embryos survive to term at near-Mendelian ratios, but a large number of these animals were found still-born at postnatal day 1 (Figure S2B). Nevertheless, by postnatal day 7, 40% of the expected Mendelian ratio of Ramp2−/− Tg pups were viable, which represents a significant survival rescue when compared with the completely penetrant lethality of Ramp2−/− ntg mice (Table 1). These surviving Ramp2−/− Tg mice were indistinguishable from Ramp2−/− Tg littermates (Figure S2C). Interestingly, there was a significant skewing in the sex ratio of the surviving Ramp2−/− Tg mice, such that 78% were female. These data demonstrate that transgenic endothelial restoration of Ramp2 is able to blunt the endothelial and edematous phenotypes observed with global Ramp2 genetic deletion, leading to significantly improved survival.

**Figure 1.** Endothelial restoration of Ramp2 partially rescues embryonic edema leading to prolonged survival of Ramp2−/− mice.

A. Representative images and (B) quantification of edema severity from e14.5 Ramp2+/+, Ramp2−/− ntg, and Ramp2−/− Tg embryos. Edema scoring system: (1) no edema, (2) mild edema, and (3) severe edema. C. Immunohistochemistry and (D) quantification of jugular lymph sac size using lymphatic vessel endothelial hyaluronan receptor-1 (Lyve1; green) and 4’6-diamidino-2-phenylindole (DAPI; magenta). E, Histology of descending aortas and (F) quantification of aortic vascular smooth muscle cell wall thickness. G, Heart histology, (H) quantification of ventricle area, and (I) whole heart relative Ramp2 expression from viable Ramp2−/− ntg, Ramp2−/− ntg, and Ramp−/− Tg e14.5 embryos. Samples were normalized to Ramp2−/− ntg and Gapdh expression. Scale bars, 100 µm. Data represented as average±SEM from n=3 to 8 mice per genotype. CA indicates carotid artery; JLS, jugular lymph sac; and JV, jugular vein. Significance determined by 1-way ANOVA with Tukey multiple comparison with *P<0.05, **P<0.01, and ***P<0.001.

**Table 1.** Endothelial Restoration of Ramp2 Can Rescue the Global Ramp2−/− Embryonic Lethality

<table>
<thead>
<tr>
<th>Mendelian Ratio</th>
<th>ntg</th>
<th>Tg(Cdh5-Ramp2)</th>
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<tr>
<td></td>
<td>Ramp2−/−</td>
<td>Ramp2−/−</td>
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<tr>
<td>Expected n</td>
<td>35</td>
<td>70</td>
</tr>
<tr>
<td>Actual n P7</td>
<td>47</td>
<td>88</td>
</tr>
<tr>
<td>Actual/expected, %</td>
<td>134</td>
<td>126</td>
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Breeding results, both expected Mendelian and actual results from Ramp2−/− Tg(Cdh5-Ramp2) crossed with Ramp2−/− ntg. n = 280 pups. Significance in survival between Ramp2−/− ntg and Ramp2−/− Tg pups determined by the Mantel–Cox test: *P<0.001.
an opportunity to evaluate how Ramp2 loss-of-function during development affects adult cardiovascular homeostasis. Diastolic, systolic, and mean arterial blood pressures were significantly reduced in \textit{Ramp2}−/− \textit{Tg} compared with \textit{Ramp2}+/− \textit{ntg} and \textit{Tg} female controls (Figure 2A). Importantly, there were no significant differences in basal blood pressure between \textit{Ramp2}+/− \textit{ntg} and \textit{Ramp2}−/− \textit{Tg} mice, indicating that the decreased blood pressure was not caused solely by the transgene. Moreover, the reduced basal blood pressure of \textit{Ramp2}−/− \textit{Tg} mice was observed without the exogenous administration of the canonical, hypotensive ligand AM, which was previously required to lower blood pressures in a VSMC-specific overexpression model of \textit{Ramp2}.21,22

Similar to the developmental defects in VSMC wall thickness of the \textit{Ramp2}−/− \textit{Tg} embryos and other models lacking AM/CLR/Ramp2 function,9,19,20 the VSMC walls were significantly thinner in \textit{Ramp2}−/− \textit{Tg} adult descending aortas than in wild-type controls (Figure 2B). Intra-arterial blood pressure measurements further confirmed the hypotensive phenotype of adult \textit{Ramp2}−/− \textit{Tg} females (Figure 2C). To test VSMC responsiveness, \textit{Ramp2}−/− \textit{Tg} and controls were challenged with intravenous injections of the \(\alpha\)-adrenergic receptor agonist, phenylephrine, and the vasodilator, AM. The \textit{Ramp2}−/− \textit{Tg} adults were capable of normal vasoconstriction and vasodilation when compared with controls (Figure 2C). So, although the thinner VSMC walls likely play a role in the hypotensive phenotype of \textit{Ramp2}−/− \textit{Tg} mice, additional systemic changes likely contribute to the hypotension.

\textbf{\textit{Ramp2}−/− \textit{Tg} Mice Develop Spontaneous Dilated Cardiomyopathy Phenotype}

Considering the hypotension and the developmental defects observed in \textit{Ramp2}−/− \textit{Tg} embryonic hearts, we next assessed how cardiac function and morphology were altered in adult \textit{Ramp2}−/− \textit{Tg} mice. Echocardiography on conscious mice revealed significantly dilated LV during both diastole and systole, with significantly larger LV volumes and LV internal diameter dimensions in the \textit{Ramp2}−/− \textit{Tg} mice compared with \textit{Ramp2}+/+ and \textit{Ramp2}−/−, with and without the transgene (Table 2). Representative M-mode echocardiograms from \textit{Ramp2}+/− \textit{ntg}, \textit{Ramp2}−/− \textit{Tg}, and \textit{Ramp2}−/− \textit{Tg} illustrate the ventricular dilation in \textit{Ramp2}−/− \textit{Tg} mice (Figure 3A), which resulted in a trending increase in calculated cardiac output in these animals (Table 2). Septum and LV posterior wall dimensions of \textit{Ramp2}−/− \textit{Tg} mice were unchanged from controls although there was a nonsignificant trend toward a thinner posterior wall. There was also a modest, but significant reduction in both ejection fraction and fractional shortening in the \textit{Ramp2}−/− \textit{Tg} dilated hearts. These data indicate that the loss of \textit{Ramp2} in nonendothelial cells leads to a spontaneous dilated cardiomyopathy (DCM)–like phenotype in adult mice, which at 6 months of age had not yet progressed to heart failure, as indicated by the elevated cardiac output and sufficient heart function.

On dissection, we observed that the hearts of \textit{Ramp2}−/− \textit{Tg} mice were grossly enlarged compared with wild-type and \textit{Ramp2}+/− \textit{ntg} mice (Figure 3B and 3C). The adult \textit{Tg} mice had no differences in body weight (\textit{Ramp2}+/− \textit{ntg}: 28.4±1.4 g, \textit{Ramp2}−/− \textit{Tg}: 28.4±1.2 g, \textit{Ramp2}−/− \textit{Tg}: 28.3±1.2 g) or TL (\textit{Ramp2}+/− \textit{ntg}: 17.8±0.2 mm, \textit{Ramp2}−/− \textit{Tg}: 17.9±0.1 mm, \textit{Ramp2}−/− \textit{Tg}: 17.8±0.2 mm) regardless of their \textit{Ramp2} genotype. Yet, total heart weights were significantly larger in the \textit{Ramp2}−/− \textit{Tg} mice when normalized to both body weight (Figure 3D) or TL (\textit{Ramp2}−/− \textit{ntg}: 7.3±0.4 mg/mm, \textit{Ramp2}−/− \textit{Tg}: 6.5±0.2 mg/mm, \textit{Ramp2}−/− \textit{Tg}: 9.0±0.3** mg/mm; **P<0.01). Moreover, when normalized to body weight, the LV (Figure 3E), right ventricle (Figure 3F), and right atria (\textit{Ramp2}−/− \textit{ntg}: 0.15±0.01 mg/g, \textit{Ramp2}−/− \textit{Tg}: 0.15±0.01 mg/g, \textit{Ramp2}−/− \textit{Tg}: 0.21±0.02* mg/g; *P<0.05) all exhibited significant enlargement in the \textit{Ramp2}−/− \textit{Tg} animals compared with all other genotypes. Similar significant trends were observed.
when the individual chamber weights were normalized to TL (LV:TL; Ramp2+/+ ntg: 5.4±0.3 mg/mm, Ramp2+/+ Tg: 4.8±0.2 mg/mm, Ramp2−/− Tg: 6.5±0.2** mg/mm; **P<0.01).

Cross-sectional area of myocytes within the LV revealed slight, but significant cardiomyocyte hypertrophy, with no changes in LV capillary density, in the Ramp2+/+ mice compared with hearts of Ramp2+/+ Tg mice (Figure 4A through 4D). Picrosirius red staining showed no differences in perivascular fibrosis, but there was significantly increased interstitial fibrosis in the Ramp2+/+ hearts compared with hearts of Ramp2+/+ and Ramp2−/− Tg mice (Figure 4E through 4H). There were elevated levels of the oxidative stress indicator, lipid peroxi-
dase evidenced by 4-hydroxynonenal staining in Ramp2−/− Tg hearts (Figure 4I through 4J). Together, the modest increases in hypertrophy, fibrosis, and oxidative stress, as well as modest decline in heart function, further support that 6-month-old Ramp2−/− Tg mice exhibit a compensated, DCM-like phenotype.

**Adult Ramp2−/− Tg Mice Develop Multiorgan Inflammation**

DCM and hypotension can lead to vascular congestion and organ dysfunction throughout the body. Consistently, we observed that the spleen:body weight ratio was significantly increased in the Ramp2−/− Tg mice compared with all other genotypes (Figure 5A). Ramp2−/− Tg mice also exhibited macroscopic vascular congestion within their livers compared with control animals (Figure 5B). Furthermore, multiorgan histology revealed a marked increase in the number of inflammatory foci, particularly surrounding the vasculature, within the liver (Figure 5C and 5D), kidney (Figure 5E and 5F), and lungs (Figure 5G and 5H). A diagnostic profile of adult serum from wild-type and Ramp2−/− Tg mice revealed few significant changes in circulating ions or enzyme levels that are typically indicative of hepatocellular or renal damage (Table S2). This supports that the end-organ inflammation in Ramp2−/− Tg mice is likely downstream of altered hemodynamics caused by DCM and hypotension, rather than primarily caused by loss-of-function of Ramp2 in end organs.

**Conditional Calcrl Deletion Does Not Lead to a DCM Phenotype.**

Because CLR and AM represent the most well-characterized, canonical pathway for RAMP2 modulation and the knockout mice for these genes recapitulate the Ramp2−/− developmental phenotypes, it is reasonable to consider that disruption of this pathway, which has been demonstrated to be cardioprotective in both animal studies and in humans, may underlie the DCM and hypotensive phenotypes in Ramp2−/− Tg mice. To test the functions of CLR in nonendothelial cells, we generated cardiac- and VSMC-specific Calcrl null mice using the SM22-Cre mediated excision. The CalcrlloxP/loxP; SM22Cre+ adult males were normotensive and had no basal changes in heart function, size, or morphology (Table
Similarly, cardiac-specific \textit{Calcrl} deletion using the αMHC-Cre+ transgenic line also failed to recapitulate the DCM phenotype, with animals surviving to adulthood with no basal cardiac dysfunction (R.T. Dackor and K.M. Caron, unpublished data, 2016). These data demonstrate that cardiac- and VSMC-specific \textit{Calcrl} expressions are not required for embryonic development or adult cardiovascular maintenance.

We have previously shown that temporal, global deletion of \textit{Calcrl} in adult \textit{Calcrl}\textsuperscript{flp/flp}\textsuperscript{P}, \textit{CAGG-CreER\textsuperscript{TM}} mice results in dilated lymphangiectasia in many lymphatic vascular beds throughout the body.\textsuperscript{13} However, these mice did not display any obvious cardiac phenotypes and they had similar heart:body weight ratio as tamoxifen-injected control mice (\textit{Calcrl}\textsuperscript{loxP/loxP} \textit{CAGG-CreERT\textsuperscript{M}}: 4.43±0.19 mg/g versus \textit{Calcrl flox/flox}: 4.13±0.14 mg/g, respectively). Furthermore, conscious echocardiography of \textit{Calcrl}\textsuperscript{loxP/loxP}\textit{CAGG-CreERT\textsuperscript{M}} female mice, even at 14 months of age, failed to reveal any significant changes in LV internal diameter, function, or heart size compared with control mice (Table S4). Collectively, these data, generated from 3 independent series of conditional deletion approaches, indicate that the global-, cardiac-, or VSMC-specific loss of \textit{Calcrl} does not recapitulate the DCM phenotype observed in the \textit{Ramp2−/− Tg} animals. Therefore, this strongly suggests that the \textit{Ramp2−/− Tg} phenotype is imparted by other RAMP2-associated GPCR pathways.

\begin{figure*}
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\caption{Ramp2\textsuperscript{−/−} Tg adults develop spontaneous dilated cardiomyopathy. A, Representative M-mode echocardiograms from left ventricles of Ramp2\textsuperscript{−/−} ntg, Ramp2\textsuperscript{−/−} Tg, and Ramp2\textsuperscript{−/−} Tg mice. Macroscopic (B) and hematoxylin-eosin (H&E; C) images of whole hearts and left ventricles from of Ramp2\textsuperscript{−/−} ntg, Ramp2\textsuperscript{−/−} Tg, and Ramp2\textsuperscript{−/−} Tg mice. Scale bars, 2 mm. Quantification of heart (D), left ventricle (LV; E), and right ventricle (RV; F) weight normalized to body weight (BW). Data represented as averagesSEM from n=4 to 10 mice per genotype. HW indicates heart weight; and N.A., not available. Significance determined by 1-way ANOVA with Tukey multiple comparison with *\textit{P}<0.05, **\textit{P}<0.01, and ***\textit{P}<0.001.}
\end{figure*}

\textbf{Ramp2\textsuperscript{−/−} Tg Hearts Exhibit Reduced Signaling Pathways and Expression of Numerous RAMP-Associated GPCRs}

The genetic reduction or downregulation of the transcriptional regulator cAMP response element-binding protein (CREB)\textsuperscript{25,31} and the crucial peroxisome proliferator-activated receptor (PPAR) pathway transcription factor, \textit{Pgc-1α},\textsuperscript{32–34} have both been shown to be involved in DCM pathogenesis. Thus as expected, the relative expression of \textit{Pgc-1α} was significantly downregulated in LV and in an enriched cardiomyocytes fraction of \textit{Ramp2−/− Tg} hearts compared with controls (Figure 6A and 6B). There was also a significant reduction in phosphorylated CREB compared with total CREB and Gapdh in the \textit{Ramp2−/− Tg} hearts compared with \textit{Ramp2+/+ ntg} and \textit{Ramp2+/+ Tg} hearts (Figure 6C). Numerous family B GPCRs signal through these pathways, including the Gcgr that has been shown to signal through cAMP to activate both CREB and PPAR transcription,\textsuperscript{35–38} and both the Pthr1 and CaSR signal through cAMP and CREB.\textsuperscript{39–41} Interestingly, the gene expression levels of these RAMP-associated GPCRs were significantly downregulated in the LV of \textit{Ramp2−/− Tg} mice compared with those of control animals, whereas genes encoding for other RAMP-associated GPCRs, like \textit{Calcr}, \textit{Vipr1}, and \textit{Gpr30}, were unchanged (Figure 6D).

Similar gene expression changes in these family B GPCR expression profiles were confirmed in isolated cardiomyocyte fractions (Figure 6E), further supporting the myocyte-specific genetic dysregulation. \textit{Calcrl} expression was significantly...
increased in LV, but not in the cardiomyocyte-enriched fraction, demonstrating that Calcrl upregulation is from a noncardiomyocyte cell type. The expression of both Pthr1 and Gcgr was significantly decreased during development in the hearts of Ramp2−/− ntg and Ramp2−/− Tg embryos (Figure 6F), supporting that these changes are specific to Ramp2 loss rather than secondary to the Cdh5-driven Ramp2 Tg or the DCM phenotype, as might be the case for CaSR. Serum analysis revealed no significant dysregulation of circulating calcium or glucose, thereby eliminating uncompensated Pthr1 or Gcgr systemic signaling as a cause for the cardiovascular phenotypes (Table S2). Collectively, these data demonstrate that genetic loss of Ramp2 in nonendothelial cells of the heart leads to downregulation of RAMP2-associated GPCRs, Gcgr and Pthr1, as an underlying mechanistic basis for the decreased pCREB and Pgc-1α responsible for the pathogenesis of the DCM-like phenotype in Ramp2−/− Tg mice (Figure 6G).

**Discussion**

In this study, we generated an endothelial-specific Ramp2 Tg mouse model to attempt to rescue the embryonic lethality caused by global loss of Ramp2. Although no Ramp2 null mice survive to birth, we observed a significant number of Ramp2−/− Tg born and able to survive into adulthood. This result further confirms that endothelial Ramp2 is essential for embryonic survival and represents, to our knowledge, the first genetic rescue of the global Ramp2 null lethality.

It remains unclear why a significant number of Ramp2−/− Tg pups survive to late-gestation, but are stillborn. Interestingly, it was recently shown that mice with endothelial excision of Ramp2 during development using a Cdh5-Cre died during late-gestation. In addition, they report that ≈5% of the endothelial Ramp2 knockouts live into adulthood and develop large hearts, hypotension, and multiorgan vasculitis. In this current study, we found that 40% of Ramp2−/− Tg survived to adulthood and also developed similar cardiovascular and inflammatory phenotypes. These 2 mouse models, as well as a previously published endothelial-specific deletion of Calcrl, demonstrate that adequate levels and timing of endothelial Ramp2/CLR/AM signaling are critical for embryonic survival.9

In addition, these studies identify potential sex-dependent mechanisms of Ramp2, or Cdh5, regulation and function,
as evidenced by the substantially reduced numbers of male Ramp2<sup>−/−</sup> Tg survivors. We have previously shown that adult Ramp2<sup>−/−</sup> females have endocrine phenotypes not present in Ramp2<sup>−/−</sup> males. In addition, Ramp3<sup>−/−</sup> males, but not females, displayed exacerbated cardiovascular phenotypes when challenged with hypertension.4,10 Thus, although RAMP2 and RAMP3 interact with both similar and different GPCRs, our observations of sex-dependent phenotypes in these genetic animals will provide an area for interesting future investigations.

The complex compensatory mechanisms through which RAMP-mediated AM/CLR signaling regulate blood pressure have not been fully elucidated. It is well documented that AM infusion acts to lower blood pressure through both VSMC and endothelium, but it remains disputed if altered AM expression using genetic models alters basal blood pressure.18,42–45 Moreover, although developmental loss of Ramp2 leads to spontaneous hypotension and adult Ramp1<sup>−/−</sup> mice develop hypertension.11,12 Calcrl deletion in VSMC and cardiomyocytes appears dispensable for regulation of basal vascular tone in adult males. It is evident that Ramp2 and AM signaling plays a role in VSMC and myocardium development and function, which likely contributes to the pathogenesis of hypotension and DCM in Ramp2<sup>−/−</sup> Tg survivors. Yet, the hypotension and DCM phenotypes occur despite maintenance of normal cardiac output. It is further possible that the developmentally induced thin VSMC walls contribute to reduced vascular tone; however, we demonstrated their ability to effectively respond to acute phenylephrine vasoconstriction. Therefore, additional studies that explore the entire repertoire of RAMP-mediated GPCRs will be required for full elucidation of the mechanistic basis for the hypotension in Ramp2<sup>−/−</sup> Tg survivors.

Loss of Ramp2 in multiple nonendothelial cardiac cells, along with altered humoral signaling could lead to cardiac dysfunction and DCM pathogenesis. The genetic dysregulation in isolated cardiomyocytes suggests roles of Ramp2/GPCRs specifically in cardiomyocytes. Interestingly, a recently published study demonstrated that a cardiomyocyte-specific Ramp2 deletion led to a DCM-like phenotype caused by mitochondrial dysfunction and irregular calcium handling, which the authors attribute to loss of CLR/AM signaling.46 Similarly, this study suggests that cardiomyocyte loss of Ramp2 in the Ramp2<sup>−/−</sup> Tg mice is likely responsible for

Figure 5. Ramp2<sup>−/−</sup> Tg have vascular congestion and multiorgan inflammation downstream of hypotension and dilated cardiomyopathy. A, Spleen:body weight ratio from Ramp2<sup>++</sup>, Ramp2<sup>−/+</sup>, and Ramp2<sup>−/−</sup> females with and without the transgene. B, Macroscopic images of liver vasculature abnormalities in Ramp2<sup>−/−</sup> Tg compared to that of Ramp2<sup>−/−</sup> ntg and Ramp2<sup>−/−</sup> Tg controls. Histology and quantification of inflammatory foci (blue arrows) in (C and D) livers, (E and F) kidneys, and (G and H) lungs from Ramp2<sup>−/−</sup> ntg, Ramp2<sup>−/−</sup> Tg, and Ramp2<sup>−/−</sup> Tg mice. Scale bars, 1 mm (B) and 100 µm (C, E, and G). N.A. indicates not available. Data represented as average±SEM from n=3 to 4 mice per genotype. Significance determined by 1-way ANOVA with Tukey multiple comparison with *P<0.05, **P<0.01, and ***P<0.001.
Moreover, our data demonstrate that neither cardiomyocyte- or VSMC-specific loss of Calcrl during development or conditional Calcrl deletion in adults recapitulate the DCM phenotype observed in the Ramp2−/− Tg and the aforementioned Ramp2flox/flox;α-MHC-MerCreMer mice. Therefore, collectively, these studies imply the involvement of other RAMP-associated GPCRs.

It is apparent that RAMPs interact with numerous GPCRs biochemically although in vivo physiological evidence of these interactions is limited. We found that lack of Ramp2 in nonendothelial cells leads to decreased expression of cardiac Pthr1 and Gcgr. Interestingly, human PTHR1 and GCGR interact specifically with RAMP2 and not with RAMP1 or RAMP3 in which RAMP2 is important in chaperoning both GPCRs to the plasma membrane.

Figure 6. Decreased signaling and expression of receptor activity-modifying protein (RAMP)-associated G-protein–coupled receptors (GPCRs) in embryonic and adult Ramp2−/− Tg hearts. Relative Pgc-1α expression from Ramp2−/+ ntg, Ramp2−/+ Tg, and Ramp2−/− Tg adult left ventricle (A) or cardiomyocytes (B). C, Quantification and representative Western blot of phosphorylated cAMP response element-binding protein (pCREB) to total CREB in Ramp2−/+ ntg, Ramp2−/+ Tg, and Ramp2−/− Tg left ventricles. Samples were normalized to Ramp2−/+ ntg and CREB with Gapdh used as a loading control. Relative expression of the family B GPCRs: Calcrl, Calcr, Pthr1, Gcgr, Vipr1, CaSR and Gpr30 in (D) whole left ventricles and (E) isolated cardiomyocytes from adult Ramp2−/− Tg mice and Ramp2−/+ controls. F, Relative GPCR expression from e14.5 Ramp2−/+ ntg, Ramp2−/+ Tg, and Ramp2−/− Tg whole hearts. Samples were normalized to Ramp2−/+ ntg and Gapdh and Rpl19 expression. E, Data represented as average±SEM from n=3 to 5 mice per genotype. Significance determined by 1-way ANOVA with Tukey multiple comparison (A, C, D, and F) or unpaired Student t test (B and E) with *P<0.05. F, Model summarizing cardiovascular phenotypes in Cdh5+ endothelium and Cdh5− cells in Ramp2−/+ ntg, Ramp2−/+ Tg, and Ramp2−/− Tg adults. 4-HNE indicates 4-hydroxynonenal; and VSMC, vascular smooth muscle cell.
plasma membrane. Furthermore, it was recently demonstrated that RAM2 was important in GCGGR ligand selectivity between glucagon and glucagon-like peptide 1, which have opposing physiological effects on glucose homeostasis and cardiovascular function. Although GCGGR has not been directly connected to DCM pathogenesis, glucagon can alter calcium signaling in myocytes and glucagon-like peptide 1 improves glucose uptake and survival of canines with DCM. Likewise PTHR1 signaling is important in vitamin D and calcium homeostasis, which have both been associated with DCM. Together, Ramp2 loss-of-function can alter not only both Pthr1 and Gcgr expressions simultaneously but also their ability to reach plasma membrane, bind ligands, and signal. In addition, we observed decreased signaling through CREB and downregulation of Pgc-1α, which both have been shown to lead to DCM. Thus, the loss of Ramp2 likely has numerous mechanisms for the development and maintenance of cardiac functions both dependent and independent of canonical AM/CLR/Ramp2 signaling.

Perspectives

The in vivo interplay between GPCR/RAMP/Ligand is highly complex and is only starting to be understood. However, a better understanding of these interactions in a spatial and temporal manner in a pathophysiologic context will help us better target the unique GPCR/RAMP interfaces to potentially treat diseases like DCM and hypertension.

Acknowledgments

We thank the University of North Carolina Animal Models Core, the University of North Carolina Rodents Advanced Surgical Models Core, the University of North Carolina Animal Models Core (NIH CA16086). We also thank Dr Lin Xiao, Dr Andrew Dudley, Dr Samantha Hoopes, John Pawlak, and other members of Caron laboratory for technical support and discussions.

Sources of Funding

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**

- Using a novel genetic mouse model, we find that endothelial expression of RAMP2 is able to rescue the embryonic lethality of RAMP2/− mice.
- RAMP2 loss-of-function leads to spontaneous hypotension, dilated cardiomyopathy, and multiorgan inflammation, which we show are not recapitulated in genetic models with loss of CLR signaling.
- In vivo loss of RAMP2 causes dysregulation of numerous RAMP-associated GPCRs, including the glucagon and parathyroid hormone receptors.

**What Is Relevant?**

- The physiological consequences of RAMP interactions with family B GPCRs have been challenging to address because of the embryonic lethality of RAMP2 mice. Here, we elucidate the importance of RAMP2 interactions with several GPCR pathways, which can ultimately provide novel targets, or predict off-target consequences, for pharmacological therapies against cardiovascular disease.

**Summary**

This study provides genetic in vivo evidence that endothelial RAMP2 expression is necessary and sufficient to rescue the lethality of global loss of RAMP2. RAMP2 expression in nonendothelial cells during development is required to maintain adult blood pressure and cardiac homeostasis—a process that involves numerous GPCR signaling pathways, including glucagon and parathyroid hormone receptor signaling. Collectively, these studies extend the functional repertoire of RAMP-associated receptors in cardiovascular physiology.
Endothelial Restoration of Receptor Activity–Modifying Protein 2 Is Sufficient to Rescue Lethality, but Survivors Develop Dilated Cardiomyopathy
Daniel O. Kechele, William P. Dunworth, Claire E. Trincot, Sarah E. Wetzel-Strong, Manyu Li, Hong Ma, Jiandong Liu and Kathleen M. Caron

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Online Supplement: Endothelial restoration of receptor activity-modifying protein 2 is sufficient to rescue lethality, but survivors develop dilated cardiomyopathy.

Kechele Ramp2 Endothelial Rescue & Cardiomyopathy

Daniel O. Kechele¹, William P. Dunworth², Claire E. Trincot², Sarah E. Wetzel-Strong¹, Manyu Li¹, Hong Ma³, Jiandong Liu³, and Kathleen M. Caron¹,²

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³Department of Pathology and Laboratory Medicine, The University of North Carolina, Chapel Hill, North Carolina, USA.
Supplemental Methods

Ramp2 Transgene Construct Generation
The mouse Ramp2 cDNA with human CD33 signal sequence, FLAG-tag epitope, bovine growth hormone polyadenylation tail in pcDNA3.1 backbone was gift from Dr. Walter Born. The mouse vascular endothelial cadherin (Cdh5) promoter with rabbit β-globin intron II was extracted from a plasmid which was gift from the Parise Lab. The pieces of the transgene were inserted into pBluescript II KS+/− backbone and confirmed by sequencing. The University of North Carolina Animals Models Core microinjected the isolated transgene into pronuclei of C57BL/6 embryos and implanted into pseudo-pregnant females using standard published procedure.1

Mouse Studies
SJL-Tg(Tagln-cre)1Her (SM22-Cre) mice were obtained from the Jackson Laboratory2. Vascular smooth muscle- specific Calcrl-null animals were generated by crossing CalcrlFlox/Flox with CalcrlFloxP/+; SM22-Cre+ mice (Calcrl+/+ x SM22Cre+) generating the experimental (CalcrlFloxP/loxP,SM22Cre+) and control (Calcrl+/loxP,SM22Cre+) littermate mice used for this study. The SM22Cre+ transgene was expressed in both control and experimental mice to ensure Cre recombinase toxicity could not contribute to differential phenotypes 3. All mice used for this study were 3-4 month old male mice on a mixed genetic background. Three to four month old female Calcrlflox/flox; CAGG CreERTM mice were injected intraperitoneal with tamoxifen (5 mg/40 g body weight, Sigma) for 5 days to excised Calcrl ubiquitously as previously reported.4 Aged matched female Calcrlflox/flox mice injected with tamoxifen were used as controls. Mice were aged between 50-60 weeks post tamoxifen administration then conscious echocardiography was used to assess heart function.

Blood Pressure
Non-invasive blood pressures were measured using the CODA 8 (Kent Scientific Corporation) tail-cuff telemeter as previously described.5 Conscious 5-8 month old Ramp2 female and 3-4 month CalcrlfloxP/loxP; SM22Cre mice were acclimated to tail-cuff apparatus measurements for 4 days prior to 3 days of collected blood pressure measurements. Systolic, diastolic, and mean arterial pressure was measured 25 times per day per mouse and averaged together with measurements from 3 days after acclimation. Measurements were excluded by software if mouse moved during measurement or if mouse heart rate was below 450 bpm or above 750 bpm. For drug studies, mice were anesthetized with 1.5-2% isoflurane inhalation and an intra-arterial catheter was inserted into the right carotid artery. Measurements were recorded using SciSense Model ADV500 PV control unit with Labscribe 2 software (iWorx Systems). Baseline blood pressure was established before drugs were injected into the left jugular vein. Phenylephrine (Sigma) was delivered at 30 µg/kg and adrenomedullin (Phoenix Pharmaceuticals) at 12 nmol/kg both diluted in separate 50 µL saline as previously reported.6, 7 The percent changes were calculated from average of measurements 20 s taken 5 s post-injection and normalized to 20 s prior to injection.
Echocardiography
Conscious echocardiography was done using a VSI 2100 high frequency ultrasound (VisualSonics) on 5-8 month old female Ramp2 mice and aged female Calcr^loxP/loxP;CAGG-CreER mice. M-mode echocardiographs of left ventricle were analyzed using three measurements of both diastolic and systolic dimensions and averaged using Vevo 2100 software (VisualSonics), which calculated heart rate, ejection fraction, fractional shortening, and left ventricular volumes. Approximate cardiac output was determined by stroke volume (LV Vol; diastolic – LV Vol; systolic) multiplied by heart rate and divided to 1000. A 30-MHz probe and Vevo 660 Ultrasonography (VisualSonics) was used for 3-4 month old male Calcr^loxP/loxP;SM22Cre mice and controls. These mice were anesthetized with 1-1.5% isoflurane and body temperature was maintained at 37°C by placing mice under a heating lamp and heart rate was closely monitored.

Tissue Collection
Whole mouse necropsy including heart, aorta, lung, kidney, and liver were weighed, imaged, and collected in 4% paraformaldehyde, flash frozen in liquid nitrogen, or stored in RNALater (Ambion). Organ weights were normalized to either body weight or tibia length. Heart chamber dissections were done to identify chamber-specific changes and collect tissue for histology, RNA, and protein from the left ventricle. Pregnant females were dissected during midgestation and all viable embryos were imaged and collected. For embryonic hearts, e14.5 whole hearts were removed and separated from lungs prior to fixation. Endothelial cells were isolated with magnetic-associated cell sorting using CD31-specific antibodies as previously described. Briefly, all lobes of lungs were collected pooled from either transgenic or wild-type adult mice and dissociated using manual mincing followed by collagenase type II (Worthington), neutral protease (Worthington), and deoxyribonuclease (Worthington). Single cell suspensions are labeled with PE-rat anti-mouse CD31 antibody (BD Pharmingen) and subsequently with anti-PE conjugated magnetic beads (Miltenyi) and run through magnetic column to isolate both CD31- and CD31+ cell fractions. Cells pellets were flash frozen and stored at -80°C until RNA extraction. Adult cardiomyocytes were isolated with collagenase II digestion using previously published protocol. Briefly, mice were injected with 100 IU of heparin (Sigma) and anaesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Hearts were dissected from thoracic cavity and cannulated to perfusion system (Radnoti). Hearts were perfused with Wittenberg Isolation Buffer with a constant flow of 4 mL/min at 37°C for 5 min, followed by Digestion Solution for another 10 min and stopped by perfusion with Stop Solution. The hearts were further teased into small pieces with forceps, pipetted into single cells, and filtered through 100 μm cell strainer. Cardiomyocytes were enriched by spinning down at low speed (50 g for 5 min) and pellets were collected in TRIzol reagent (Invitrogen) and stored at -80°C. Descending aorta was collected and the adventitia and endothelial layers were removed by enzymatic digestion with collagenase type II (Worthington) to obtain purified VSMC as previously described.

Gene Expression Analysis
Tissues were either snap frozen in liquid nitrogen and stored at -80°C or stored at -20°C in RNALater (Ambion). RNA was extracted using TRIzol reagent and a bead
homogenizer (Percellys) according to standard procedures, and then DNase treated (Promega RQ1) and reverse transcribed with M-MLV (Invitrogen) or iScript cDNA Synthesis Kit (BioRad). Semi-quantitative gene expression analysis was carried out using an in-house purified PFu polymerase and products were run out on a 3% agarose gel. Quantitative gene expression was assayed with Taqman master mix with ROX (Applied Biosystems or Biobasic) and run on a StepOne Plus (Applied Biosystems). RT-PCR primers and probes are listed in Supplemental Table S1. Biological N was 3-5 mice per genotype and the relative expression levels were determined by the ΔΔCt and normalized to Gapdh or Rpl19 expression.

Whole Mount, Histology, and Immunohistochemistry
Fixed whole mount tissue was washed and imaged using a Leica MZ16FA dissecting stereoScope outfitted with a QImaging Micropublisher 5.0 RTV color CCD camera using QCapture imaging software (QImaging). Paraffin embedded tissues were sectioned and stained for hematoxylin & eosin and Picrosirius Red using standard procedures. H&E and Picrosirius Red slides were imaged using a Leitz Dialux 20 microscope outfitted with a QImaging Micropublisher 5.0 RTV color CCD camera. For immunohistochemistry, paraffin sections were deparaffinized, hydrated, permeabilized and blocked with 5% normal donkey serum. Slides were then stained with primary antibodies including, rabbit anti-LYVE1 (1:300, Fitzgerald), mouse anti-4-HNE (1:40, ABCAM) overnight at room temperature. Sections were rinsed, blocked, and incubated with secondary antibodies including, Dylight 594 isolectin B4 (1:200, Vector Laboratories), rhodamine wheat germ agglutinin (WGA) (1:1000, Vector Laboratories), donkey anti-rabbit Cy5 (1:200, Jackson ImmunoResearch), donkey anti-mouse Cy3 (1:100, Jackson ImmunoResearch), and Hoechst 33258 (1:1000, Sigma-Aldrich) in the dark for 90 minutes at room temperature. Images were acquired on a Nikon E800 fluorescence microscope with a Hammamatsu Orca CCD camera and DAPI, Cy2, Cy3, Cy5, Texas Red filter cubes with Metamorph software (Molecular Devices Corp.). Image brightness and contrast changes were made equally across all comparable images using Photoshop CS4 and color images were pseudo-colored using ImageJ (imageJ.nih.gov).

Western Blot
Tissues were either snap frozen in liquid nitrogen and stored at -80°C. Protein was extracted using Lens Homogenization Buffer with DTT and cOmplete protease inhibitor cocktail tablets (Roche) and a bead homogenizer (Percellys) according to standard procedures. Protein concentrations were determined using Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) read on a Mithras LB 940 (Berthold Technologies) plate reader. Protein ran on Mini-Protean TGX SDS-PAGE gel (BioRad) and transferred onto nitrocellulose (GE Healthcare) membrane. Blots were blocked and stained in 5% BSA or 5% nonfat milk diluted in tris-buffered saline with 0.1% Tween 20. Blots were incubated in primary antibodies including, rabbit anti-DYKDDDDK FLAG M2 (1:750, Cell Signaling), rabbit anti-phosphorylated CREB Ser133 87G3 (1:1000, Cell Signaling), rabbit anti-CREB 48H2 (1:1000, Cell Signaling), and mouse anti-GAPDH (1:4000, Novus Biologicals) overnight at 4°C. Blots were rinsed, blocked, and incubated with secondary antibodies including, goat anti-rabbit Dylight 680 (1:15,000, Thermo Scientific) and goat anti-mouse Dylight 800 (1:15,000, Thermo Scientific) in the dark for
60 minutes at room temperature. An Odyssey CLx (Li-COR) was used for imaging. Image brightness and contrast changes were made equally across all comparable images using Photoshop CS4 and quantification by densitometry was done using thresholds by ImageJ.

**Serum Analysis**
Adult females were anesthetized using isoflurane and whole blood was collected by retro-orbital bleeds and separated using Microtainer SST tubes (BD Scientific) and serum was stored at -20°C. Whole serum (100 μL) was loaded onto VetScan Comprehensive Diagnostic Profile rotors (Abaxis) and analyzed using VetScan V2 Chemistry Analyzer (Abaxis) according to manufacturer’s protocol.

**Morphometric Quantification**
Embryonic e13.5 jugular lymph sacs were measured from both sides of embryos taken from relatively the same sectional plane. Embryonic e14.5 cardiac ventricles area was measured using images from whole hearts extracted during dissection and qualitatively confirmed using histological sections. Adult and e14.5 descending aorta wall thickness was determined by measuring the distance from lumen to edge of vascular smooth muscle cell excluding the adventitia with 10-15 measurements made around the circumference of the vessel in cross section. Hypertrophy was assessed by two blinding researchers measuring the area of 15 individual cardiomyocytes in cross section from three fields per H&E and WGA stained left ventricles. Capillary density quantification has been previously described. Briefly, left ventricles were stained with isolectin and WGA and 30 to 50 cardiomyocytes and bordering capillaries were counted using ImageJ and averaged from 3 fields per left ventricle. Perivascular fibrosis was quantified by measuring the area of Picrosirius Red staining surrounding 3-4 equivalent coronary vessels per left ventricle and normalizing it to the area of the vessel lumen. The amount of left ventricle interstitial fibrosis and 4-HNE oxidative stress were determined by threshold analysis of the signal normalized to the total section area in a 3-4 20x fields per mouse from equivalent regions of the left ventricle. Generalized inflammation was assessed by averaging the number of hematoxylin dense foci larger than 0.02 mm² per section.
Supplemental References


### Supplemental Tables

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’ – 3’)</th>
<th>Reverse Primer (5’ – 3’)</th>
<th>Taqman Probe (5’-[6-FAM] – [TAMRA–6-FAM]-3’)</th>
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<td><strong>Ramp2 (Wild-type Allele)</strong></td>
<td>GAAGTCAGGCA GTCAGGGGTG</td>
<td>TCTGTCTGGA TGCTGCCCTTG C</td>
<td>ATGGAAGACTACGAAACAC ATGTCTACCTTG</td>
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<td>GACGAGTTCTT CTGAGGGGA</td>
<td>TCTGTCTGGA TGCTGCCCTTG C</td>
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<td><strong>Ramp2</strong></td>
<td>CAGAATCAATC TCAATCACACTG AG</td>
<td>GCCATGCAA CTCTTGACT CATACC</td>
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<td><strong>FLAG-Ramp2 (Transgene)</strong></td>
<td>GACTACAAAGA CGATGACGACA AGC</td>
<td>GCCATGCAA CTCTTGACT CATACC</td>
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<td><strong>Calcrl (Wild-type Allele)</strong></td>
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<td>GACGAGTTCT TCTGAGGGGA</td>
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<td><strong>Calcrl (Flox Allele)</strong></td>
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<td>GAATAAGTGTTGAATGCTGGAAG</td>
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<td><strong>SM22-Cre</strong></td>
<td>GCTGCCACGAC CAAGTGACAGCA AATG</td>
<td>GTAGTTATTC GGATCATACAG CTACAC</td>
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<table>
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<tr>
<th>Gene</th>
<th>Taqman Probe (ABI/Thermo/Fisher)</th>
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<td><em>Pthr1</em></td>
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<td><em>Gcgr</em></td>
<td>Mm00433536_m1 (ABI)</td>
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<tr>
<td><em>CaSR</em></td>
<td>Mm00443375_m1 (ABI)</td>
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<tr>
<td><em>Calcrl</em></td>
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<td><em>Ctr (Calcrl)</em></td>
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<td><em>Vip1r</em></td>
<td>Mm00449214_m1 (ABI)</td>
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<tr>
<td><em>Pgc-1α</em></td>
<td>Mm01208835_m1 (Life Tech)</td>
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<td><em>(Ppargc1α)</em></td>
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<td><em>Rpl19</em></td>
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Supplemental Table S1: Genotype and RT-PCR Gene Expression Primers and Probes
<table>
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<tr>
<th>Serum Analyte</th>
<th>$\text{Ramp2}^{+/+}$ ntg</th>
<th>$\text{Ramp2}^{-/-}$ Tg</th>
<th>P value</th>
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<tbody>
<tr>
<td>Albumin (g/dL)</td>
<td>$3.8 \pm 0.1$</td>
<td>$3.6 \pm 0.2$</td>
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<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>$53 \pm 7$</td>
<td>$50 \pm 6$</td>
<td>0.72</td>
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<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>$53 \pm 3$</td>
<td>$45.8 \pm 2$</td>
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</tr>
<tr>
<td>Amylase (U/L)</td>
<td>$631 \pm 8$</td>
<td>$713 \pm 23$</td>
<td>0.02</td>
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<tr>
<td>Total bilirubin (mg/dL)</td>
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<td>$0.3 \pm 0.0$</td>
<td>1</td>
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<tr>
<td>Blood Urea Nitrogen (mg/dL)</td>
<td>$22 \pm 1$</td>
<td>$18 \pm 2$</td>
<td>0.06</td>
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<td>Calcium (mg/dL)</td>
<td>$8.6 \pm 0.1$</td>
<td>$8.7 \pm 0.2$</td>
<td>0.82</td>
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<tr>
<td>Phosphorus (mg/dL)</td>
<td>$6.9 \pm 0.3$</td>
<td>$8.2 \pm 0.4$</td>
<td>0.06</td>
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<tr>
<td>Creatinine (mg/dL)</td>
<td>$0.2 \pm 0.0$</td>
<td>$0.3 \pm 0.0$</td>
<td>0.09</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>$252 \pm 11$</td>
<td>$260 \pm 18$</td>
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<tr>
<td>Sodium (mmol/L)</td>
<td>$147 \pm 1$</td>
<td>$148 \pm 0$</td>
<td>0.82</td>
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<tr>
<td>Potassium (mmol/L)</td>
<td>$5.6 \pm 0.5$</td>
<td>$5.8 \pm 0.7$</td>
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<td>Total protein (g/dL)</td>
<td>$5.1 \pm 0.2$</td>
<td>$4.8 \pm 0.2$</td>
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<td>Globulin (g/dL)</td>
<td>$1.2 \pm 0.1$</td>
<td>$1.2 \pm 0.1$</td>
<td>0.72</td>
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**Supplemental Table S2:** Relatively similar systemic circulating factors in $\text{Ramp2}^{+/+}$ ntg and $\text{Ramp2}^{-/-}$ Tg serum. Comprehensive diagnostic profile of 100 μL of serum from adult female $\text{Ramp2}^{+/+}$ ntg and $\text{Ramp2}^{-/-}$ Tg mice. N = 4-5 mice per genotype. Data represented as averages ± SEM with significance determined by p<0.05 using the unpaired student T-test.
## Cardiovascular Parameters

<table>
<thead>
<tr>
<th></th>
<th>Calcrl&lt;sup&gt;loxP/+&lt;/sup&gt;; SM22-Cre+</th>
<th>Calcrl&lt;sup&gt;loxP/loxP&lt;/sup&gt;; SM22-Cre+</th>
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<tbody>
<tr>
<td>Mouse Number</td>
<td>5</td>
<td>6</td>
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<tr>
<td>Mouse Age (wks)</td>
<td>12-16</td>
<td>12-16</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>Male</td>
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<tr>
<td>Body Weight (g)</td>
<td>33.3 ± 1.6</td>
<td>32.6 ± 0.7</td>
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<tr>
<td>Mean Arterial Pressure (mmHg)</td>
<td>128.6 ± 4.0</td>
<td>121.6 ± 4.2</td>
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<tr>
<td>Anesthesia</td>
<td>1.5% Isoflurane</td>
<td>1.5% Isoflurane</td>
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<tr>
<td>Heart Rate (BPM)</td>
<td>510 ± 12</td>
<td>478 ± 8</td>
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<tr>
<td>LVID, d (mm)</td>
<td>3.75 ± 0.16</td>
<td>4.00 ± 0.11</td>
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<td>LVID, s (mm)</td>
<td>2.86 ± 0.15</td>
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<td>LVPW, d (mm)</td>
<td>1.07 ± 0.08</td>
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<tr>
<td>LVPW, s (mm)</td>
<td>1.28 ± 0.08</td>
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<tr>
<td>Ejection Fraction (%)</td>
<td>25.8 ± 3.1</td>
<td>23.6 ± 1.7</td>
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<td>Fractional Shortening (%)</td>
<td>48.8 ± 6.2</td>
<td>47.4 ± 2.9</td>
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<td>Cardiac Output (mL/min)</td>
<td>16.0 ± 2.0</td>
<td>15.3 ± 0.7</td>
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<td>HW:BW (mg/g)</td>
<td>5.48 ± 0.34</td>
<td>5.31 ± 0.21</td>
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<tr>
<td>LV:BW (mg/g)</td>
<td>4.04 ± 0.27</td>
<td>3.78 ± 0.16</td>
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</tbody>
</table>

Supplemental Table S3: Vascular smooth muscle cell conditional Calcrl deletion does not lead to hypotension or dilated cardiomyopathy phenotype in adult males mice. Tail-cuff telemetry, echocardiography, and dissection measurements from 12-16 week old male Calcrl<sup>loxP/+</sup>; SM22-Cre and Calcrl<sup>loxP/loxP</sup>; SM22-Cre. Mice were conscious for blood pressure measurements, but under isoflurane anesthesia for echocardiography. BPM, beats per minute; LV, left ventricle; LVID, left ventricle internal diameter; LVPW, left ventricle posterior wall, HW, heart weight; BW, body weight. Data represented as averages ± SEM with significance determined by the unpaired student T-test.
### Heart Parameters

<table>
<thead>
<tr>
<th></th>
<th>Calcrl&lt;sup&gt;lox/lox&lt;/sup&gt;</th>
<th>Calcrl&lt;sup&gt;loxP/loxP&lt;sup&gt;+&lt;/sup&gt;&lt;/sup&gt;; CAGG-CreER&lt;sup&gt;TM&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse Number</strong></td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td><strong>Mouse Age (wks)</strong></td>
<td>70.4 ± 1.8 (+TAM 12-16)</td>
<td>68.2 ± 2.5 (+TAM 12-16)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td><strong>Anesthesia</strong></td>
<td>Conscious</td>
<td>Conscious</td>
</tr>
<tr>
<td><strong>Heart Rate (BPM)</strong></td>
<td>577 ± 29</td>
<td>614 ± 29</td>
</tr>
<tr>
<td><strong>LV Volume, d (µL)</strong></td>
<td>51.7 ± 6.0</td>
<td>47.9 ± 3.5</td>
</tr>
<tr>
<td><strong>LV Volume, s (µL)</strong></td>
<td>12.5 ± 2.3</td>
<td>12.2 ± 1.8</td>
</tr>
<tr>
<td><strong>Cardiac Output (mL/min)</strong></td>
<td>23.0 ± 2.0</td>
<td>22.3 ± 1.0</td>
</tr>
<tr>
<td><strong>Ejection Fraction (%)</strong></td>
<td>73.1 ± 4.3</td>
<td>74.9 ± 2.6</td>
</tr>
<tr>
<td><strong>Fractional Shortening (%)</strong></td>
<td>42.3 ± 3.6</td>
<td>43.0 ± 2.4</td>
</tr>
<tr>
<td><strong>IVS, d (mm)</strong></td>
<td>1.13 ± 0.08</td>
<td>1.13 ± 0.04</td>
</tr>
<tr>
<td><strong>IVS, s (mm)</strong></td>
<td>1.72 ± 0.09</td>
<td>1.63 ± 0.08</td>
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<tr>
<td><strong>LVID, d (mm)</strong></td>
<td>3.63 ± 0.20</td>
<td>3.40 ± 0.10</td>
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<tr>
<td><strong>LVID, s (mm)</strong></td>
<td>2.13 ± 0.24</td>
<td>1.94 ± 0.11</td>
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<tr>
<td><strong>LVPW, d (mm)</strong></td>
<td>0.93 ± 0.14</td>
<td>0.98 ± 0.10</td>
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<tr>
<td><strong>LVPW, s (mm)</strong></td>
<td>1.50 ± 0.11</td>
<td>1.55 ± 0.13</td>
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<tr>
<td><strong>HW:BW (mg/g)</strong></td>
<td>3.89 ± 0.31</td>
<td>4.13 ± 0.20</td>
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<tr>
<td><strong>LV:BW (mg/g)</strong></td>
<td>2.84 ± 0.19</td>
<td>2.97 ± 0.14</td>
</tr>
</tbody>
</table>

**Supplemental Table S4:** Global conditional *Calcrl* deletion does not lead to dilated cardiomyopathy phenotype in aged females. Echocardiography and dissection measurements from conscious aged female *Calcrl<sup>lox/lox</sup>* and *Calcrl<sup>loxP/loxP<sup>+</sup></sup>*; *CAGG-CreER<sup>TM</sup>* mice injected with TAM when 12-16 weeks old. BPM, beats per minute; LV, left ventricle; d, diastole; s, systole; IVS, interventricular septal; LVID, left ventricle internal diameter; LVPW, left ventricle posterior wall, HW, heart weight; BW, body weight. Data represented as averages ± SEM with significance determined by the unpaired student T-test.
Supplemental Figure S1: Endothelial-specific overexpression of murine Ramp2.

(A) Transgene construct utilizing the vascular-specific Cdh5 promoter to drive FLAG-tagged murine Ramp2 cDNA. (B) Ramp2-FLAG transgene protein was detected in both transgenic founders 1 and 2 in the heart (H), intestine (I), kidney (K), and lung (L). FLAG protein was not detected in wild-type tissue. (C) Semi-quantitative RT-PCR of FLAG transgene transcript in isolated lung CD31+ endothelium from transgenic and wild-type mice. RT-PCR for Ramp2 expression from (D) isolated CD31+ endothelium and whole lungs and (E) isolated cardiomyocyte and whole left ventricles from adult Ramp2 Tg and wild-type controls. Samples normalized to wild-type whole tissue expression and Gapdh.
Supplemental Figure S2: Generation of gene-targeted Ramp2 deficient mice with and without Ramp2 Tg. (A) Ramp2+/+ Tg(Cdh5-Ramp2)1 mice were bred to Ramp2+/- ntg animals to generated Ramp2+/- Tg. PCR representing the six genotypes possible at e14.5 generated from the parent cross of Ramp2+/- Tg mice with Ramp2+/- ntg. (B) Kaplan-Meier survival plot of Ramp2+/- embryos with and without the Tg. (C) Representative images surviving Ramp2+/+ Tg and Ramp2+/- Tg pups. Significance were determined by Mantel-Cox and Gehan-Breslow-Wilcoxon tests with ***p<0.001.
Supplemental Figure S3: Generation of VSMC-specific Calcr deficient mice. (A) PCR representing the four genotypes generated from the parent cross of Calcr^{Flox/Flox} x Calcr^{LoxP/+}; SM22Cre^{+}. The recombined “loxP” allele is only detected in SM22Cre^{+} mice and all mice were born at the expected Mendelian ratios. (B) Relative Calcr gene expression in aortic (Ao.) VSMCs, bladder, heart, skeletal muscle (Sk. Ms.), liver, and lung from adult Calcr^{LoxP/LoxP}; SM22Cre^{+} and Calcr^{LoxP/+}; SM22Cre^{+} controls. (C) Representative images of H&E stained left ventricles from adult males of both genotypes. Scale bar: 1 mm. Data represented as averages ± SEM with *p < 0.05 determined by the unpaired student T-test and was normalized to individual tissues Calcr^{LoxP/+};SM22Cre^{+} expression.