Neuronal Calcium Sensor-1 Promotes Immature Heart Function and Hypertrophy by Enhancing Ca\(^{2+}\) Signals

Tomoe Y. Nakamura, Andreas Jeromin, Katsuhiko Mikoshiba, Shigeo Wakabayashi

**Rationale:** Neuronal calcium sensor-1 (NCS-1) regulates various neuronal functions. Although it is expressed in the heart, very little is known about its cardiac functions.

**Objective:** This study aimed to identify the physiological and pathological roles of NCS-1 in the heart.

**Methods and Results:** We characterized the cardiac functions of knockout mice (NesI\(^{-/-}\)) and identified NCS-1 as a novel regulator of cardiac Ca\(^{2+}\) signaling, specifically in immature and hypertrophic hearts. NCS-1 was highly expressed in young hearts, and its deletion decreased survival and contractile function in young mice. Intracellular Ca\(^{2+}\) levels and sarcoplasmic reticulum Ca\(^{2+}\) content were significantly lower in NesI\(^{-/-}\) myocytes than in wild-type cells. This was due to reduced Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) activity in NesI\(^{-/-}\) myocytes, which led to reduced sarcoplasmic reticulum Ca\(^{2+}\) uptake and release. NCS-1 physically and functionally interacted with inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs) in the heart. In addition, IP\(_3\)R stimulation resulted in phosphorylation of CaMKII-δ, which was enhanced by NCS-1 overexpression. These results suggest that a functional link exists between NCS-1, IP\(_3\)R function, and CaMKII activation that may affect global Ca\(^{2+}\) signals in the immature heart. Furthermore, NCS-1 was upregulated in hypertrophic hearts, and hormone-induced hypertrophy was largely prevented in NesI\(^{-/-}\) hearts. Inhibitors of IP\(_3\)Rs, CaMKII, and calcineurin all prevented NCS-1–induced hypertrophy, which suggests the involvement of these pathways.

**Conclusions:** NCS-1 is an important regulator of immature heart function and hypertrophy, and it functions in part by promoting IP\(_3\)R function, followed by CaMKII-dependent signal activation. (Circ Res. 2011;109:512-523.)

**Key Words:** calcium signaling ■ excitation-contraction coupling ■ hypertrophy ■ mouse ■ neuronal calcium sensor-1

Calcium regulates various cellular processes and is responsible for activating myocyte contraction via excitation-contraction (EC) coupling and stimulating the gene transcription underlying hypertrophy. In adult ventricular muscles, propagation of the action potential initiates sarcoplasmic Ca\(^{2+}\) entry through voltage-gated L-type Ca\(^{2+}\) channels, which triggers Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) Ca\(^{2+}\) stores. These whole-cell transients can be described by spatiotemporal summation of the local Ca\(^{2+}\) transients that arise from SR Ca\(^{2+}\)-release channel ryanodine receptors (RyR2s) at T-tubule SR junctions, which leads to contraction.

Myocytes in the immature heart are structurally and functionally different from those in the mature heart: The T-tubule network is largely absent, the SR is sparse, and expression levels of Ca\(^{2+}\) regulatory proteins are different. Although L-type Ca\(^{2+}\) channel activity and RyR expression are low in the fetal heart and increase during development, T-type Ca\(^{2+}\) channel activity and Na\(^{+}/Ca\(^{2+}\) exchanger 1 (NCX1) expression peak near birth and decline postnatally. RyR2s mainly mediate the release of SR Ca\(^{2+}\), whereas cardiomyocytes also express inositol 1,4,5-trisphosphate receptor (IP\(_3\)R), a Ca\(^{2+}\) release channel. Because IP\(_3\)R expression is considerably lower than RyR expression in myocytes, the role of inositol 1,4,5-trisphosphate (IP\(_3\))–mediated Ca\(^{2+}\) signaling in cardiomyocytes is yet unknown; however, recent evidence suggests that IP\(_3\)Rs play an important role in cardiac function, especially in immature and diseased hearts. For example, IP\(_3\)Rs play a crucial role in heart development in early embryonic mice by enabling the initiation of pacemaking activity, thereby promoting cardiogenesis. Thus, the mechanism of EC coupling in the immature heart differs from that observed in adult hearts; however, the detailed molecular mechanisms of this event are not yet completely understood.

Hypertrophic enlargement of the heart occurs during development and in response to physiological stimuli or pathological insults. Progression of hypertrophy is commonly accompanied by complex changes in the expression of genes...
such as “fetal program” genes, which show higher expression levels in immature hearts. Although the detailed mechanisms of cardiac hypertrophy have been reported, molecules involved in its regulation may still be missing.

Here, we identified neuronal calcium sensor-1 (NCS-1) as a previously unrecognized regulator of EC coupling in young hearts and of hypertrophy in adults. NCS-1 is a small (22 kDa) N-terminal myristoylated Ca\textsuperscript{2+}-binding protein that contains 4 EF-hand motifs, 3 of which (EF2 through EF4) bind to Ca\textsuperscript{2+} in the physiological range. On binding Ca\textsuperscript{2+}, NCS-1 undergoes large conformational changes and regulates a variety of its target proteins, such as phosphatidylinositol 4-hydroxykinase, some voltage-gated ion channels, and the D2 (dopamine) receptor. Although low NCS-1 levels are found in almost all tissues, NCS-1 is mainly expressed in the brain and heart. NCS-1 has been studied extensively for its functions in neurons and neuroendocrine cells, and studies have demonstrated its importance for neurotransmitter release, synaptic plasticity, learning and memory, and neuronal survival. Recently, NCS-1 was reported to be involved in paclitaxel-induced spontaneous Ca\textsuperscript{2+} oscillation in cardiomyocytes. However, little evidence is available for its physiological and pathological roles in cardiac functions, possibly because of its relatively low expression in the adult heart. We previously detected high expression levels (comparable to the levels in neurons) of NCS-1 in immature hearts and hypertrophic hearts overexpressing Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (data not shown); therefore, we hypothesized that NCS-1 has a specific role in cardiac function, particularly at the immature stage and during progression of hypertrophy. To validate these hypotheses, we investigated the function of NCS-1 in the heart using Ncs1-knockout (Ncs1\textsuperscript{-/-}) mice. NCS-1 was found to increase IP,R-dependent Ca\textsuperscript{2+} signaling, which in turn activates Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII)-dependent pathways, consequently promoting contraction in young hearts. Our results also demonstrated that NCS-1 promotes receptor stimulation–induced cardiac hypertrophy by activating both CaMKII- and calcineurin-dependent pathways in adults. These observations suggest additional mechanisms of EC coupling in young hearts and regulation of hypertrophy.

Methods
An expanded Methods section is available in the Online Supplement at http://circres.ahajournals.org. It includes a detailed description of the generation of Ncs1\textsuperscript{-/-} and transgenic mice overexpressing the NCS-1 mutant (E120Q); echocardiography; osmotic minipump implantation; quantitative reverse transcription–polymerase chain reaction; histological analysis; cell isolation; intracellular and SR Ca\textsuperscript{2+} measurements; immunoprecipitation and immunoblotting; sources of antibodies, adenoviruses, and other materials; and statistical analysis.

Results
NCS-1 at the Immature Stage
We investigated NCS-1 expression during heart development in detail. We found higher NCS-1 expression at the fetal and neonatal stages, which gradually decreased after 2 weeks of birth (Figure 1A). Expression levels were comparable in the atria and ventricles at the same age (Figure 1B). Immunofluorescence analysis revealed that NCS-1 was predominantly localized to the plasma membrane and perinuclear regions, particularly at the nuclear envelope and SR, in α-actinin–positive cardiac myocytes (Online Figure I).

Diminished Systolic Function in Young Ncs1\textsuperscript{-/-} Mice
To determine the cardiac role of NCS-1, we obtained global Ncs1\textsuperscript{-/-} mice that exhibited a complete absence of NCS-1 protein in the heart (Figure 1C). These mice had a high mortality rate, specifically at the neonatal stage (approximately 4 days after birth; Figure 1D). Furthermore, the body weights of these mice were higher than those of wild-type (WT) mice after 2 months (Figure 1E); however, the underlying mechanisms for this weight increase are unknown. To analyze cardiac functions at different developmental stages, we selected 2-week-old juveniles (immature but that could be reliably measured echocardiographically), 6-week-old young adults (similar body weights), and 4-month-old adults (significantly different body weights). The overall whole-heart structures were similar among the mice at all ages (Figure 1F); however, echocardiographic analysis revealed considerably diminished ventricular function in the 2-week-old Ncs1\textsuperscript{-/-} mice, which resulted in a reduced percentage of fractional shortening and ejection fraction (Figure 1G; Online Table I). However, such reduced ventricular functions were observed to a lesser extent in the 6-week-old and 4-month-old mice (Figure 1H; Online Figure II; Online Table I).

Intracellular Ca\textsuperscript{2+} Levels of Ncs1\textsuperscript{-/-} Myocytes
To elucidate the cellular mechanism of diminished systolic function in immature Ncs1\textsuperscript{-/-} hearts, we measured the intracellular Ca\textsuperscript{2+} transient in spontaneously beating cultured neonatal mouse ventricular myocytes (NMVMs). As shown in Figure 2A, Ncs1\textsuperscript{-/-} cardiomyocytes had significantly lower systolic Ca\textsuperscript{2+} levels than WT cardiomyocytes, with little decrease in diastolic Ca\textsuperscript{2+} levels, and consequently, they also had lower Ca\textsuperscript{2+} amplitude (difference between diastolic and systolic Ca\textsuperscript{2+} levels). Furthermore, the peak amplitude of the caffeine-induced Ca\textsuperscript{2+} transient was used as an index of the SR Ca\textsuperscript{2+} content. This value in the Ncs1\textsuperscript{-/-} myocytes was half of that in the WT myocytes (Figure 2B),
which suggests reduced SR Ca\(^{2+}\) pumping as a possible mechanism of decreased intracellular Ca\(^{2+}\) transient in \textit{Ncs1}\(^{-/-}\) myocytes.

Although major Ca\(^{2+}\) regulatory proteins such as the voltage-gated Ca\(^{2+}\) channels CaV1.2 and CaV3.1 or NCX1, calsequestrin, and the SR Ca\(^{2+}\) pump showed similar expression in the hearts of 1-week-old WT and \textit{Ncs1}\(^{-/-}\) mice (Figure 3A), phosphorylation levels of CaMKII-\(\delta\) (Figure 3B) and CaMKII-dependent phosphorylation of phospholamban at Thr17, a Ca\(^{2+}\)-pump regulatory protein, in the \textit{Ncs1}\(^{-/-}\) hearts were almost half those of the WT mice, with no difference at Ser16, which is a protein kinase A phosphorylation site (Figure 3C). Furthermore, the rate of decline in the field stimulation–elicited Ca\(^{2+}\) transient was significantly slower in the \textit{Ncs1}\(^{-/-}\) myocytes (Figure 3D), wherein the time constant
increased to 150% of the WT, which indicates slower Ca\textsuperscript{2+} removal, mainly by SR Ca\textsuperscript{2+} pumping. Therefore, fewer CaMKII-δ and phospholamban activities, leading to less SR Ca\textsuperscript{2+} loading and release, would be the mechanism underlying the decrease in systolic Ca\textsuperscript{2+} levels in Ncs\textsuperscript{1}\textsuperscript{-/-} myocytes at the immature stage. On the other hand, there were few differences in the total and phosphorylated CaMKII and phospholamban activities, leading to less Ca\textsuperscript{2+} pumping. Therefore, fewer NCS-1 myocytes at the immature stage. On the other hand, there were few differences in the total and phosphorylated CaMKII and phospholamban levels in 6-week-old adult hearts between the WT and Ncs\textsuperscript{1}\textsuperscript{-/-} mice (Figures 3B and 3C). Furthermore, the total CaMKII-δ and phosphorylated CaMKII expression levels were significantly higher at the immature stage (Figure 3E), which suggests that CaMKII signaling is more important in immature hearts, and NCS-1 may be involved in such signaling. In addition, when NMVMs were pretreated by the CaMKII inhibitor KN93 for WT-basal, KO-basal and WT with KN93, and KO with KN93, respectively. n.s. Indicates not significant. B, Caffeine-induced Ca\textsuperscript{2+} transient was measured with Fluo-4 AM to estimate the SR Ca\textsuperscript{2+} content (n=12 cells each from 3 WT and 3 Ncs\textsuperscript{1}\textsuperscript{-/-} hearts). *P<0.05.

Physical and Functional Interaction of NCS-1 With Cardiac IP\textsubscript{3}Rs
To identify the cardiac Ca\textsuperscript{2+} source that activates CaMKII in WT immature hearts, we searched for proteins that interact with NCS-1. As previously reported in neurons\textsuperscript{22} and recently in hearts,\textsuperscript{23} we also detected that both IP\textsubscript{3}R1 and IP\textsubscript{3}R2 immunoprecipitated and colocalized with NCS-1 in HEK293 cells that overexpressed NCS-1 plus IP\textsubscript{3}R1 or IP\textsubscript{3}R2 (Online Figure III, B and C) and in primary cultured NMVMs (Online Figure III, D and E). NCS-1 proteins colocalized with IP\textsubscript{3}Rs predominantly at the perinuclear region. Furthermore, endogenous NCS-1 interacted with IP\textsubscript{3}Rs in native heart homogenates from WT mice (but not from NCS-1\textsuperscript{-/-} mice) in the presence of Ca\textsuperscript{2+} (50 μmol/L), whereas no interaction was detected in the absence of Ca\textsuperscript{2+} (Figure 4A), which demonstrates that the NCS-1-IP\textsubscript{3}R association is Ca\textsuperscript{2+} dependent. Furthermore, no interaction was detected in transgenic mice that overexpressed the NCS-1 mutant E120Q, which has little affinity for Ca\textsuperscript{2+} binding (Figure 4A). Such a Ca\textsuperscript{2+} dependent association exists at least in part because the function and the conformation of NCS-1 is Ca\textsuperscript{2+} dependent. Similar to NCS-1 and CaMKII, the expression level of IP\textsubscript{3}R2 was significantly higher at the neonatal stage (Figure 4B),
which suggests that all NCS-1, CaMKII, and IP3Rs may have a crucial role in immature heart function.

We next examined the effect of knocking out the NCS-1 gene on cardiac IP3R function. When ATP (which induces IP3 release) was applied to spontaneously beating NMVMs, it potently increased both the diastolic and systolic levels of Ca2+ in WT myocytes. This effect was significantly reduced in Ncs1−/− myocytes (Figure 4C). Similar results were obtained in the absence of extracellular Ca2+ (Figure 4D), in which the effect of ATP was mainly mediated via IP3R activation and not via sarcolemmal Ca2+ entry, or with the membrane-permeable IP3 ester adenophostin A. These effects were also blocked by the IP3R inhibitor 2-aminoethoxydiphenyl borate (Figure 4E). These results...
Figure 4. Physical and functional interactions of NCS-1 with IP₃Rs were diminished in Ncs1⁻/⁻ hearts. A, NCS-1 coimmunoprecipitated with IP₃Rs in heart homogenates prepared from 1-week-old WT mice in 50 μmol/L Ca²⁺ solution; however, no interactions were detected in the absence of Ca²⁺ (20 mmol/L EGTA solution) in WT mice or in Ncs1⁻/⁻ (KO) mice or adult transgenic mice overexpressing NCS-1 mutant E120Q-HA. The expression level of the E120Q protein in the transgenic mouse heart was confirmed by use of anti-HA and anti-NCS-1 antibodies. B, IP₃R2 expression levels in mouse ventricles during development. C and D, Intracellular Ca²⁺ transient in spontaneously beating NMVMs from WT and Ncs1⁻/⁻ mice before and after ATP (100 μmol/L) application. Representative traces of the Indo-1 fluorescence ratio with (C) or without (nominal Ca²⁺-free solution plus 1 mmol/L EGTA) extracellular Ca²⁺ (D) and summarized data are illustrated in the bar graphs (n=6/group). E, Summarized data of intracellular Ca²⁺ transient amplitude of cardiomyocytes after exposure to 10 μmol/L adenophostin A (AP; AdePhos) or AP plus 2-aminoethoxydiphenyl borate (2-APB; n=6/group). *P<0.05.
demonstrate that NCS-1 enhances Ca\(^{2+}\) release from intracellular stores through IP\(_3\)R activation.

**NCS-1–Mediated Hormone-Induced Cardiac Hypertrophy**

Genes expressed at high levels at the immature stage are often upregulated in hormone-induced cardiac remodeling.\(^1\) When NMVMs were treated with the \(\alpha_1\)-adrenergic receptor agonist phenylephrine (PE) or endothelin-1, NCS-1 expression was increased (Figure 5A). In addition, NCS-1 overexpression induced by adenoviral infection resulted in morphological changes in the cultured NMVMs, as observed when hypertrophic stimuli were applied; myocytes grouped together (Figure 5B), and their contractions became stronger (data not shown). Furthermore, NCS-1 overexpression induced the increased expression of the hypertrophy-related gene ANP (atrial natriuretic peptide), and this effect was largely prevented by pretreatment of the myocytes with or without 2-aminoethoxydiphenyl borate (2-APB), KN93, or FK506 (inhibitors for IP\(_3\)Rs, CaMKII, and calcineurin, respectively) under serum-free conditions for 12 hours. They were then infected with Ad-NCS-1, and immunostaining was performed after 2 days (n=5 dishes/group). *P<0.05.

**Figure 5. NCS-1 overexpression resulted in hypertrophy of NMVMs.**

A, Treatment of NMVMs with PE (20 \(\mu\)mol/L) and endothelin-1 (ET-1; 5 nmol/L) for 3 days resulted in increased NCS-1 expression. NCS-1 expression was normalized to the GAPDH level. Con indicates control.

B, NCS-1 overexpression in NMVMs with adenovirus (Ad) showed morphological changes (n=12 dishes/group).

C, Immunostaining of NMVMs for atrial natriuretic peptide (green) and \(\alpha\)-actinin (red). NMVMs were pretreated with or without 2-aminoethoxydiphenyl borate (2-APB), KN93, or FK506 (inhibitors for IP\(_3\)Rs, CaMKII, and calcineurin, respectively) under serum-free conditions for 12 hours. They were then infected with Ad-NCS-1, and immunostaining was performed after 2 days (n=5 dishes/group). *P<0.05. Scale bars=20 \(\mu\)m.

**mice were treated with constant PE infusion, they exhibited marked cardiac hypertrophy, which was revealed by the increased heart weight-to-body weight ratio, fibrosis, myocyte area (Figures 6A through 6C, respectively), and expression of several hypertrophy markers, including ANP, brain-type natriuretic peptide, and \(\beta\)-myosin heavy chain (Figure 6D). In contrast, these PE-induced effects were markedly prevented in the Ncs\(_1\)\(^{-/-}\) hearts (Figures 6A through 6D). In addition, NCS-1 expression levels (both mRNA and protein) were increased significantly (Figure 6E). These results suggest that \(\alpha_1\)-adrenergic receptor stimulation–induced cardiac hypertrophy is mediated at least in part by NCS-1.**

Furthermore, although the level of phosphorylated CaMKII was markedly increased in PE-treated WT hearts, no increment was detected in Ncs\(_1\)\(^{-/-}\) hearts (Figure 7A). Similarly, protein and mRNA levels of MCIP/RCAN1 (modulatory calcineurin-interacting protein 1 regulator of calcineurin 1), which was used as a sensitive indicator of calcineurin activity, were elevated in PE-treated WT hearts but not in Ncs\(_1\)\(^{-/-}\) hearts (Figure 7B). In addition, overexpression of NCS-1 significantly increased the activity of NFAT (nuclear factor of activated T cell) in myocytes carrying the NFAT-luciferase reporter transgene (Online Figure IV, C). These
results indicate that both the CaMKII- and calcineurin-dependent pathways are activated in the hypertrophic heart, and the extent of their activation depends on NCS-1 levels. This notion was further supported by the evidence that both CaMKII and calcineurin inhibitors prevented NCS-1–induced hypertrophy, which was evaluated by expression of atrial natriuretic factor (Figure 5C). However, other signal transduction pathways such as ERK42/44 and p38 mitogen-activated protein kinases remained unchanged by NCS-1 expression (Online Figure IV, D).

To further confirm the link between NCS-1, IP3R, and CaMKII activation, we used a heterologous overexpression system. When HEK293 cells were transfected with CaMKII-H9254 with or without IP3R2 and NCS-1, the levels of phosphorylated CaMKII were significantly increased in the cells that overexpressed NCS-1 and IP3R2 (Figure 7C).
Furthermore, when these cells were exposed to the IP3R agonist adenophostin A, CaMKII phosphorylation increased in a time-dependent manner, which was prevented by pre-treatment with 2-aminoethoxydiphenyl borate (Figure 7D). These results illustrate a functional correlation between NCS-1 and CaMKII, as well as between IP3R stimulation and CaMKII activation.

Discussion
In the present study, we identified 2 novel roles of NCS-1 in cardiac tissues: positive regulation of contraction in immature hearts and of hypertrophy in adults. The Ca2+ regulatory mechanism in the immature heart is considered to be different from that in the adult heart. The embryonic heart is considered more dependent on transsarcolemmal Ca2+ flux, eg, through an Na+/Ca2+ exchanger and T-type Ca2+ channel. However, accumulated evidence suggests that SR maturation during neonatal development positions it as the primary source of Ca2+ for contraction. Therefore, additional factors might enhance SR-dependent EC coupling, particularly in the postnatal stage. We found that NCS-1, which is a cardiac Ca2+ signaling regulator, enhances EC coupling in young
hearts. Because of the decreased intracellular Ca\(^{2+}\) transient amplitude and SR Ca\(^{2+}\) content in the heart, Nes\(^{1/-}\) mice exhibited diminished systolic functions, specifically at the young stage. Moreover, phosphorylation of CaMKII, CaMKII-dependent phosphorylation of phospholamban, and Ca\(^{2+}\) pump activity also decreased in Nes\(^{1/-}\) myocytes. These results indicate that NCS-1 activates CaMKII signaling, thereby enhancing SR-dependent EC coupling, which is otherwise inefficient because of the structural immaturity of the young heart. In line with this hypothesis, we observed significantly higher levels of total and phosphorylated CaMKII in the hearts of immature mice than in those of adult mice (Figure 3E), and the T-tubule network was still largely absent in both WT and Nes\(^{1/-}\) mice at this age (Online Figure V).

In healthy adult hearts, however, the EC coupling mechanism largely depended on the L-type Ca\(^{2+}\) channel and RyRs, with mature SR and T-tubule structures (Online Figure V), and this mechanism may be less dependent on the functions of NCS-1, IP\(_3\)Rs, and CaMKII, because low expression levels of these molecules were detected. This could explain in part the small difference observed in ventricular function between adult WT and Nes\(^{1/-}\) mice (Online Table I).

NCS-1 was associated with IP\(_3\)Rs and promoted IP\(_3\)-induced Ca\(^{2+}\) release in cardiac cells. This finding is consistent with those of previous reports that have demonstrated that NCS-1 enhances IP\(_3\)R activity when monitored as the phosphorylation, and increases in human hypertrophic hearts at the transcriptional level. The expression level of NCS-1 was increased significantly in hypertrophic hearts at the transcriptional level. The increased protein was detected as early as 2 days after PE infusion in the in vivo hearts (Online Figure IV, A), which suggests that NCS-1 has a role in the early stage of hypertrophy. As expected, PE-induced hypertrophy was largely attenuated in Nes\(^{1/-}\) mouse hearts. Because NCS-1 physically and functionally interacted with cardiac IP\(_3\)Rs, which reportedly function as hypertrophic effectors in vivo, we hypothesized that PE-induced hypertrophy is mediated at least in part by NCS-1–induced enhancement of Ca\(^{2+}\) release from IP\(_3\)Rs. Numerous signaling pathways are known to coordinate hypertrophy, including the Ca\(^{2+}\)-dependent prohypertrophic signal molecule calcineurin and CaMKII, both of which are activated by the IP\(_3\)R-mediated Ca\(^{2+}\) source.

In the present study, we observed that PE-induced activation of CaMKII and calcineurin signals were largely attenuated in Nes\(^{1/-}\) hearts. Furthermore, NCS-1–induced hypertrophy was prevented by IP\(_3\)R, CaMKII, and calcineurin inhibitors. These data strongly indicate that NCS-1 enhances the Ca\(^{2+}\) signal by activating IP\(_3\)Rs, consequently mediating cardiac hypertrophy through both CaMKII and calcineurin pathways.

However, we cannot exclude the possibility that NCS-1 modulates other Ca\(^{2+}\) signals in addition to these via IP\(_3\)Rs. Indeed, numerous lines of evidence suggest that other Ca\(^{2+}\) sources, such as those via transient receptor potential canonical (TRPC) channels, are important mediators of pathologic hypertrophy. In addition to the perinuclear region and SR, NCS-1 is also localized in the sarcolemma (Online Figure I) and is known to bind to several other proteins, including phosphatidylinositol 4-hydrox kinase, and ion channels such as Kv4 K\(^+\) channel, TRPC5, and voltage-gated Ca\(^{2+}\) channels, some of which are expressed in the heart. It is therefore possible that NCS-1 directly or indirectly affects sarcolemmal Ca\(^{2+}\) entry through some ion channels. Indeed, we observed that the ATP-induced Ca\(^{2+}\)-transient amplitude was significantly higher in WT myocytes not only without (IP\(_3\)R-mediated Ca\(^{2+}\) release) but also with extracellular Ca\(^{2+}\) (Figures 4D and 4C, respectively), which suggests the involvement of other Ca\(^{2+}\) signals. It has been reported that the expression of TRPC5, one of the proteins that interact with NCS-1, increases in human heart failure, which suggests that NCS-1 might also be involved in human heart disease. In addition, another target of NCS-1, phosphatidylinositol 4-hydrox kinase, is the key supplier of the phospholipase C substrate phosphatidylinositol 4,5-bisphosphate and can therefore regulate Ca\(^{2+}\) signaling mediated by G-protein–coupled receptors and various ion channel functions. Such interactions may be an additional factor in the regulation of receptor stimulation–induced hypertrophy.

We proposed that NCS-1, coordinated with IP\(_3\)Rs and CaMKII, is involved both in the EC coupling in immature hearts and in hypertrophy. Many of the prohypertrophic effects of Ca\(^{2+}\) are regulated by small EF-hand Ca\(^{2+}\) sensor proteins, such as calmodulin, chromogranin B, and integrin-binding protein-1 (CIB1). Interestingly, the
expression pattern of CIB1 is high at the immature stage and reinduced in the adult heart during pathological hypertrophy,34 similar to NCS-1, as well as IP3Rs35 and CaMKII.1 The coordinated manner of expression of these proteins implies that NCS-1, IP3Rs, and CaMKII may belong to a common set of genes for Ca2+ regulation that control immature heart function and hypertrophy.

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Disclosures

A.J. is a full-time employee of Banyan Biomarkers, Inc. The remaining authors report no conflicts.

References


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**Novelty and Significance**

**What Is Known?**

- The EF-hand Ca2+-binding protein neuronal Ca2+ sensor-1 (NCS-1) is recognized as an important regulator of neuronal functions such as synaptic plasticity.
- Although NCS-1 is also expressed in the heart, especially at high levels during the embryonic period, little is known about its cardiac functions.
- Mechanisms of excitation-contraction (EC) coupling in fetal and neonatal hearts are considered to be different from those in the adult heart because of the structural immaturity of the sarcoplasmic reticulum (SR) in young heart, but the detailed molecular mechanism for this is not completely understood.

**What New Information Does This Article Contribute?**

- We identified NCS-1 as a novel regulator of EC coupling in fetal and neonatal hearts.
- NCS-1 increases Ca2+ signals through activation of inositol 1,4,5-trisphosphate receptor (IP3R), followed by Ca2+/calmodulin-dependent protein kinase II (CaMKII)–dependent signaling.
- NCS-1 expression increases in hypertrophic hearts and mediates the progression of hormone-induced hypertrophy in adult hearts.

Although NCS-1 is an important regulator of neuronal functions, its physiological and pathological roles in the heart have not yet been identified. By characterizing the cardiac phenotypes of knockout (Ncs1−/−) mice, we identified 2 novel functions of NCS-1 in cardiac tissues: It is a positive regulator of contraction in the developing heart and of hypertrophy in adults. In fetal and neonatal hearts, the structure and function of SR are immature; nonetheless, it is considered a primary source of Ca2+ for contraction, which suggests the existence of missing factors that promote SR-dependent EC coupling in the postnatal stages.

We showed that NCS-1 enhances Ca2+ signals in the developing heart, mainly by promotion of IP3R function, followed by CaMKII signaling, which results in a large increase in the SR Ca2+ content. In addition, NCS-1 expression increases in the early stages of hypertrophy in the adult heart. NCS-1 promotes progression of hypertrophy at least in part through IP3R activation. To the best of our knowledge, this is the first report that describes the phenotypes of Ncs1−/− mice. Our results reveal a previously unrecognized mechanism of EC coupling in the developing heart and another regulatory mechanism for the progression of receptor stimulation–elicited cardiac hypertrophy.
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Neuronal calcium sensor-1 promotes immature heart function and hypertrophy by enhancing $\text{Ca}^{2+}$ signals

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Detailed Methods

All animal experiments were performed according to the Animal Welfare Committee guidelines of the National Cerebral and Cardiovascular Center Research Institute.

Generation of Ncs1<sup>−/−</sup> mice. Frozen embryos of Ncs1<sup>−/−</sup> mice (C57BL/6-NCR), originally generated by Horst Bluethmann and coworkers (MGI accession ID: 3525199), were re-derived at Charles River Laboratory. Ncs1<sup>−/−</sup> hetero mice were generated by crossing Ncs1<sup>−/−</sup> and C57BL/6-NCR mice at our facility, and WT, hetero, and Ncs1<sup>−/−</sup> mice were obtained by crossing hetero mice. The mice were genotyped by PCR analysis of genomic DNA using primers 5′-CCGAGCATGGGGAAATCCAAC-3′, 5′-GCACGCTGACAGGCGACACC-3′, and 5′-GCTGACCGCTTCTCCTGCTTTAC-3′, and the genotypes were characterized by western blotting (Figure II-A).

Transgenic mice overexpressing the NCS-1 mutant (E120Q). A hemagglutinin (HA)-tagged E120Q NCS-1 mutant was generated with a conventional PCR protocol by using WT human NCS-1 (accession number: AF134479) as a template and cloned into an expression plasmid containing the Myh6 (encoding α-MHC) promoter. Genotyping was performed by PCR with primers 5′-CCTACGCGACGTGCACATGGGGAAATCCAACAGCAAGTTGAAG-3′ and 5′-CTAAGCGTAATCTGGAACATCGTATGGGTATCCTCCTACCAGCCCCTGCTTAGG-3′. NCS-1 expression was revealed by immunohistochemistry and western blot analysis using antibodies to HA tag (Sigma, St. Louis, MO) or NCS-1.

Echocardiography. Standard echocardiograms were performed on anesthetized mice (1–1.5% isoflurane at 1 L/min). The mice were placed on a heated platform and continuously monitored via electrocardiograms (ECGs). M-mode images were obtained by transthoracic echocardiography using a 30-MHz MS-550D transducer (Vevo 2100; Visual Sonics, Toronto, ON, Canada) for 2-week-old mice, an MS-400 transducer for adult and fetal hearts and an MS-700 for neonatal hearts. The percentage of fractional shortening (FS) was calculated as [(LVIDd − LVIDs)/LVIDd] × 100, where LVIDd and LVIDs are the left ventricular internal end-diastolic and end-systolic dimensions (mm), respectively.

Osmotic mini-pump implantation. Alzet osmotic mini-pumps (1007D; Durect Corporation, Cupertino, CA) filled with either PE (60 mg/(kg·day)<sup>−1</sup>) or saline were implanted into the intrascapular subcutaneous space in WT or Ncs1<sup>−/−</sup> mice. One week later, their hearts were removed and used for further analysis.
**Quantitative RT-PCR.** Total RNA was extracted from snap-frozen whole hearts of mice subjected to osmotic mini-pump infusion with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA was reverse transcribed into cDNA by using QuantiTect Reverse Transcription kit (Qiagen). Real-time quantitative PCR was performed according to the manufacturer’s protocol. The primers and probe sets for ANP, BNP, RCAN1, MHC, and L7 were purchased from Applied Biosystems, and the reactions were run in a LightCycler II (Roche Molecular Biochemicals). The data were collected using LightCycler software Ver. 3.5 (Roche Molecular Biochemicals). Quantified mRNA were normalized to L6 mRNA levels and expressed relative to the WT control.

**Histological analysis.** Hearts from WT and Ncs1^-/-_ mice were fixed in 10% formalin and embedded in paraffin. Serial 5-μm heart sections were stained with hematoxylin and eosin (H&E) or Masson’s trichrome, and images were acquired using a digital camera (Olympus BX41) equipped with image filing software (Flovel FLVFS-LS, Tokyo, Japan). Fibrosis was quantified by image analysis using Image-Pro Plus software (Media Cybernetics, Bethesda, MD). Multiple photographs of each muscle section were used to represent the entire section. After selecting the area of interest, the fibrotic areas were extracted with the threshold range of 150 to 213 (for blue), and the sum of the extracted areas was calculated as the percentage of the total area.

**Primary culture.** Ventricular myocytes were isolated from 2-day-old mice and dissociated into single cells by trypsinization, as described previously. After excluding non-myocytes by differential adhesion treatment, myocytes were seeded into collagen-coated glass-bottom dishes and incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum.

**Adenoviral vectors.** Plasmids for generating adenoviruses were constructed by using Gateway Technology (Invitrogen, Carlsbad, CA), and the adenoviruses were obtained through standard procedures. The viral titers were measured by using a QuickTiter adenovirus titer immunoassay kit (Cell Biolabs, Inc., San Diego, CA) and determined as 2.3 × 10⁹ plaque-forming units (pfu)/ml for NCS-1 and 1.2 × 10⁹ pfu/ml for NCS-1-HA.

**Measurement of NFAT activity.** Myocytes were plated on 24-well dishes, and 18 hours after incubation, they were infected with individual adenoviruses (multiplicity of infection (moi): 100). Two hours later, the cells were co-transfected with 0.45 μg of pNFAT-Luc (Stratagene) and 0.05 μg of Renilla-Luc control plasmid (Promega) using Lipofectamine 2000 (Invitrogen). One day after the transfection, the medium was replaced with serum-free MEM and further incubated for 24 hours. Six hours after the stimulation, cells were lysed in reporter lysis buffer (Promega) followed by freezing and thawing, and luciferase activity was measured using the.
Dual-Glo luciferase assay system (Promega). The light intensity of pNFAT-Luc was normalized to that of Renilla-Luc and expressed relative to the WT control.

**Intracellular Ca**²⁺ **transient measurement.** Intracellular Ca**²⁺** transient was measured as described previously. Isolated myocytes were loaded with indo-1 acetoxyethyl ester (Invitrogen; 10 µmol/L for 5 min) and 0.25% cremophor and continuously superfused with modified Tyrode’s solution containing NaCl (137 mmol/L), KCl (5.4 mmol/L), MgCl₂ (1 mmol/L), CaCl₂ (1.8 mmol/L), and N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonic acid (HEPES; 10 mmol/L) (pH 7.4). The intracellular Ca**²⁺** transient was measured at 34°C by using a cooled fast CCD camera (EMCCD; Hamamatsu Photonics K.K., Shizouka, Japan) with a 340-nm excitation wavelength and emission at 405 and 480 nm. The data were analyzed with AQUACOSMOS (Hamamatsu Photonics K.K.), and the 405/480 fluorescence ratio was considered to be indicative of the intracellular Ca**²⁺** level.

**Estimation of SR Ca**²⁺ **content.**
To obtain strong fluorescence images, in order to manipulate experiments with ease, we used Fluo-4 AM as the Ca**²⁺** indicator. NMVMs were loaded with 1 µmol/L Fluo4-AM (Invitrogen) and 0.25% cremophor and incubated for 30 minutes at 37°C, followed by 30 minutes in dye-free solution to allow intracellular de-esterification of the dye. Dishes were mounted on an inverted microscope (Olympus 1X81, equipped with ×60 objective) attached to a confocal laser-scanning unit (Olympus Fluoview FV1000; argon laser), although the confocal aperture was set to 800 µm in order to obtain whole-cell fluorescence images. The myocytes were superfused with Tyrode solution containing 1.8 mmol/L CaCl₂ at 20°C to arrest their spontaneous beating, and electrically stimulated for 2 minutes at 1 Hz through parallel platinum field electrodes to obtain the steady-state SR Ca**²⁺** loading. Three seconds after stopping the stimulation, the myocytes were briefly (1.5 s) exposed to 10 mmol/L caffeine to monitor SR Ca**²⁺** release. The excitation wavelength was 488 nm and fluorescent emission was measured at >510 nm. Images were acquired using FV10-ASW imaging software (Olympus Optical Co., Tokyo, Japan). Myocytes that showed similar dye-loading were selected for analysis. Intracellular calcium images were calibrated according to the following:

\[
[K_{a}^{2+}] = K_d x R / (K_d / [Ca^{2+}]_{rest} - R + 1),
\]

where \( R \) is the normalized fluorescence (\( F/F_0 \)), and \( K_d \) is the dissociation constant of the Ca**²⁺**-fluor-4 complex. We used a \( K_d \) for fluo-4 in the cytoplasmic environment of muscle cells of 1.1 µmol/L. \(^3\) \([Ca^{2+}]_{rest}\) is the resting Ca**²⁺** concentration, which was determined using Indo 1 AM as previously described. \( R_{min} \) and \( R_{max} \) were determined using saturating (20 mmol/L) and Ca**²⁺**-free (10 mmol/L EGTA) Tyrode solution, respectively, in the presence of the non-fluorescent ionophore bromo-A-23187 (10 µmol/L, Sigma-Aldrich) and butanedione monoxime (3 mmol/L, Sigma-Aldrich) to avoid hypercontracture. The SR Ca**²⁺** contents were determined as 720 ± 25 nmol/L and 350 ± 23 for WT and KO myocytes, respectively.
Antibodies and other materials. Rabbit polyclonal antibodies against NCS-1 and NCX1 were prepared as described previously. We used rabbit polyclonal antibodies against Cav1.2 and Cav3.1 (Millipore, Billerica, MA), phospho-CaMKII (Thr286) (Cell Signaling Technology, Danvers, MA), phospho-PLB (Ser16) (Upstate Biotechnology, Waltham, MA), phospho-PLB (Thr17) (Santa Cruz Biotechnology, Santa Cruz, CA), IP₃R1-3 (Santa Cruz Biotechnology) and IP₃R2 (GeneTex, Inc., Irvine, CA) as well as mouse monoclonal antibodies against NCS-1 and LAP2 (BD Transduction Laboratory, Franklin Lakes, NJ), calsequestrin and PLB (Upstate Biotechnology), α-actinin and SERCa2a (Sigma), GAPDH (Chemicon, Billerica, MA) and CaMKII-δ (L-14) (Santa Cruz Biotechnology). Phenylephrine hydrochloride, ET-1, and 2-aminoethoxydiphenyl borinate were from Sigma-Aldrich. Adenophostin A and hexasodium salt were purchased from Calbiochem. All other reagents were of special grade.

Immunoprecipitation and immunoblotting. HEK293 cells or cultured NMVMs were solubilized in modified RIPA buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.4), 1% Triton X-100, and protease inhibitors (Roche Diagnostics, Basel, Switzerland). The hearts of WT or Ncs1⁻/⁻ mice were homogenized in the modified RIPA buffer. After centrifugation (15,000 rpm for 20 min), total cell lysates or homogenates were immunoprecipitated with the appropriate antibodies and protein A Sepharose in the presence of 50 μM Ca²⁺ or Ca²⁺-free conditions (30 mM EGTA in Ca²⁺-free solution). For general immunoblotting, mouse heart homogenates were prepared in urea buffer containing 2 mol/L urea, 10 mmol/L NaHCO₃, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 2% sodium dodecyl sulfate (SDS), and protease inhibitors followed by centrifugation (15,000 rpm for 10 min). Then, the immunoblots were analyzed using the same amounts of proteins after quantification (Pierce Chemical Co., Rockford, IL). The band density was quantified using Image-Pro Plus (Media Cybernetics).

Immunofluorescence. Cultured NMVMs or HEK293 cells treated for transfection or infection for 2–3 days were subjected to immunofluorescence analysis by using standard procedures. The images of stained samples were acquired by confocal microscopy (Fluoview FV1000; Olympus). The expression levels of ANF were quantified by the color extraction method using Image-Pro software, as described for histological analysis.

Ventricular myocyte isolation and T-tubule imaging. Single ventricular myocytes were freshly isolated from adult male mouse hearts through standard enzymatic techniques by using Langendorff’s perfusion apparatus. Ventricles were also isolated from 1-week-old mice; after sectioning, they were enzymatically dissociated into single cells. For T-tubule imaging, myocytes were incubated in the non-permeable, plasma membrane-selective fluorescent dye
1-(3-sulfonatopropyl)-4-[beta[2-(di-n-octylamino)-6-naphthyl]vinyl]pyridinium betaine (di-8-ANEPPS; 5 µmol/L for 5 min; Invitrogen) followed by washing in Tyrode’s solution. The stained samples were observed by confocal microscopy (Fluoview FV1000; Olympus).

**Statistical analysis.**

Differences between the 2 groups were analyzed by unpaired or paired Student’s t test. Differences between more than two groups were analyzed using one-way ANOVA followed by Dunnett’s test (Figure 5C, Figure 7D, and Supplemental Figure V-C) and two-way ANOVA followed by Student-Newman-Keuls test. Repeated measure of ANOVA followed by Student-Newman-Keuls Method was used for Figure 4C and log rank test was used for Figure 1D. A probability value of less than 0.05 was considered significant.

**Supplemental References**


**Online Table I**

Echocardiographic analyses of C57BL/6 N-CR wild-type (WT) and Ncs-1\(^{-/-}\) (KO) mice at 2 weeks (juvenile), 6 weeks (young adult), and 4 months (adult) of age.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Juvenile</th>
<th>Young adult</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n = 6)</td>
<td>KO (n = 6)</td>
<td>P</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>2.61 ± 0.06</td>
<td>2.79 ± 0.04</td>
<td>0.0823</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>1.45 ± 0.08</td>
<td>1.94 ± 0.10*</td>
<td>0.0058</td>
</tr>
<tr>
<td>FS (%)</td>
<td>47.4 ± 0.8</td>
<td>30.6 ± 3.3**</td>
<td>0.0015</td>
</tr>
<tr>
<td>LV diastolic volume (mL)</td>
<td>24.6 ± 1.4</td>
<td>29.2 ± 1.1*</td>
<td>0.0292</td>
</tr>
<tr>
<td>LV systolic volume (mL)</td>
<td>4.8 ± 0.3</td>
<td>12 ± 1.5**</td>
<td>0.0017</td>
</tr>
<tr>
<td>%EF</td>
<td>80.7 ± 0.8</td>
<td>59.1 ± 4.8**</td>
<td>0.0029</td>
</tr>
</tbody>
</table>

Abbreviations: LVIDd, left ventricular internal dimension at end diastole; LVIDs, left ventricular internal dimension at end systole; FS, fractional shortening; EF, ejection fraction. Values are means±SEM. *P<0.05 versus WT; **P<0.01 versus WT. The parameters were measured at a heart rate of around 420 bpm at all ages.
Online Figure I

Expression pattern of NCS-1 protein. (A) Subcellular localization pattern of NCS-1 in frozen longitudinal sections (left) and cross-sections (right) of cardiac muscle obtained from the hearts of 11-day-old mice. NCS-1 localized at plasma membrane (yellow arrow) and perinuclear region (white arrow). The specificity of the NCS-1 antibody is shown in Online Figure III-A. (B) NCS-1 is predominantly expressed in the α-actinin-positive cardiomyocytes but not in fibroblasts. (C and D) NCS-1 colocalization with LAP2 (C) and SERCa2 (nucleus and SR markers, respectively) (D) in cultured neonatal mouse ventricular myocytes (NMVMs). Scale bars = 10 μm.
Online Figure II
Characterization of heart functions in 4-month-old wild-type (WT) and Ncs1<sup>−/−</sup> (KO) mice. (A) Comparisions of heart weight (HW), HW-to-body weight (BW) ratio and HW-to-tibial length (TL) ratio. (B) Representative M-mode echocardiographic images obtained at a heart rate (HR) of around 420 bpm, adjusted by using 1%–1.5% isoflurane anaesthesia. The data on the left ventricular internal dimension (LVID) at end diastole and end systole and left ventricular fractional shortening (FS) are summarized in the bar graphs and Online Table 1. No differences in the cardiac functions were detected in 4 month-old mice.
Online Figure III
Neuronal calcium sensor-1 (NCS-1) co-immunoprecipitated and co-localized with inositol 1,4,5-trisphosphate receptors (IP3R1 and IP3R2) in HEK293 cells and cardiomyocytes. (A) Specificity of NCS-1 antibody. Total homogenate in urea buffer was prepared from WT mouse ventricle and 25 μg of protein was loaded on the 4-12% gel. (B) Co-immunoprecipitation (IP) in HEK293 cells transfected with NCS-1-HA and IP3R1 or NCS-1-HA and IP3R2 followed by IP of total cell lysates with HA matrix. The immunoprecipitants were immunoblotted with pan anti-IP3R antibody. (C) Immunofluorescence analysis in HEK293 cells transfected with NCS-1 plus IP3R1, NCS-1 plus IP3R2 or E120Q-HA plus IP3R2. Cells were double-stained with rabbit anti-IP3R and mouse anti-NCS-1(or anti HA for E120Q-HA transfected cells) antibodies. Little co-localization of E120Q and IP3R2 was detected at the perinuclear region. (D) Co-IP in cultured NMVMs infected with adeno-NCS-1-HA followed by IP of total lysate with rabbit anti NCS-1 antibody. The immunoprecipitants were immunoblotted with pan anti-IP3R or IP3R2 specific antibodies. Reciprocal analysis was also performed. (E) Immunofluorescence analysis in cultured myocytes infected with or without adeno-NCS-1-HA. Scale bars = 10 μm.
Online Figure IV

Involvement of NCS-1 in cardiac hypertrophy and its possible signaling pathways. (A) Phenylephrine (PE)-treatment increases NCS-1 expression from early stage. WT mice at 7 weeks of age were subjected to chronic PE or PBS infusion for 2, 4, 6 and 8 days, and heart homogenates were subjected to Western blot analysis. (B) Mean values of heart weight and body weight of WT and NCS-1−/− mice 1 week after infusion of PE or saline. (C) NFAT-dependent luciferase activity in cultured NMVMs treated with Ad-NCS-1, 100 μM PE, 10% serum or 1 μM FK506, a calcineurin inhibitor (n = 4/group). *P < 0.05, versus control group (determined by one-way ANOVA followed by Dunnett’s test). Expression level of NCS-1 and GAPDH with or without Ad-NCS-1 infection is also shown. (D) Immunoblot analysis of the levels of total and phosphorylated ERK and p38 MAP kinases in heart homogenates from WT or Ncs1−/− mice subjected to PE or saline infusion for 1 week. Phosphorylated protein levels were normalized to the total levels and summarized in the bar graphs (n = 8/group).
Online Figure V
T-tubule staining with di-8-ANEPPS in ventricular myocytes from adult and 1-week-old mice. The T-tubule density was less in immature myocytes, but there was little difference between WT and Ncs1−/− mice at all ages. Scale bars = 10 μm.
Online Figure VI
No physical interaction occurred between CaMKII-δ and NCS-1. (A) HEK293 cells were transfected with CaMKII-delta and NCS-1 HA. Two days later, total cell lysate were immunoprecipitated with anti-HA, anti-CaMKII-delta or anti-phospho CaMKII and immunoprecipitants were processed to Western blot analysis. (B) HEK293 cells were transfected with CaMKII-delta/GFP and NCS-1 (or its mutant E120Q or G2A)/Ds-Red. Two days later, cells were observed for fluorescence microscopy. CaMKII-δ and NCS-1 were not extensively co-localized, while unmyristoylated NCS-1 mutant G2A, which is known to be inhibited membrane association were co-localized.
Online Figure VII
Possible mechanism through which NCS-1 mediates EC coupling in the immature heart and cardiac hypertrophy.