The Cardiac Ca^{2+}-Deficient EF-Hand Governs the Phenotype of the Cardiac-Skeletal TnC-Chimera in Solution by Sr^{2+}-Induced Tryptophan Fluorescence Emission†

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ABSTRACT: In the development of force during Sr^{2+} activation, phenotypically cardiac muscle is more sensitive than fast-twitch skeletal muscle, and TnC is central in this mechansim. The uncertainty has remained, however, whether such functional manifestations *in situ* relied critically on protein–protein interactions in the fiber or whether the Sr^{2+} sensitivities were governed intrinsically within the TnC molecule. To resolve this, we substituted a tryptophan for phenylalanine-26 in both rabbit sTnC (sTnC.W26) and in a chimera (c1/s.W26) where the 41 N-terminal amino acid residues were of bovine cTnC and the remaining 42–160 residues of rabbit sTnC. The metal ion dependent fluorescence emissions of the constructs could be examined in solution isolated from the protein–protein interactions found *in situ*. The Sr^{2+} sensitivities of these proteins differed by 0.55 ± 0.02 pSr unit, but Ca^{2+} sensitivities were indistinguishable, as in the fiber. In another mutant, where the ^{27}VLGA^{30} cluster was replaced with D-AD to enable site 1 to coordinate metal ion binding despite closely preserved cardiac structure, the Sr^{2+}-sensitivity response was transformed into the skeletal-type. The Hill coefficients were also characteristically distinct for the various constructs. The findings indicate that cardiac N-terminal 41 residues define TnC performance in solution similar to that *in situ*. Moreover, the study provides unambiguous evidence that TnC isoforms intrinsically dominate the phenotype in the switching mechanisms for both cardiac and skeletal contractilities.

Ca^{2+} ion is the physiological trigger for contraction in both cardiac and skeletal muscles. It switches on the contractile mechanism by binding to troponin C (TnC[1]), which is a subunit of troponin. Another subunit troponin I (TnI) inhibits contraction in the absence of Ca^{2+}, but such inhibition is overcome by the Ca^{2+}-saturated TnC complex (Leavis & Gergely, 1984; Zot & Potter, 1987). This mechanism is similar in cardiac and skeletal muscles. But, there are important amino acid sequence differences within the N-terminal regions of cardiac(c) and skeletal(s) TnC isoforms, resulting in decrease of maximal Ca^{2+}-binding in cTnC. Thus, whereas each TnC isoform comprises four putative EF-hand domains (sites 1 and 2 reside in the N-terminal regulatory domain, and sites 3 and 4 reside in the C-terminal nonregulatory domain of the TnC crystal structure; see Strynadka & James, 1989; Kretsinger, 1980), the site 1 in cTnC fails to bind Ca^{2+} (van Eerd & Takahashi, 1976; Leavis & Kraft, 1978). The consequences of a single Ca^{2+}-binding regulatory site in cTnC are of longstanding interest in studies of the activation mechanism in cardiac muscle.

A major phenotype difference between cardiac and skeletal muscles is unmasked with Sr^{2+} ion as surrogate for Ca^{2+} in the contractions of skinned fibers. The Sr^{2+} sensitivity for half maximal activation concentration in the pSr-force relation is 4-fold or more greater for cardiac muscle than for skeletal muscle (Kitazawa, 1976; Kerrick et al., 1980; Babu et al., 1987). A number of studies have indicated that these cardiac/skeletal phenotypes can be manipulated by TnC isoforms. For instance, the exchange of skeletal TnC in replacement for endogeneous cTnC reduced the Sr^{2+} sensitivity of skinned trabeculae to the value of skeletal muscle (Babu et al., 1987). With a cardiac-skeletal chimera (with 1–41 amino acid residues of cTnC and 42–160 residues of sTnC), moreover, the cardiac type Sr^{2+} response was nearly preserved, indicating that the N-terminal 41 residues in cTnC contained sufficient information for phenotype conversion in the fiber (Gulati et al., 1992). Nevertheless, the uncertainty remained whether the Sr^{2+} sensitivity was intrinsically different in the various TnC moieties, or whether such differences could be manifest only in the fiber where protein–protein interactions would prevail. For an unambiguous resolution of this, it was important to distinguish the phenotypes of TnC isoforms in solution. This is attempted here by generating rabbit TnC variants with a tryptophan substituting for phenylalanine-26. The three TnC constructs were (1) wild-type sTnC.W, (2) chimera c1/s.W, and (3) a novel chimera in which the cardiac residues ^{27}VLGA^{30} were replaced by D-AD to introduce metal ion coordination in cardiac site 1. The fluorescence emissions by the tryptophan were measured as functions of Sr^{2+} and Ca^{2+} ion concentrations to differentiate the underlying phenotypes in solution.

EXPERIMENTAL PROCEDURES

The Construction of TnC Mutants. The rabbit fast-twitch skeletal TnC-encoding synthetic cDNA, as well as the cDNA for c1/s chimera combining the 41 residues of cardiac and the

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[1] Abbreviations: TnC, troponin C; sTnC, rabbit skeletal fast-twitch muscle troponin, C; cTnC cardiac TnC; c1/s, cardiac-skeletal chimeric TnC construct; CBc1/s, calcium binding derivative of c1/s; sTnC.W, c1/s.W, and CBc1/s.W, corresponding variants in which W replaced F26; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreiotol; SEM, standard error of the mean; *F*, the measured peak of the fluorescence emission.

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rest of sTnC, described earlier (Babu et al., 1992; Gulati et al., 1992), were used as the precursors to generate tryptophan-containing derivatives. The original gene included 20–22 unique restriction sequences to facilitate mutagenesis and was cloned in pT7 plasmid to facilitate bacterial expression of the resultant proteins (Babu et al., 1992).

Tryptophan was substituted for Phe-26; this position was selected in rabbit sTnC because a similar construct of chicken sTnC was found to be effective in an earlier study (Trigo-Gonzalez et al., 1992; Pearlstone et al., 1992). Because no tryptophan existed in the rabbit TnC, Trp-26 provided a ready spectroscopic marker which could be followed with ease. The present mutagenesis was directed in PCR by an appropriate oligonucleotide representing the 5′ sequence. The 492-basepair product coding for sTnC.W26 was reinserted into the plasmid between the NdeI/SacI sites (see Babu et al., 1992). For a Trp derivative of the chimera c1/s, the same position was selected for mutation to facilitate comparison of the results with sTnC.W. A separate oligomer was prepared to effect the mutation in c1/s.

The c1/s chimeric precursor was further altered to amend site 1 for metal ion binding, by replacing ^{27}VLGA^{30} with D-AD. A similar strategy was earlier successful for cTnC (see Sweeney et al., 1990). By convention, the present construct is termed CBc1/s. The corresponding tryptophan derivative was produced as above. All mutated cDNAs were painstakingly sequenced prior to protein expression.

Since tryptophan was always inserted in the position of Phe-26, these constructs herefrom are referred to simply as sTnC.W, etc.

A total of four new mutant variants (CBc1/s, CBc1/s.W, sTnC.W, and c1/s.W), as well as two previous bacterially-generated proteins (sTnC and c1/s), were thus utilized in this study.

The bacterial expressions and purifications of the proteins, normal and Trp derivatives, were similar to those described previously (Babu et al., 1992, Gulati et al., 1993b). The Trp insertion had no discernible effect on the purification profile or the yield of the proteins.

All purified proteins were routinely tested on 15% SDS-PAGE with EGTA (2 mM) and $CaCl_2$ (2 mM) for homogeneity and their Ca^{2+} responsiveness. A single band was observed in EGTA (coomassie brilliant blue staining), indicating more than 95% purity. With Ca^{2+}, all bands migrated with higher mobility, characteristic of TnC and calmodulin. No mobility differences were observed between the normal and their Trp derivatives in EGTA or Ca^{2+}. As a further check, the Ca^{2+} binding on proteins was also determined using radiolabelled $^{45}Ca^{2+}$ (see below).

The purified proteins were stored at −20 °C in a solution containing 10 mM Tris (pH 7.5) and 10 mM DTT. The protein concentrations were determined as needed using the BioRad assay using an appropriate TnC standard (see Babu et al., 1992).

$^{45}Ca^{2+}$ Binding Capacities of the Proteins. The $^{45}Ca^{2+}$ binding measurements were carried out on both normal and Trp derivatives of sTnC, c1/s, and CBc1/s. The radiolabel equilibration for at least 48 h was carried out by microdialysis in an eight-well unit (BRL) as described earlier (Gulati et al., 1992).

Measurements of the Fluorescence Emissions. The fluorescence emissions were determined on a Shimadzu RF5000U spectrofluorophotometer equipped with a temperature-controlled cuvette chamber. The excitation and emission bandwidths were 5 nm. The scan speed was set at 1.7 nm/s.

Table 1: Maximal $^{45}Ca^{2+}$ Binding to Purified Proteins in pCa 3.5 Solution[a]

normal variants		tryptophan variants	
protein	bound Ca^{2+}, mol/mol	protein	bound Ca^{2+}, mol/mol
sTnC	3.90 ± 0.1	sTnC.W	4.16 ± 0.1
c1/s	2.97 ± 0.1	c1/s.W	3.13 ± 0.1
CBc1/s	3.70 ± 0.2	CBc1/s.W	3.78 ± 0.2

[a] In each case the data is the mean of six experiments.

The excitation wavelength was set at 295 nm to selectively excite the Trp residue and avoid the contribution from Tyr residues naturally present in the proteins (Y10 & Y109, and Y4 & Y110 in c1/s; see, Gulati et al., 1993a). The entire emission spectrum between 300 and 400 nm was scanned in each case and the emission maxima was measured. The maximal emission wavelengths were in the range of 339–342 nm.

The Ca^{2+} and Sr^{2+} titrations were performed on samples (2 mL) contained in a 1-cm path length quartz cuvette. All buffers were prepared from reagent-grade water (17-18 MOhm, Milli-Q Millipore Corp). The buffer (prior to protein addition) was run through a Chelex-100 column and stored in plasticware. The proteins were routinely dialyzed overnight against the chelex-treated solution.

The Ca^{2+} and Sr^{2+} titrations were made in a buffer solution containing 100 mM MOPS (pH 7.0), 100 mM KCl, 1 or 5 mM EGTA, and 10 mM DTT. The protein amount was usually 0.1 mg/mL. Free metal ion concentrations were computed using the appropriate binding constants (Martell & Smith, 1974). The calculated amounts of stock $CaCl_2$ were added directly to the cuvette for titrations between pCa 7 and pCa 4. The pH was checked after each addition and readjusted. Similarly, the Sr^{2+} titrations were made between pSr 6 and 3. All measurements of fluorescence emissions were made at 25 °C. The titration data were computer fitted with the Hill equation as before (Babu et al., 1987). The results are expressed as mean ± SEM.

RESULTS AND DISCUSSION

A. Ca^{2+} Binding Capacity of the Trp-Containing TnC Derivatives. To investigate the influence of cardiac N-terminal EF-hand in differentiating the phenotype between cardiac and skeletal muscles, three bacterially synthesized TnC constructs (sTnC.W, c1/s.W, and CBc1/s.W) were used. At the outset, we measured their Ca^{2+} binding capacities in relation to their tryptophan-free precursors.

Table 1 indicates the saturable amounts of radiolabeled $^{45}Ca^{2+}$ bound (mol/mol of protein) on these proteins. These amounts were determined by equilibrating the proteins in a dialysis chamber with a physiological buffer solution with a free Ca^{2+} level of pCa 3.5. The wildtype sTnC and its Trp variant both bound close to 4 mol of Ca^{2+}/mol of protein.

The chimeras c1/s and c1/s.W constructs each bound close to 3 mol of Ca^{2+}/mol of protein, as expected due to the structure of cardiac site 1 and the lack of sufficient coordinating residues therein (Gulati et al., 1992). The CBc1/s and CBc1/s.W derivatives bound close to 4 mol, mol of protein, confirming that site 1 was functionally amended.

These results indicate that the insertion of Trp-26 replacing Phe-26 preserved the Ca^{2+} binding in the recombinant rabbit sTnC and its various derivatives.

B. The Steady-State Fluorescence Spectra of Trp-26: The Effect of Ca^{2+} Ion. Figure 1 shows two typical fluorescence emission of Trp-26 in all three (sTnC.W, c1/s.W, & CBc1/

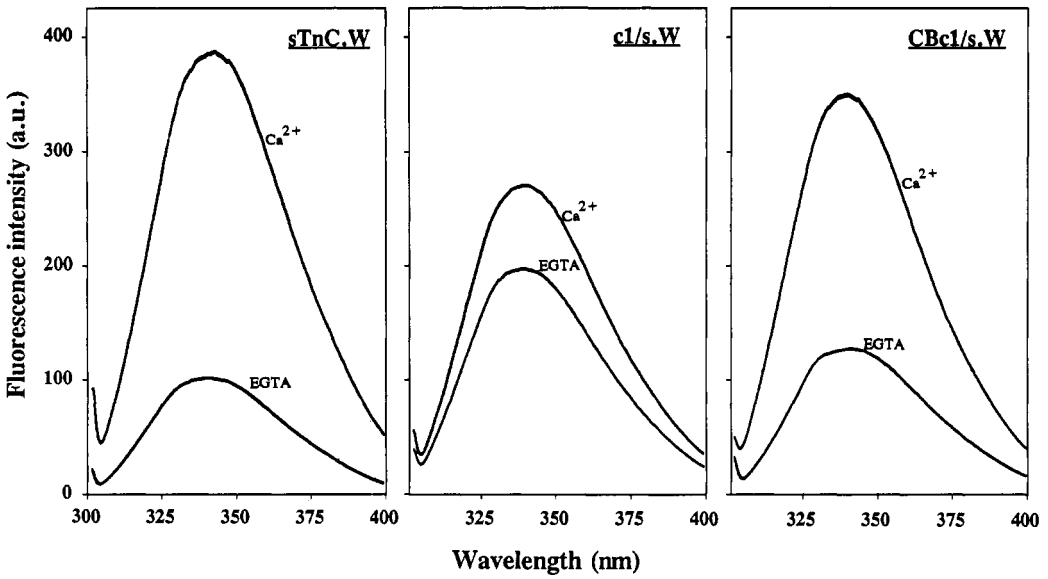

FIGURE 1: Fluorescence emission spectra of sTnC.W, c1/s.W, and CBc1/s.W before (bottom) and after (upper) the addition of saturable amount of calcium chloride, at 25 °C. The spectra were recorded at the excitation wavelength of 295 nm for tryptophan. The emission maxima for the three proteins were 342, 339, and 341 nm, respectively. Protein concentration was 0.1 mg/mL.

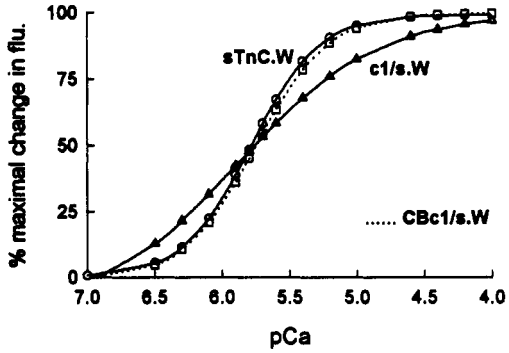

FIGURE 2: The calcium dependence of Trp-26 fluorescence in sTnC.W (circles), c1/s.W (triangles), and CBc1/s.W (squares). The percentage of maximal change in fluorescence peak value is plotted as a function of pCa.

s.W) derivatives determined under identical conditions (0.1 mg/mL). The lower curve in each case was recorded in a buffer solution containing 1 mM EGTA and no added Ca^{2+} (see the Experimental Procedures); the upper curves were recorded in the presence of pCa 4. The emission values are indicated in arbitrary units (au), and the peak emission of the wildtype in EGTA (F_{EGTA}) was normalized to 100. In saturating amount of Ca^{2+} (pCa 4), the peak value (F_{pCa4}) for sTnC.W was nearly 4-fold higher (377 ± 12 au for seven measurements). The peak fluorescence emissions were also measured at intermediate Ca^{2+} concentrations and the differential ($\Delta F = F_{Ca} - F_{EGTA}$) at all Ca^{2+} concentrations (pCa range 7–4) was normalized to the peak fluorescence in pCa 4 (i.e., $\Delta F/(F_{pCa4} - F_{EGTA})$). This is plotted in Figure 2 (circles).

The fluorescence emission spectra for the chimera c1/s.W (0.1 mg/mL) differ from sTnC.W in both EGTA as well as in the presence of Ca^{2+}. The F_{EGTA} value for the chimera was 195 ± 7 au and the peak pCa 4 value (F_{pCa4}) was 264 ± 9 au ($n = 4$). The effects on fluorescence emissions at intermediate Ca^{2+} values and normalized to the pCa 4 peak value, as above, are plotted in Figure 2 (triangles).

The pCa values for half-maximal fluorescence (pCa_{50}) are very similar in c1/s.W (5.72 pCa units) and sTnC.W (5.76 pCa units). However, the rise in the normalized curve is less steep in c1/s.W and the corresponding Hill coefficient is lower

Table 2: Apparent Dissociation Constants and Hill Coefficients Determined by Titrations of Fluorescence[a]

	calcium		strontium	
protein	pCa_{50}	n_H	pSr_{50}	n_H
sTnC.W	5.76 ± 0.03	1.4 ± 0.05	3.85 ± 0.03	2.04 ± 0.10
c1/s.W	5.72 ± 0.06	0.9 ± 0.05	4.40 ± 0.01	1.40 ± 0.14
CBc1/s.W	5.75 ± 0.04	1.6 ± 0.10	4.00 ± 0.02	2.10 ± 0.02

[a] In each case the data is the mean of four experiments.

than that found for sTnC.W (n_H = 1.4 for sTnC.W and 0.9 for c1/s.W). These parameters are listed in Table 2.

For comparison with fibers, it should be noted that sTnC and c1/s (Gulati et al., 1992) in cardiac muscle had previously indicated that the half-maximal Ca^{2+} concentrations were also indistinguishable for the two proteins (fiber pCa_{50} = 5.40 for sTnC and 5.45 for c1/s). The Hill coefficients in the trabecuale were 4.9 (for sTnC) and 3.1 (for c1/s) (Gulati et al., 1988). Thus with respect to activations with Ca^{2+} ion, these proteins indicate a similar trend in solution as in the fiber. Moreover, the findings indicate that despite the absence of metal ion binding in site 1 in c1/s.W, the Trp placed there still serves as a good probe for Ca^{2+} binding to other EF-hands in the protein molecule.

To study the specific effect of metal ion binding in cardiac site 1, residues ^{27}VLGA^{30} in c1/s.W were replaced by D-AD. The new construct was called CBc1/s.W. The F_{EGTA} (128 ± 5 au) and F_{pCa4} (357 ± 9 au; n = 4) values similar to the corresponding values for sTnC.W (Figure 1). Figure 2 depicts a typical titration curve with Ca, and Table 2 lists the derived values for pCa_{50} and the Hill coefficient. The current pCa_{50} value (5.75 pCa units) is indistinguishable from the values listed for sTnC.W and c1/s.W. The Hill coefficient value in solution for the present mutant (n_H = 1.6), however, is closer to sTnC.W than to c1/s.W. This suggests that for Ca^{2+} ion activations, CBc1/s.W mimics sTnC.W.

C. The Effect of Sr^{2+} Ions. Since Sr^{2+} ion yields a large separation between the sensitivities of cardiac and skeletal muscles for tension development (Babu et al., 1987), the solution studies with the mutants were next performed using this metal ion. We first compared the results between c1/s.W and sTnC.W. The spectral changes with Sr^{2+} ion were

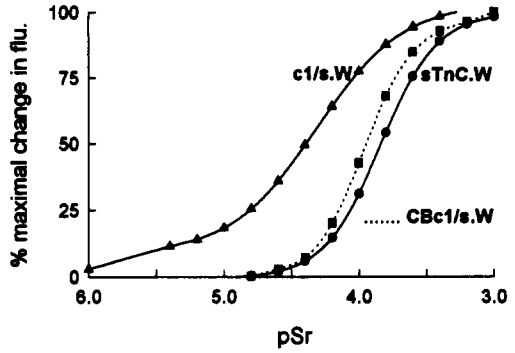

FIGURE 3: The strontium dependence of Trp-26 fluorescence changes in sTnC.W (circles), c1/s.W (triangles), and CBc1/s.W (squares).

determined by the procedures similar to Ca^{2+}. That is, the peak values for fluorescence emissions were measured first in EGTA (F_{EGTA}) and then in maximal pSr3 (F_{pSr3}). The corresponding increments at intermediate Sr^{2+} ion concentrations were normalized to the maximal fluorescence. These normalized data (i.e., $(F_{Sr} - F_{EGTA})/(F_{pSr3} - F_{EGTA})$) are plotted in Figure 3.

The circles in Figure 3 depict the data points for sTnC.W, and the line joining the circles is a Hill plot. The curve for c1/s.W (triangles) is shifted toward lower [Sr^{2+}] concentration. The values for the half-maximal shifts and the Hill coefficients derived from this set of data are listed in Table 2. The ΔpK shift (i.e., pSr_{50} for c1/s.W – pSr_{50} for sTnC.W) is 0.55 ± 0.02 pSr unit. The published data (see Gulati et al., 1992) on cardiac muscle indicated at ΔpK shift of 0.62 ± 0.02 pSr unit as derived by comparing the pSr–force curves with c1/s (four independent measurements) and sTnC (eight measurements).

The Sr^{2+} results with CBc1/s.W in solution are also listed in Table 2 (third row). Both the derived pSr_{50} as well as the Hill coefficient for CBc1/s.W are close to the corresponding values for sTnC.W, which indicates that in the chimera, comprising 41 (of the total 160) amino acid residues of cardiac TnC, the metal ion binding capability in site 1 is sufficient to convert the Sr^{2+} phenotype from cardiac to the skeletal type. This was true for the Hill coefficient, indicating that N-terminal 41 residues of cTnC also govern intrinsic TnC cooperativity. Evidently, much of the relevant information for TnC-dominated phenotype interconversion resides within these 41 residues of cardiac TnC.

D. Comparison with Other Results. The first 41 cardiac type residues in c1/s mutant are indicated to be critical for the skeletal to cardiac phenotype interconversion in solution, in agreement with results in the fiber (Gulati et al., 1992). This conclusion was supported further by the results on the calcium-binding derivative of the chimera (CBc1/s), which indicated that metal ion binding to cardiac site 1 in these 41 residues was sufficient to convert the response to skeletal type. This also extends the earlier findings on cardiac muscle (Babu et al., 1989; Gulati et al., 1991) and on slow-twitch fibers (Sweeney et al., 1990), which were tested with a metal ion binding derivative of cTnC (CBM1). These studies had shown that the cardiac phenotype for Sr^{2+} activation was converted to fast-twitch skeletal muscle phenotype, but the question had remained whether the entire cTnC structure was necessary in effecting the phenotype conversion, or whether the metal ion binding to the first EF-hand was the more critical part. This ambiguity is now well resolved in the present study.

E. Concluding Remarks. The present study provides the first direct observations of fluorescence changes in the cardiac metal ion-deficient N-terminal site. The fluorescence emissions by the tryptophan in this site recorded significant effects when metal ions (Ca^{2+} or Sr^{2+}) bound to other EF-hands in the molecule (Figures 2 and 3). This suggests that the various regions including the putative metal ion-deficient site in cTnC perform interactively during the Ca^{2+}-induced switching in muscular contraction.

The dominant role of TnC in both cardiac and skeletal contractile switching mechanisms is also established unequivocally on the strength of present findings. By scrutinizing the half-maximal metal ion concentrations for fluorescence emissions of the cardiac-skeletal chimera (c1/s.W) and sTnC.W (Figure 3 and Table 2) in light of the known information from force development in cardiac muscle (Gulati et al., 1992), the cardiac/skeletal phenotype differences of Sr^{2+} sensitivities are explained by the intrinsic properties of TnC moieties. The protein–protein interactions which must occur within the reglatory complex in the fibers are nonetheless seen to be unimportant for specifying the Sr^{2+} phenotype.

Interestingly, there is evidence suggesting that under pathophysiological and some physiological conditions TnT and TnI may modify cardiac Ca^{2+} sensitivity (Solaro et al., 1981; Nassar et al., 1991; Babu et al., 1994). The mechanism for this deserves to be explored in light of the fact that protein–protein interactions seem to play little role in determining the Sr^{2+} phenotype.

Furthermore, the remarkable conversion in the phenotype made possible with only a small modification in the structure between sTnC and the chimeras raises the possibility that the normal cardiac EF-hand has high versatility, which may be especially important for the heart to respond to the varied myocardial stimuli. Future structure–function studies of such chimeric constructs and their variants should also address these implications.

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