Calsequestrin Accumulation in Rough Endoplasmic Reticulum Promotes Perinuclear Ca\(^{2+}\) Release *

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Running title: CSQ2-mediated perinuclear Ca\(^{2+}\) signaling

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Background: Calsequestrin is a high-capacity Ca\(^{2+}\)-binding protein that stores Ca\(^{2+}\) within the sarcoplasmic reticulum.

Results: Calsequestrin retention in rough endoplasmic reticulum promotes perinuclear Ca\(^{2+}\) release and spontaneous Ca\(^{2+}\) wave initiation from perinuclear regions.

Conclusion: Subcellular redistribution of calsequestrin affects spatial distribution of Ca\(^{2+}\) signals and myocyte function.

Significance: Our data provide a new perspective of calsequestrin in perinuclear Ca\(^{2+}\) homeostasis.

SUMMARY

Molecular mechanisms underlying Ca\(^{2+}\) regulation by perinuclear endoplasmic / sarcoplasmic reticulum (ER/SR) cisternae in cardiomyocytes remain obscure. To investigate the mechanisms of changes in CSQ2 trafficking on perinuclear Ca\(^{2+}\) signaling, we manipulated the subcellular distribution of CSQ2 by overexpression of CSQ2-DsRed, which specifically accumulates in perinuclear rough ER. Adult ventricular myocytes were infected with adenoviruses expressing CSQ2-DsRed, wild-type CSQ2 (CSQ2-WT), or empty vector. We found that perinuclear-enriched CSQ2-DsRed but not normally distributed CSQ2-WT enhanced nuclear Ca\(^{2+}\) transients more potently than it did cytosolic Ca\(^{2+}\) transients. Overexpression of CSQ2-DsRed produced more actively propagating Ca\(^{2+}\) waves from perinuclear regions than CsQ2-WT. Activities of the SR/ER Ca\(^{2+}\)-ATPase (SERCA) and ryanodine receptor (RYR2) but not type 2 IP\(_3\) receptor (IP\(_3\)R2) were required for the generation of these perinuclear-initiated Ca\(^{2+}\) waves. In addition, CSQ2-DsRed was more potent than CSQ2-WT in inducing cellular hypertrophy in cultured neonatal cardiomyocytes. Our data demonstrate for the first time that CSQ2 retention in rough ER / perinuclear region promotes perinuclear Ca\(^{2+}\) signaling, and predisposes to RyR2-mediated Ca\(^{2+}\) waves from CSQ2-enriched perinuclear compartments and myocyte hypertrophy. These findings provide new insights into the mechanism of CSQ2 in Ca\(^{2+}\) homeostasis, suggesting that rough ER-localized Ca\(^{2+}\) stores can operate independently in raising levels of cytosolic / nucleoplasmic Ca\(^{2+}\) as a source of Ca\(^{2+}\) for Ca\(^{2+}\)-dependent signaling in health and disease.

Calcium (Ca\(^{2+}\)) is the most universal signal used by different cell systems to encode diverse information, which is decoded from the temporal and spatial dynamics of Ca\(^{2+}\) signals by specific mechanisms. It has been recognized that the subcellular locations of Ca\(^{2+}\) events determine the specific biological outcomes of Ca\(^{2+}\) signaling.(1) In cardiomyocytes, Wu et.al showed that type 2 IP3 Receptors (IP3R2) mediated nuclear envelope Ca\(^{2+}\) signals have a primary effect on regulating gene transcription.(2) More recently, it was demonstrated that nuclear Ca\(^{2+}\) elevations, but not the global contraction-associated Ca\(^{2+}\) elevations, induce cardiomyocyte hypertrophy.(3) However, mechanisms regulating the nuclear or perinuclear Ca\(^{2+}\) events still remain obscure.
During cardiac excitation-contraction (EC) coupling, sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release occurs primarily through the type 2 ryanodine receptors (RyR2s), which are located at junctional SR sites.(4-6) RyR2 functionally associates with three additional SR proteins: the luminal SR protein cardiac calsequestrin (CSQ2) and two smaller SR transmembrane proteins, triadin and junctin.(6-9) CSQ2 is a low affinity, high-capacity Ca\textsuperscript{2+}-binding protein that can store Ca\textsuperscript{2+} within the SR.(10) Each molecule of CSQ2 can bind 18 to 50 Ca\textsuperscript{2+} ions. CSQ2 is also believed to regulate the activity of RyR2 Ca\textsuperscript{2+} release channels by controlling the local luminal Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in the vicinity of the RyR2 channels.(11-13).

The junctional SR appears to be contiguous with the nuclear envelope in cardiomyocytes.(14) Meanwhile, cardiac rough ER exists in adult cardiomyocytes as perinuclear cisternae, perhaps including the outer leaf of the nuclear envelope, from where CSQ2 is synthesized and traffics anterogradely along an as-yet unidentified pathway.(15) Well-defined changes in CSQ2 co-translocation processing during cardiac hypertrophy or heart failure, suggest that retention of CSQ2 in the rough ER of the perinuclear cisternae is greatly augmented.(16) We postulated that the accumulation of CSQ2 in the perinuclear rough ER could play a role in the regulation of local Ca\textsuperscript{2+} events, which may lead to pathophysiological response.

To test this hypothesis, we used adenovirus-mediated expression of a CSQ2 fusion protein, CSQ2-DsRed, in cultured cardiomyocytes. When CSQ2 is overexpressed as a fusion protein with DsRed, it is mostly retained in rough ER.(15) This CSQ2 accumulation in rough ER/perinuclear cisternae allows us to study the effects of CSQ2 retention on myocyte perinuclear Ca\textsuperscript{2+} handling and its implication for cardiac disease. We compared triggered and spontaneous Ca\textsuperscript{2+} release in cultured adult cardiomyocytes expressing CSQ2 in either the junctional SR (CSQ2-WT) or the perinuclear region (CSQ2-DsRed). Our data show that enrichment of CSQ2 in the perinuclear cisternae was sufficient to enhance nuclear Ca\textsuperscript{2+} transients, promoting Ca\textsuperscript{2+}-dependent transcription and myocyte hypertrophy, and also led to the shift of spontaneous arrhythmogenic Ca\textsuperscript{2+} waves emanating from the perinuclear region instead of the junctional SR.

**EXPERIMENTAL PROCEDURES**

*Adult ventricular myocyte culture and adenoviral infection of CSQ2 constructs* - Rat and mouse ventricular myocytes were isolated and cultured as described.(17) Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee at the University of Iowa. IP\textsubscript{3}R2\textsuperscript{-/-} and wildtype blackswiss mice were kindly provided by Dr. Ju Chen (University of California at San Diego).(18) Two hours after plating cells, adenoviruses were applied at a multiplicity of infection (MOI) of 100. Adenoviruses containing cDNAs for CSQ2 (Ad-CSQ2), CSQ2-DsRed fusion protein (Ad-CSQ2-DsRed) were described previously.(15) Empty Ad5 vector (Ad-empty) was purchased from Gene Transfer Vector Core of University of Iowa. Experiments were performed 40-48 hours after infection.

**Western blotting analysis and immunofluorescence of CSQ2 and RyR2 proteins** - Western blotting was performed as previously described.(17) Mouse anti-CSQ2 was used to detect endogenous and exogenous CSQ2. GAPDH (Cell Signaling Technology, Inc) was used as a loading control. Immunofluorescence of CSQ2 and RyR2 proteins was performed as previously described.(15, 19) CSQ2-DsRed was detected as DsRed fluorescence. Nuclear staining was performed with TO-PRO-3 (Invitrogen Inc).

**Confocal Ca\textsuperscript{2+} imaging of cultured adult ventricular myocytes** - Confocal Ca\textsuperscript{2+} imaging was performed as described.(19) Briefly, cells were loaded with Fluo-4 AM at 37 °C for 30 minutes. Then the cells were washed with Tyrode’s solution at room temperature for 15 min before Ca\textsuperscript{2+} imaging. Confocal images were acquired using a 63X, 1.3 NA oil immersion objective mounted on a Zeiss LSM 510 confocal microscope (Carl Zeiss MicroImaging Inc., Germany). Confocal line scan or 2-D frame scan was used to record Ca\textsuperscript{2+} signals. Steady state Ca\textsuperscript{2+} transients were measured in Tyrode’s solution containing 1.8 mM Ca\textsuperscript{2+} under field stimulation of 1 Hz. Ca\textsuperscript{2+} waves were examined after field stimulation was halted and each cell was imaged for 24-40 sec. In some
experiments, ouabain (100 μM), isoproterenol (100 nM), or 10 mM [Ca$^{2+}$], were added to induce Ca$^{2+}$ waves. The fluorescence of CSQ2-DsRed was also recorded to identify the infected myocytes and perinuclear-localized protein. For CSQ2-WT and Ad-empty infected myocytes, the nuclear region was readily identified by the strong Ca$^{2+}$ indicator staining of the nuclear envelope and immediate surrounding SR, and also by delayed Ca$^{2+}$ transients when compared with adjacent cytosolic regions. SR Ca$^{2+}$ content was measured with as the maximum Ca$^{2+}$ release induced by a local spritz of 10 mM caffeine. 

**Primary culture of neonatal cardiomyocytes and immunostaining assay** - Neonatal myocytes were prepared as previously described, (20) and infected with viral construct at MOI of 50. Cells were used 48 hours after adenovirus infection. α-actinin (sigma) immunofluorescence was used to detect cardiomyocytes. Cell surface area was measured using ImageJ 1.43 (NIH Imaging).

**Quantitative RT-PCR** - Total RNA extraction from cell samples was performed by using TRIZOL (invitrogen). 0.6 μg of DNase-treated total RNA samples for each group was reverse transcribed using Superscript II (Invitrogen). qPCR using iQ SYBR Green Supermix (Bio-Rad) was performed on a Bio-Rad iQ5 real time PCR cycler. Primers were specific for mouse sequences. The sequences were as follows: ANF: forward, GTCTTGGCCTTTTGGCTC, reverse: TTCCTCAGTCTGCTCCTC. GAPDH: forward, CATTTTCCTGATGACATTGAATACG, reverse, TCCAGGGTTTCTTACTCCTGGA. Relative transcript quantities of target (ANF) and endogeneous (GAPDH) genes were determined using the comparative C$\text{_{T}}$ method. The amount of ANF was normalized to the amount of GAPDH for each group.

**Statistics** - Data were expressed as mean ± SE. ANOVA, Student’s t test, and χ$^2$ test were applied when appropriate. A p value of <0.05 was considered statistically significant.

**RESULTS**

**Perinuclear CSQ2 increases nuclear Ca$^{2+}$ transients** - Western blotting assay indicated that infection of myocytes with Ad-CSQ2-WT and Ad-CSQ2-DsRed resulted in comparable expression levels of CSQ2-WT (increased by 150±20% (n=3) compared to Ad-empty) and CSQ2-DsRed (increased by 143±21% (n=3) compared to endogenous CSQ2 of the Ad-CSQ2-DsRed infected myocytes) (**Fig. 1**). Confocal immunofluorescence showed that wildtype CSQ2 was overexpressed with brighter CSQ2 fluorescence and normally distributed throughout the junctional SRs; whereas exogenously expressed CSQ2-DsRed localized to the perinuclear region of cardiomyocytes in both rats (**Fig. 2A-F**) and mice (Online Supplementary Data, Figure S1). Confocal duo-imaging of RyR2 immunofluorescence and CSQ2 DsRed fluorescence in Ad-CsQ2-DsRed infected myocytes showed that, RyR2 was present in perinuclear SR puncta that co-localized with CSQ2-DsRed (**Fig. 2G-L**). RyR2 was evenly distributed in both junctional SR and perinuclear region, whereas CsQ2-DsRed was confined to the perinuclear region (**Fig. 2G-L**).

We next investigated how expression of perinuclear-enriched CSQ2-DsRed and WT CSQ2 affected Ca$^{2+}$ transients in rat ventricular myocytes. Steady state, field stimulated Ca$^{2+}$ transients (1 Hz) were acquired using linescan confocal imaging, with the scan line across the center of the nucleus (nuclei) longitudinally (**Fig. 3A**). Cytosolic Ca$^{2+}$ ($[\text{Ca}^{2+}]_{\text{cyt}}$) and nuclear Ca$^{2+}$ ($[\text{Ca}^{2+}]_{\text{nu}}$) transients were obtained, normalized, and plotted against time (**Fig. 3A**). The $[\text{Ca}^{2+}]_{\text{nu}}$ transients were distinguished from corresponding $[\text{Ca}^{2+}]_{\text{cyt}}$ transients by a smaller magnitude and prolonged time-to-peak and decay kinetics. Overexpression of CSQ2-WT significantly increased the amplitudes of $[\text{Ca}^{2+}]_{\text{cyt}}$ transients and $[\text{Ca}^{2+}]_{\text{nu}}$ transients proportionally (**Fig. 3B,C,D**). Overexpression of CSQ2-DsRed also increased the amplitudes of $[\text{Ca}^{2+}]_{\text{cyt}}$ transients to a similar level as that of CSQ2-WT even though CSQ2-DsRed was predominantly localized around the nucleus. These data imply that the rough ER can serve as a Ca$^{2+}$ store and contributes to bulk $[\text{Ca}^{2+}]_{\text{cyt}}$ signals, as previously suggested,(14) In contrast to effects of WT-CSQ2 and CSQ2-DsRed on $[\text{Ca}^{2+}]_{\text{nu}}$, CSQ2-DsRed expression induced a much greater effect on $[\text{Ca}^{2+}]_{\text{nu}}$ when normalized to the amplitude of $[\text{Ca}^{2+}]_{\text{cyt}}$ transients (**Fig. 3D**). Assessment of SR Ca$^{2+}$ stores demonstrated that overexpression of CSQ2-WT and CSQ2-DsRed similarly increased the bulk Ca$^{2+}$ loading of caffeine-sensitive Ca$^{2+}$ stores (**Fig. 3E**). These data
indicate that overexpression of CSQ2-WT enhances [Ca\textsuperscript{2+}]\textsubscript{cyt} and [Ca\textsuperscript{2+}]\textsubscript{nu} equally, whereas perinuclear-enriched CSQ2 increases [Ca\textsuperscript{2+}]\textsubscript{nu} signal more potently than it does [Ca\textsuperscript{2+}]\textsubscript{cyt}, suggesting that CSQ2 plays a critical role in mobilizing Ca\textsuperscript{2+} release from ER/SR lumens.

**CSQ2-DsRed promotes Ca\textsuperscript{2+} waves originating from the perinuclear region** - Based on our finding that perinuclear CSQ2 favorably increases nuclear Ca\textsuperscript{2+}, we next investigated whether overexpression of CSQ2-DsRed promotes spontaneous Ca\textsuperscript{2+} release events in a similar spatial pattern. We therefore compared spontaneous Ca\textsuperscript{2+} waves originating from cells expressing either wildtype or perinuclear CSQ2. Perinuclear-originated Ca\textsuperscript{2+} waves were defined as those for which the firing spot fell within the region of the nucleus and the 4 \( \mu \)m flanking regions in linescan images (Fig. 4A, right panel. For more details, see Online Supplementary Data, Figures S2 & S3). Both perinuclear- and non-perinuclear-originated Ca\textsuperscript{2+} waves were detected in the presence and absence of CSQ2 overexpression (Fig. 4A). In empty virus-infected myocytes, the perinuclear-originated Ca\textsuperscript{2+} waves accounted for 30% of the total recorded waves (Fig. 4B). Overexpression of CSQ2-WT slightly increased the percentage to 39%. By sharp contrast, in CSQ2-DsRed overexpressing myocytes, the perinuclear-originated Ca\textsuperscript{2+} waves were significantly increased (to 86%). To promote the generation of spontaneous Ca\textsuperscript{2+} waves, the myocytes were also exposed to high extracellular Ca\textsuperscript{2+} (10 mM), 100 \( \mu \)M ouabain, or 100 nM isoproterenol. Under each of these conditions, overexpression of CSQ2-DsRed but not CSQ2-WT maintained this increased percentage of perinuclear-originated Ca\textsuperscript{2+} waves of more than 80% (Fig. 4C-E). Overexpression of CSQ2-WT only slightly increased the percentage of perinuclear-originated Ca\textsuperscript{2+} waves in the presence of ouabain or excess extracellular Ca\textsuperscript{2+} (Fig. 4D-E), but not isoproterenol. Taken together, these data indicate that the localization of CSQ2 was the critical factor for spatial distribution of spontaneous Ca\textsuperscript{2+} waves such that increased expression of CSQ2 in the perinuclear region promotes Ca\textsuperscript{2+} wave generation from this area.

**Ryanodine receptors but not IP\textsubscript{3} receptors are necessary for the generation of perinuclear-originated Ca\textsuperscript{2+} waves** - IP\textsubscript{3} receptors (IP\textsubscript{3}-Rs), especially type 2 IP\textsubscript{3}-Rs (IP\textsubscript{3}R2s), are localized to nuclear envelope (21) where they contribute to nuclear Ca\textsuperscript{2+} signaling (22-25). To examine whether the CSQ2-DsRed induced spatial redistribution of Ca\textsuperscript{2+} wave firing requires IP\textsubscript{3}Rs activity, we first tested whether inhibition of IP\textsubscript{3}Rs with Xestospongin C (XeC) and 2-aminoethoxydiphenyl borate (2-APB) affects the generation of Ca\textsuperscript{2+} waves from the perinuclear region. Figure 5A and Figure S4) show a sequence of 2D images displaying representative non-perinuclear- and perinuclear-originated Ca\textsuperscript{2+} waves from myocytes infected with either Ad-CSQ2-WT (left panels) or Ad-CSQ2-DsRed (right panels). Unexpectedly, neither the generation of non-perinuclear-originated Ca\textsuperscript{2+} waves nor the perinuclear-originated Ca\textsuperscript{2+} waves was blocked by XeC and 2-APB. Although the initiation of Ca\textsuperscript{2+} waves was not affected by XeC or 2-APB, application of high concentration 2-APB reduced the size and amplitude of Ca\textsuperscript{2+} waves (Fig. S4-B), possibly due to the non-specific effects of 2-APB, e.g., its partial inhibition on SERCA pump. (26)

To further test the contribution of the perinuclear-localized IP\textsubscript{3}R2 to the generation of perinuclear-originated Ca\textsuperscript{2+} waves, ventricular myocytes from wildtype or IP\textsubscript{3}R2 knockout (IP\textsubscript{3}R2\textsuperscript{-/-}) mice were used. Similar to the results from rat myocytes (Fig. 4), overexpression in wildtype myocytes of CSQ2-DsRed but not CSQ2-WT, resulted in mostly (95%) perinuclear-originated Ca\textsuperscript{2+} waves (Fig. 5B, D). The percentage of Ca\textsuperscript{2+} waves generated in the perinuclear region remained unchanged (90%) in IP\textsubscript{3}R2\textsuperscript{-/-} myocytes that expressed CSQ2-DsRed (Fig. 5C, E). These data indicate that the enhanced release of perinuclear Ca\textsuperscript{2+} that occurs with CSQ2-DsRed in rough ER / perinuclear cisternae occurs through a mechanism not involving IP\textsubscript{3}R2.

To further understand the mechanism of CSQ2-mediated perinuclear Ca\textsuperscript{2+} wave generation, we then evaluated the effects of RyR2 inhibitor on perinuclear-originated Ca\textsuperscript{2+} waves. Treatment with the RyR2 inhibitor tetracaine (100 \( \mu \)M, Fig. 6A) or more specific RyR2 blocker ryanodine (5 \( \mu \)M, Fig. 6B) of rat ventricular myocytes expressing either CSQ2-WT or CSQ2-DsRed, completely blocked both the perinuclear- and non-perinuclear-originated Ca\textsuperscript{2+} waves. Removal of the inhibitor tetracaine restored Ca\textsuperscript{2+} wave generation from both regions following either CSQ2-WT or CSQ2-
DsRed overexpression. In addition, the irreversible SERCA inhibitor thapsigargin completely abolished all Ca\(^{2+}\) waves initiated from any regions in both Ad-CSQ2-WT- and Ad-CSQ2-DsRed-infected myocytes (Fig. 6C). These data, taken together, indicate that IP\(_3\)Rs contribute minimally to CSQ2-DsRed-induced re-distribution of Ca\(^{2+}\) waves, whereas RyR2-mediated Ca\(^{2+}\) release is necessary for the generation of both perinuclear- and non-perinuclear-originated Ca\(^{2+}\) waves, consistent with the co-localized pattern of RyR2 with CSQ2-DsRed in a confined perinuclear region. Our data also suggest that SERCA-regulated Ca\(^{2+}\) storage is necessary for the generation of either type of Ca\(^{2+}\) wave, independent of CSQ localization.

Overexpression of CSQ2-DsRed and CSQ2-WT enhances the hypertrophy of neonatal cardiomyocytes - Nuclear Ca\(^{2+}\) plays an important role in the regulation of gene expression. Enhanced nuclear Ca\(^{2+}\) signals induced by CSQ2 redistribution could affect hypertrophic processes in cardiomyocytes. We therefore studied the consequence of overexpressing CSQ2-WT and CSQ2-DsRed in mouse neonatal cardiomyocytes, which display autonomous hypertrophy in culture. Using an antibody specific for \(\alpha\)-actinin to define myocyte dimension, we found that overexpression of CSQ2-DsRed increased the surface area of neonatal cardiomyocytes by 27\(\pm\)2\%, whereas equal overexpression of CSQ2-WT (Fig. 1) produced a modest increase in surface area (14\(\pm\)3\%, Fig. 7A, B). These data were consistent with our findings on quantitative analysis of hypertrophic marker ANF in neonatal myocytes under these treatments (Fig. 7C). Taken together, our results demonstrate that overexpression of CSQ2 enhances hypertrophy of neonatal cardiomyocytes, and the perinuclear-enriched form of CSQ2 produces a more pronounced effect as compared to CSQ2-WT, consistent with their differential contributions to nuclear Ca\(^{2+}\) levels.

**DISCUSSION**

We recently developed a method to direct CSQ2 localization to the perinuclear rough ER by appending the fluorescent protein DsRed to CSQ2. (15) CSQ2-DsRed overexpression thus provides an interesting model for studying the biological consequences of the perinuclear-enriched form of CSQ2. In the present study, by overexpressing CSQ2-WT or CSQ2-DsRed in rat and mouse ventricular myocytes, we found that 1) CSQ2-DsRed increases the bulk Ca\(^{2+}\) storage in ER/SR, similarly to CSQ2-WT; 2) CSQ2-DsRed but not CSQ2-WT preferentially enhances the nuclear Ca\(^{2+}\) transients and promotes active, propagated Ca\(^{2+}\) waves initiated from the perinuclear region; 3) Perinuclear-originated Ca\(^{2+}\) waves are mediated by RyR2 instead of IP\(_3\)R2 and 4) CsQ2-DsRed induces a greater degree of cellular hypertrophy in cultured neonatal cardiomyocytes compared to CSQ2-WT.

The increases in cytosolic [Ca\(^{2+}\)] were initially thought to be transmitted passively to the nucleus via Ca\(^{2+}\) diffusion through nuclear pore complexes. More recent studies show that cardiomyocytes contain a separate membrane source around and within the nucleus that is sensitive to IP3. IP\(_3\)R2 receptors localized to the inner membrane surface of the nuclear envelope can function as a source of Ca\(^{2+}\) that leads to hypertrophic growth. Other studies have described a nucleoplasmic reticulum that contains IP\(_3\)-Rs and RyR2s, which is likely due to nuclear envelope membrane extension or in-foldings deep into the nucleoplasm, as revealed earlier by Vaux and colleagues and more recently from Franzini-Armstrong group with high magnification of electron micrographs. Escobar et al. described perinuclear dyads in close proximity to the nuclear envelope, aligned along a perinuclear basket of longitudinal T-tubules. These authors suggested that Ca\(^{2+}\) microdomains emanating from these RyR-sensitive sites could support or augment IP3-dependent activation. In the present study, we found that CSQ2 localized to the rough ER preferentially enhanced [Ca\(^{2+}\)]\(_{nu}\) transients as opposed to [Ca\(^{2+}\)]\(_{cyt}\) transients, while the more widely-distributed (junctional SR localized) CSQ2 proportionally enhanced [Ca\(^{2+}\)]\(_{nu}\) and [Ca\(^{2+}\)]\(_{cyt}\) transients. This differential effect is observed despite similar increases in the general SR Ca\(^{2+}\) stores by the expression of the two Ca\(^{2+}\) binding proteins. These findings suggest that both passive Ca\(^{2+}\) diffusion and active Ca\(^{2+}\) release may contribute to [Ca\(^{2+}\)]\(_{nu}\) homeostasis, and the contribution of these two modes of [Ca\(^{2+}\)]\(_{nu}\) regulation may depend on the compartmentalization of Ca\(^{2+}\) storage and Ca\(^{2+}\) binding proteins.
(compartmentalized Ca\(^{2+}\) stores). Increasing the Ca\(^{2+}\) storage in the junctional SR may therefore affect the \([\text{Ca}^{2+}]_{\text{nu}}\) by enhancing the passive flooding of cytosolic Ca\(^{2+}\) into the nucleoplasm, whereas recruiting Ca\(^{2+}\) to perinuclear ER/SR Ca\(^{2+}\) stores may directly, and more effectively, increase the Ca\(^{2+}\) released into nucleoplasm.

Recent studies reported that CSQ2 regulates the activity of Ca\(^{2+}\) release by controlling the local luminal \([\text{Ca}^{2+}]_l\) in the vicinity of the RyR channels.(11,13,35) Their data showed that overexpression of CSQ2-WT attenuates the generation of Ca\(^{2+}\) waves,(11,35) indicating its important role for the luminal Ca\(^{2+}\) sensing of RyR2. Thus, we initially predicted that myocytes overexpressing perinuclear CSQ2 would have fewer Ca\(^{2+}\) waves originating around nucleus than control myocytes. Surprisingly, we observed that the fraction of perinuclear-originated Ca\(^{2+}\) waves in CSQ2-DsRed-expressing myocytes is dramatically higher than control myocytes. Two possible mechanisms may be responsible for these apparently contradictory results: First, the RyR2 luminal Ca\(^{2+}\) sensing mechanism in rough ER may be different from that in the junctional SR. The junctional SR is a specialized subcellular compartment where RyR2 co-localizes with and acts in concert with CSQ2, triadin-1, and junctin to regulate of RyR2 by luminal Ca\(^{2+}\).(6,7,13,36,37) However, this complex has not been shown to exist in the rough ER. The second possible mechanism is that the ER form of CSQ2 in the heart is more fully phosphorylated, whereas junctional SR forms of CSQ2 are relatively dephosphorylated.(38) The result of increased levels of CSQ2 phosphorylation levels is thought to be increased rough ER retention.(15,39) This mechanism is predicted to underlie increased levels of perinuclear CSQ2 in tissue from tachycardia-induced canine heart failure.(16)

It is possible that the frequent perinuclear Ca\(^{2+}\) waves in myocytes expressing CSQ2-DsRed may be simply due to the overloading of Ca\(^{2+}\) in rough ER as a consequence of overexpression of a Ca\(^{2+}\) buffer in this region. New approaches that can spatially resolve the differences in Ca\(^{2+}\) levels between the rough ER and junctional SR are warranted and will provide a further understanding of the findings observed in this study. Our findings regarding the significance of perinuclear CSQ2 have three implications. (1) Our data suggest that the regulation of RyR2 by luminal Ca\(^{2+}\) is environment-dependent and determined differently by accessory proteins such as CSQ2. (2) The functional role of CSQ2 in cardiomyocytes is dependent on its spatial distribution. (3) If CSQ2 is redistributed through a regulated retention to rough ER, as is observed in failing hearts, Ca\(^{2+}\) homeostasis is likely to undergo a redistribution as well, from a normal contractile to an arrhythmogenic, or transcriptional phenotype. These conclusions are supported by our observations that CSQ2-DsRed preferentially enhanced perinuclear Ca\(^{2+}\) release, promotes arrhythmogenic Ca\(^{2+}\) waves from perinuclear region and stimulates myocyte hypertrophy to a greater extent than CSQ-WT. It is worthwhile to note that our findings of CsQ2-DsRed-mediated transcriptional and hypertrophic changes in cultured neonatal cardiomyocytes are interesting but limited. Future studies using alternative experimental systems, including a transgenic mouse approach may be necessary to further examine this postulation.

IP\(_3\)R2 was shown to be localized to the nuclear envelope of ventricular myocytes,(21) supporting its potential role as a regulator of perinuclear Ca\(^{2+}\) release. Several groups have demonstrated in neonatal, atrial and ventricular cardiomyocytes that IP\(_3\)-dependent Ca\(^{2+}\) release contributes to nuclear Ca\(^{2+}\) signals.(2,3,22-25) However, in the present study, using pharmacological tools and genetic mouse models, we found that IP\(_3\)R2 does not contribute to the generation of CSQ2-enhanced, perinuclear-originated Ca\(^{2+}\) waves. Instead, RyR2 is necessary for the generation of Ca\(^{2+}\) waves by perinuclear CSQ2. These data do not exclude the possibility that IP\(_3\)-dependent Ca\(^{2+}\) release may contribute to delayed responses such as Ca\(^{2+}\)-dependent transcriptional regulation.(2)

In summary, by manipulating the subcellular distribution of CSQ2, this study reveals several new aspects of its biology. Specifically, our data demonstrate that perinuclear CSQ2 accumulation enhances \([\text{Ca}^{2+}]_{\text{nu}}\) transients disproportionate to the increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) transients. CSQ2-DsRed promotes the generation of Ca\(^{2+}\) waves predominantly from the perinuclear region. The spatial redistribution of Ca\(^{2+}\) waves mediated by perinuclear CSQ2 requires RyR2s but not IP\(_3\)R2s. Thus, two Ca\(^{2+}\) microdomains for
regulation of Ca\(^{2+}\)-dependent transcription may exist in the adult cardiomyocytes: the IP\(_{3}\)-sensitive nuclear envelope and the perinuclear rough ER (Fig. 8). Finally, our data provide a new perspective of CSQ2 in perinuclear Ca\(^{2+}\) homeostasis and Ca\(^{2+}\)-dependent transcription in health and heart disease.

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FOOTNOTES
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* The abbreviations used are: CSQ2, type 2 calsequestrin; EC, excitation-contraction; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; RyR2, type 2 ryanodine receptor; IP₃R²: type 2 IP3 receptor.

FIGURE LEGENDS
Figure 1. CSQ2 (WT) and CSQ2-DsRed expression levels and localization in adenovirus-infected adult rat ventricular myocytes. A. Representative Western blots of CSQ2 expression 40-50 hrs after infection of cells with empty virus (Ad-empty) or adenovirus expression wildtype CSQ2 (Ad-CSQ2-WT) or CSQ2-DsRed fusion protein (Ad-CSQ2-DsRed). GAPDH serves as a loading control. B. Relative CSQ2 expression levels normalized to GAPDH. n=3 for each group; * p<0.01, between indicated groups by Student’s t-test.
Figure 2. A-F. CSQ2 and CSQ2-DsRed localization in adenovirus-infected adult rat ventricular myocytes. A & C are representative examples of CSQ2 immunofluorescence (detected with anti-CSQ2 antibodies, green) from myocytes infected with empty vector and CSQ2-WT, respectively. B & D are zoom-in (2x) images from C & E, respectively. Nuclei (Nu) were stained with TO-PRO-3 (blue). E & F are typical example of DsRed fluorescence showing CSQ2-DsRed subcellular distribution in the perinuclear region from a cell infected with CSQ2-DsRed. F is zoom-in (2x) image of panel E. G-L, Co-localization of RyR2 with CSQ2-DsRed in perinuclear region. G-I, RyR2 immunofluorescence (green), nuclear (Nu, blue), and CSQ2-DsRed fluorescence from adult myocytes infected with Ad-CSQ2-DsRed. J-L are zoom-in images of G-I, respectively. RyR2 was present in perinuclear SR puncta that co-localized with CSQ2-DsRed.

Figure 3. Effects of exogenous (cardiac) CSQ2 and CSQ2-DsRed on cytosolic and nuclear Ca\(^{2+}\) transients. A, Representative cytosolic and nuclear Ca\(^{2+}\) transients (1Hz) from rat ventricular myocytes infected with Ad-empty, Ad-CSQ2-WT, or Ad-CSQ2-DsRed. The scanning lines were set to cross the nucleus as indicated in the figure by the green dashed lines. Cytosolic (Cyt) and nuclear (Nu) regions were defined as indicated. The cytosolic and nuclear Ca\(^{2+}\) transients were described as F/F\(_0\). B, Cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\_cyt) transient peak amplitudes from ventricular myocytes infected as in (A). Number of cells (n)=28, 43, 34 for each group. C, Nuclear Ca\(^{2+}\) ([Ca\(^{2+}\)]\_nu) transient peak amplitudes from ventricular myocytes infected as in (A). n=27, 40, 34 for each group. D, Ratio of the amplitudes of nuclear Ca\(^{2+}\) transients to cytosolic Ca\(^{2+}\) transients. Note the significant difference between CSQ2-DsRed and the other two groups. n=27, 40, 34 for each group. E, SR Ca\(^{2+}\) content determined by 10 mM caffeine-induced Ca\(^{2+}\) release. ** p<0.01, between indicated groups by Student’s t-test.

Figure 4. CSQ2 accumulation promotes Ca\(^{2+}\) wave initiation in the perinuclear region. A, Examples of Ca\(^{2+}\) waves from cultured rat ventricular myocytes infected with adenovirus expressing CSQ2-DsRed, CSQ2-WT, or empty adenovirus. Ca\(^{2+}\) waves were recorded by confocal line scanning. The nuclear regions were defined by the red dashed lines as indicated. Two types of Ca\(^{2+}\) waves that originated from perinuclear regions or not are shown. B - E, percentages of perinuclear-originated (Peri-Nu) and non-perinuclear-originated (non-Peri-Nu) Ca\(^{2+}\) waves in normal extracellular Ca\(^{2+}\) (1.8 mM [Ca\(^{2+}\)]\_o) or after treatments as indicated (C-E). Note that Ad-CSQ2-DsRed-infected myocytes always have a significantly higher percentage of nucleus-originated Ca\(^{2+}\) waves than the other viral-infected myocytes. Number of waves detected is denoted as numbers within the bars. n=39, 25, 48 cells for Ad-empty, Ad-CSQ2-WT, Ad-CSQ2-DsRed infected myocytes, respectively, measured under 1.8 mM [Ca\(^{2+}\)]\_o; n=9, 31, 19 cells for Ad-empty, Ad-CSQ2-WT, Ad-CSQ2-DsRed infected myocytes, respectively, in 10 mM [Ca\(^{2+}\)]\_o; n=10, 25, 27 cells for Ad-empty, Ad-CSQ2-WT, Ad-CSQ2-DsRed infected myocytes, respectively, in 100 µM ouabain treatment (5 min); n=6, 16, 21 cells for Ad-empty, Ad-CSQ2-WT, Ad-CSQ2-DsRed infected myocytes, respectively, in 100 nM isoproteanol treatment (5 min) *, p<0.05, ** p<0.01 between indicated groups by \(\chi^2\) test.

Figure 5. IP\(_3\) receptors are not necessary for the initiation of perinuclear-initiated Ca\(^{2+}\) waves. A, Potent IP\(_3\)R inhibitor, Xestospongin C (XeC, IC50=358 nM) did not abolish either the perinuclear- or the non-perinuclear-originated Ca\(^{2+}\) waves. The Ca\(^{2+}\) waves were recorded by 2-D confocal imaging before and after treatment with 3 µM XeC for 10 min. Note that the Ca\(^{2+}\) wave firing spots were not affected by XeC. B-C, Either WT (B) or IP\(_3\)R2\(^{-/-}\) (C) mouse ventricular myocytes were infected with Ad-CSQ2-WT or Ad-CSQ2-DsRed. Ca\(^{2+}\) waves recorded as in (A) but in line-scan mode, with scanning line run across the nuclear center longitudinally. D-E, Summary of the percentage of perinuclear-originated and non-perinuclear-originated Ca\(^{2+}\) waves. Note that deletion of IP\(_3\)R2 did not alter the effect of CSQ2-DsRed on the spatial redistribution of Ca\(^{2+}\) waves. Number of waves detected is denoted as numbers within the bars. n=8, 23, 20 for Ad-empty, Ad-CSQ2-WT, Ad-CSQ2-DsRed in WT myocytes. n=15, 17, 28 for Ad-empty, Ad-CSQ2-WT, Ad-CSQ2-DsRed in IP\(_3\)R2\(^{-/-}\) myocytes. ** p<0.01 between indicated groups by \(\chi^2\) test.
Figure 6. Both perinuclear and non-perinuclear Ca\textsuperscript{2+} waves are RyR2 and SERCA-dependent. A, Rat ventricular myocytes infected with Ad-CSQ2-WT or Ad-CSQ2-DsRed were treated with 100 μM tetracaine for 2 min, followed by washout. Ca\textsuperscript{2+} waves were recorded by line-scan confocal imaging. Tetracaine reversibly prohibited Ca\textsuperscript{2+} wave occurrence in both types of myocytes. B, Ryanodine at 5 μM irreversibly blocked spontaneous Ca\textsuperscript{2+} waves in both Ad-CSQ2-WT and Ad-CSQ2-DsRed transfected myocytes. C, SERCA inhibitor thapsigargin (1 μM, 5 min) abolished both the nuclear- and non-nuclear-originated Ca\textsuperscript{2+} waves. n=5-8 cells per experiment.

Figure 7. Overexpression of CSQ2-DsRed and CSQ-WT enhances neonatal cardiomyocyte hypertrophy. A, Confocal micrographs of neonatal myocytes following infection with Ad-empty, Ad-CSQ2-WT, or Ad-CSQ2-DsRed and staining with α-actinin (green). B, Summary data of (A) in which cell surface area was measured in three independent experiments. The results are presented as mean±SE relative to the Ad-empty control, which was assigned a value of 1. Statistical significance was first tested by one-way ANOVA, then the differences between groups were determined by t-test. n=257, 284, 418 for Ad-empty, Ad-CSQ2-WT, and Ad-CSQ2-DsRed respectively. ** p<0.01, between indicated groups by Student’s test. C. Quantitative PCR analysis of hypertrophy marker ANF in neonatal myocytes. n=3 batches of cells per group. *, p<0.05.

Figure 8. Model of perinuclear Ca\textsuperscript{2+} microdomain. The Ca\textsuperscript{2+} microdomain in the perinuclear / rough ER is distinct from nuclear envelope associated IP\textsubscript{3}R2-mediated Ca\textsuperscript{2+} release. CSQ2 enrichment in perinuclear cisternae affects Ca\textsuperscript{2+} homeostasis in both perinuclear and nuclear regions, contributing to Ca\textsuperscript{2+}-dependent arrhythmogenesis and transcription.
Figure 1

A

| Condition          | CSQ2-DsRed (75kd) | CSQ2-WT (50 Kd) | GAPDH (37 kd) |
|--------------------|-------------------|-----------------|--------------|
| Ad-empty           |                   |                 |              |
| Ad-CSQ2-WT         |                   |                 |              |
| Ad-CSQ2-DsRed      |                   |                 |              |
| Ad-CSQ2-WT         |                   |                 |              |
| Ad-empty           |                   |                 |              |

B

Graph showing relative level of CSQ2 protein (GAPDH) in different conditions:
- Ad-empty
- Ad-CSQ2-WT

Graph showing relative level of endogenous and exogenous CSQ2 and CSQ2-DsRed:
- Endogenous CSQ2
- Exogenous CSQ2
- CsQ2-DsRed
Figure 2

Anti-CSQ2

Ad-empty

10 μm

C

Ad-CSQ2-WT

CSQ2-DsRed

10 μm

E

Ad-CSQ2-DsRed

10 μm

A

B

G. Anti-RyR2

J

H. CSQ2-DsRed

K

I. Overlap

L

1 μm

D

Nu

Nu

F
Figure 3.

A

Ad-empty

Ad-CSQ2-WT

Ad-CSQ2-DsRed

B

[Ca\textsuperscript{2+}]\textsubscript{cyt} transients at 1 Hz

[Ca\textsuperscript{2+}]\textsubscript{cyt} transients at 1 Hz

C

[Ca\textsuperscript{2+}]\textsubscript{nu} transients at 1 Hz

[Ca\textsuperscript{2+}]\textsubscript{nu} transients at 1 Hz

D

Ratio of [Ca\textsuperscript{2+}]\textsubscript{nu} to [Ca\textsuperscript{2+}]\textsubscript{cyt}

Ratio of [Ca\textsuperscript{2+}]\textsubscript{nu} to [Ca\textsuperscript{2+}]\textsubscript{cyt}

E

Caffeine-induced Ca\textsuperscript{2+} release

Caffeine-induced Ca\textsuperscript{2+} release
Figure 4.

A) non-Peri-Nu originated waves
   - Ad-empty
   - Ad-CSQ2-WT
   - Ad-CSQ2-DsRed

B) Ca^{2+} waves at 1.8 mM [Ca^{2+}]_o
   - Percentage
   - Ad-empty: 47, 68, 24
   - Ad-CSQ2-WT: 115, 68, 24
   - Ad-CSQ2-DsRed: 144, 68, 24

C) Ca^{2+} waves at 10 mM [Ca^{2+}]_o
   - Percentage
   - Ad-empty: 9, 46, 61
   - Ad-CSQ2-WT: 22, 65, 7
   - Ad-CSQ2-DsRed: 144, 68, 24

D) Ca^{2+} waves at 100 μM ouabain
   - Percentage
   - Ad-empty: 13, 40, 99
   - Ad-CSQ2-WT: 25, 36, 21
   - Ad-CSQ2-DsRed: 14, 40, 21

E) Ca^{2+} waves at 100 nM ISO
Figure 5.

A

MS ID#: JBC/2012/340927-R1

CSQ2-mediated perinuclear Ca²⁺ signaling

B

WT mouse myocytes
Ad-CSQ2-WT

C

IP3R2⁻/⁻ mouse myocytes
Ad-CSQ2-WT

D

WT mouse myocytes

E

IP3R2⁻/⁻ mouse myocytes

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Figure 6.

A

Before tetracaine (100 µM)  
After tetracaine (100 µM)  
Washout

Ad-CSQ2-WT

Ad-CSQ2-DsRed

B

Before Ryanodine (5 µM)  
After Ryanodine (5 µM)

Ad-CSQ2-WT

Ad-CSQ2-DsRed

C

Before thapsigargin (1 µM)  
After thapsigargin (1 µM)

Ad-CSQ2-WT

Ad-CSQ2-DsRed
Figure 7.

A

B

C

Cardiomyocytes Surface Area (normalized)

ANF mRNA (normalized with Gapdh)

Ad-empty | Ad-CSQ2-WT | Ad-CSQ2-DsRed

Ad-empty | Ad-CSQ2-WT | Ad-CSQ2-DsRed

** | ** | *
Figure 8.
Calsequestrin accumulation in rough endoplasmic reticulum promotes perinuclear Ca2+ release
Ang Guo, Steven E. Cala and Long-Sheng Song

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