Constructing a structural model of troponin using site-directed spin labeling: EPR and PRE-NMR

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Abstract

The relative ease of introducing a paramagnetic species onto a protein, and advances in electron paramagnetic resonance (EPR) over the past two decades, have established spin labeling as a vital structural biology technique for revealing the functional workings of the troponin muscle regulatory complex—an ~80 kDa heterotrimeric protein switch for turning on striated muscle contraction. Through the site-directed spin labeling (SDSL) of cysteine residues at key sites in troponin, a molecular-level understanding of the troponin muscle regulatory system across all levels of structural hierarchy has been achieved. Through the application of EPR, mobility and accessibility trends in the EPR signals of the spin labels attached to consecutive residues can reveal the secondary structure of troponin elements and also help map the interaction between subunits. Distance restraints calculated from the interspin interactions between spin label pairs have helped with building a structural model of the troponin complex. Further, when SDSL is paired with NMR, paramagnetic relaxation enhancement (PRE)-NMR has been used to obtain high-resolution structural detail for both intra- and interdomain interactions in troponin and revealed details of protein conformational changes and dynamics accompanying troponin function. In this review, we provide an overview of the SDSL labeling methodology and its application towards building a dynamic structural model of the multi-subunit troponin complex which details the calcium-induced conformational changes intimately linked to muscle regulation. We also describe how the SDSL method, in conjunction with EPR or NMR, can be used to obtain insights into structural perturbations to troponin caused by disease-causing mutations.

Keywords Cardiac troponin · Site-directed spin labeling · Electron paramagnetic resonance (EPR) · Paramagnetic relaxation enhancement NMR (PRE-NMR) · Protein dynamics

Introduction

It is becoming clear that many key physiological processes are controlled at a molecular level by large multi-protein complexes. In these complexes, intricate interactions between the individual subunits allow small conformational changes, produced by an effector molecule, to be amplified into large-scale structural changes involving protein domain and subunit motions. These large-scale motions then control other macromolecular interactions, resulting in control at the macroscopic

level of the cell. While providing exquisite control in normal tissue, these regulatory complexes are also often prone to disease-producing mutations. An intimate knowledge of the structure and dynamics of such complexes is therefore a prerequisite for understanding the molecular basis for disease-causing mutations and the possibility of rational therapeutic intervention. Troponin (Tn) (~80 kDa) is a prime example of one such regulatory protein complex where the simple binding of Ca^{2+} to Tn triggers a well-coordinated cascade of structural events to result in striated muscle contraction.

In striated muscle, contraction occurs as a result of the interaction between two filamentous systems; the thin filament composed of actin, tropomyosin (Tm), and the Tn complex, and the myosin thick filament (Fig. 1). When the myosin heads of the thick filament interact with the actin thin filament, they form a cross-bridge (Gordon et al. 2000). Cross-bridge formation is coupled to the simultaneous hydrolysis of ATP by myosin and results in the relative sliding of the thin filament over the thick filament. This shortens the length of the

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Fig. 1 Schematic view of the mechanism of striated muscle contraction. The actin thin filament is represented in blue and the myosin thick filament in green. The Z-disc defines the boundaries of the sarcomere contractile unit. Contraction is initiated by the influx of Ca^{2+} and results in the sliding of the thin filament over the thick filament which shortens the length of the sarcomere. Boxed region—an expanded view of the Tn complex (black) located with Tm (cyan) on the thin filament in the region

of the acto-myosin interaction site. In the “relaxed” state, the myosin binding site is blocked by Tn and Tm (left). Upon Ca^{2+} binding (red), Tn undergoes a series of conformational changes where the inhibitory region of Tn is moved away from the myosin binding site. Tm is then “rolled” into the deep groove of the actin filament helix. This movement leaves the myosin binding sites exposed and the acto-myosin cross-bridge interaction can then occur (right)

sarcomere, leading to muscle contraction. The sliding process is initiated upon the influx of Ca^{2+} across the muscle fiber membrane which occurs upon nervous excitation. Under conditions of low intracellular Ca^{2+} , the Tm molecule on the actin thin filament effectively blocks the formation of the acto-myosin cross-bridges. When Ca^{2+} levels increase to $\sim 10^{-5}$ M, the binding of Ca^{2+} to Tn triggers a series of conformational changes that relieve the inhibition on cross-bridge formation by Tm. In addition to several large scale conformational changes, other changes in the dynamic nature of the Tn molecule also contribute to the finely controlled regulation of

muscle contraction. While important for function, it is however this intrinsic flexibility and dynamic nature of the Tn complex that has thwarted efforts to obtain a complete and accurate view of the whole Tn complex.

The troponin complex—an incomplete atomic picture

The Tn complex is a heterotrimeric protein situated on the muscle thin filament. It is composed of three subunits:

TnC—an ~18-kDa dumbbell-shape protein which binds Ca^{2+} to initiate contraction; TnI—a ~24-kDa regulatory subunit which inhibits the myosin binding site on the actin thin filament; and TnT—a ~36-kDa flexible anchoring protein which adheres the whole complex to the muscle thin filament. Positioned at regular intervals along the thin filament, its role in muscle contraction is to transmit the Ca^{2+} signal to the thin filament via the positioning of Tm (Fig. 1). Due to the importance of Tn in the control of striated muscle contraction, it is not surprising that many malfunctions in Tn have been found to lead to devastating consequences, especially for the cardiac isoform. For example, there are now more than 60 mutations in Tn to date that have been linked to cardiomyopathies such as Hypertrophic and Dilated Cardiomyopathies (Gomes and Potter 2004; Willott et al. 2010). Cardiomyopathy is one of the leading causes of death in otherwise healthy people. Disappointingly, for such an important protein in human health, our understanding of the structure and function of the Tn regulatory complex is still far from complete and insights into structural perturbations to Tn caused by such disease-causing mutations has remained a challenge.

Unsurprisingly, our understanding of Tn has been inherently linked to advances in structural biology and the dependence of this field on the technology and instrumentation available to scientists to study molecular structure. While we have considerable details of the structure of the Tn complex, much of our current molecular understanding of muscle regulation has been derived from static X-ray crystallographic structures of isolated subunits or of truncated core regions of the binary (TnC-TnI) and ternary (TnC-TnI-TnT) complexes. The first of these Tn atomic structures were solved in the mid-eighties where, the structure of the smallest of the three subunits, TnC, was reported by two independent laboratories (Herzberg and James 1985; Sundaralingam et al. 1985). Both atomic resolution structures (PDB: 4TnC, 5TnC) of the skeletal isoform of this TnC Ca^{2+} -binding subunit were groundbreaking. They revealed, for the first time, the dumbbell-shaped nature of the TnC subunit and a detailed atomic description of the metal-binding domains and also provided a foundation for helping understand the function of other Ca^{2+} -regulated proteins. The next decade was a period where NMR was extensively used and provided additional high-resolution structural information of each of the isolated domains of TnC which served to define the transition between closed and open domain structures, particularly for the skeletal isoform (Findlay et al. 1994; Gagne et al. 1995; Sia et al. 1997; Tsuda et al. 1999) (PDB: 1TNP, 1TNQ, 1SPY, 1AJ4). These structural studies and associated kinetics, energetics, and dynamics of the TnC are reviewed in further detail in (Gagne et al. 1998).

A next key milestone in this field was the atomic level determination of the crystal structure of the N-terminal peptide region (residues 1–47) from the skeletal TnI subunit bound to the C-lobe fragment of TnC (Vassilyev et al. 1998) (PDB:

1A2X). This was the first reporting of atomic resolution interactions between the two Tn subunits. This X-ray structure also showed a “bent” or collapsed conformation for TnC which was in stark contrast to the elongated dumbbell-shape structure previously revealed in the uncomplexed TnC structures (Herzberg and James 1985; Sundaralingam et al. 1985). Using this structure, the authors proposed a model, later shown to be correct, for how Tn functions by suggesting that a unique regulatory segment of TnI would also bind to the N-lobe of TnC under conditions of saturating Ca^{2+} .

It was not until 2003 that the same group then solved the X-ray structures of the major core domain of the cardiac isoform of troponin (Takeda et al. 2003) (Fig. 2a). Two constructs of Tn were crystallized successfully in this pivotal study; a 52-kDa unit (PDB 1J1E) comprising full-length TnC (1–161), TnI (31–210), and the TnT T2 domain (residues 183–288). The second, slightly smaller, complex (46 kDa, PDB 1J1D) possessed a further truncated form of TnI (residues 31–163). This was the first time the architecture of all three components of Tn was revealed; and the earlier prediction of the interaction of a regulatory switch region of TnI with the TnC N-lobe verified. However, missing from this structure were several key functional regions of the Tn complex which were either intentionally removed in order to aid crystallization (~22 kDa TnT-TnI domain and the 30 residue cardiac specific TnI N-extension) or for which a structure could not be ascribed due to a lack of electron density (TnI inhibitory region and the TnI C-terminal mobile domain). Soon after, several X-ray structures of the skeletal isoform of the Tn core were solved. Importantly, these X-ray structures were solved under conditions of both high and low Ca^{2+} states (PDB: 1YTZ and 1YVO, respectively) (Vinogradova et al. 2005). This was significant as the structures revealed the large structural changes between the TnC and TnI subunits due to Ca^{2+} binding; in particular, the release of the regulatory switch region from the N-lobe of TnC in the low Ca^{2+} state was a key feature. These structures were influential in the building of today’s model of muscle regulation where changes induced in TnC then propagate via the other troponin subunits, through Tm and actin, all the way to the active site of myosin to accelerate the hydrolysis of ATP (Fig. 2b).

Construction of a troponin regulatory model

Figure 2 shows the current model for force activation in striated muscle. The model of regulation by Ca^{2+} binding, as presented in Fig. 2b, c, is built mostly from available X-ray structure data of the Tn core of both the cardiac (Fig. 2a) and skeletal muscle isoforms. While such structures constitute our most complete and current high-resolution model of Tn, they essentially provide us with only a snapshot of a highly dynamic system. Absent in the crystal structures are several mobile

Fig. 2 Structure of the cardiac isoform of Tn and model for muscle regulation. **a** Crystal structure of the cardiac core Tn complex (TnC (gray), TnI (green), and TnT (yellow)) solved under conditions of saturating Ca^{2+} (red spheres) (PDB: 1J1E) (Takeda et al. 2003). There are no available X-ray structures of the cardiac Tn core in the absence of Ca^{2+} . **b** Schematic structural model of the Tn complex and its interactions with the thin filament (actin (dark blue), Tm (cyan)), as based on the core crystal structure shown in **a**, and the available skeletal Tn X-ray structures (Vinogradova et al. 2005). Under conditions of low Ca^{2+} , the myosin-

binding sites on the actin filament are blocked (white star). The binding of Ca^{2+} to the N-lobe of TnC triggers a cascade of conformational changes which ultimately leads to the exposure of the myosin-binding site (Takeda et al. 2003). Image adapted from Cordina et al. (2014). **c** Cartoon representation of the proposed Ca^{2+} switch mechanism and the steric block model of muscle regulation (adapted from Sykes (2003)). Tm is a two-chain α -helical coiled coil that binds along the length of the actin thin filament and is repeated every 7 actin monomers

and unstructured regions of Tn which are known to also play important roles in the regulatory process. These missing components of Tn are included in the schematic model shown in Fig. 2b.

The model of the Tn complex (Fig. 2b) summarizes the proposed conformational changes that occur upon binding of Ca^{2+} to the regulatory N-lobe of TnC. Upon activation with Ca^{2+} , the TnI regulatory switch peptide region (residues 148–158) binds to the open hydrophobic cavity of the N-lobe of TnC. In doing so, the switch peptide pulls the TnI inhibitory peptide region (residues 136–147) away from actin. This movement of the inhibitory region from actin allows the Tm molecule to reposition itself on actin so as to allow the myosin heads to bind, thereby initiating contraction. The propagation of these structural changes through Tn to the thin filament, as initiated by the simple binding of Ca^{2+} to TnC, triggers the movement of Tm to a different position on the actin filament (Fig. 2c). This is referred to as the steric block model (Vibert et al. 1997).

Despite these earlier structural studies being important contributors to our current understanding of the interactions between the subunits in Tn, there has since remained a dearth of high-resolution structural information for the complex since 2005. This is particularly true for the cardiac isoform where we still do not have a high-resolution structure of a Ca^{2+} -free state (Fig. 2a). As such, many questions have remained unanswered about the structure of the key components that transmit the information between the Tn subunits and the nature of the

involved conformational changes. Many of these key regions of Tn were not revealed in the crystal structures. We are also limited in our understanding of the role dynamics plays in fine tuning the function of Tn, as well the effect that other key physiological events, such as phosphorylation of the TnI subunit (Layland et al. 2005; Solaro et al. 2013), may have on muscle regulation.

Resolving both the structure and dynamics of the whole Tn complex is still prerequisite for not only understanding the regulation of striated muscle, but also for understanding the molecular basis of many of the disease-causing mutations identified in Tn. A complete dynamic structure is also essential to help with the development of rational therapeutic interventions and new drugs to treat heart disease. While X-ray crystallography and NMR both continue to be the gold standard for obtaining atomic resolution structural information, neither method alone appears likely to be able to provide the much needed complete and “moving” picture of the whole Tn complex due to its large size, its dynamic complexity, and its interactions with the other important thin filament proteins. Thus, alternative structural tools are needed to fill in this knowledge gap to describe the structure and structural changes of regions missing from the crystal structures and which are important for facilitating Tn function. In the past, these alternative tools that have been used to support and build upon our current model of muscle regulation by Tn have included the low-resolution approaches of EPR spectroscopy, fluorescence spectroscopy including fluorescence resonance energy

transfer (FRET) (Dong et al. 1996; Dong et al. 2001), and electron microscopy (Narita et al. 2001; Behrmann et al. 2012; Risi et al. 2017). EPR and fluorescence probe-based labeling approaches can also enable the observation of Tn structural behavior in situ, such as within an intact working muscle fiber (Arata et al. 2007; Li et al. 2014). The EPR studies are the focus of this review. Together, these low-resolution approaches can also provide an appreciation of the structural transitions, and the role of structural heterogeneity and protein dynamics, that are keys for understanding the function of Tn.

In this review, we will focus on one of these alternative approaches—site-directed spin labeling (SDSL) as used in conjunction with EPR, and also NMR spectroscopy. SDSL has enabled EPR measurements in Tn across all levels of structural hierarchy, from isolated subunits to whole muscle fibers. More recently, SDSL has also pushed the size limits of NMR through the application of paramagnetic relaxation enhancement (PRE)-NMR on samples including the ~42-kDa Tn binary complex (TnC-TnI). As both the EPR and PRE-NMR approaches utilize spin labels, this review will begin with a discussion of the procedure for modifying the Tn subunits with spin labels. We will then follow with EPR and PRE-NMR exemplars from the literature which we believe have made a solid contribution to our current understanding of the manner through which the Ca^{2+} signal is propagated through the muscle thin filament.

Site-directed spin labeling of troponin

EPR and NMR are fundamentally similar magnetic resonance techniques. While NMR involves observing nuclear spin transitions, EPR involves observing the spin-state transitions of a paramagnetic species or unpaired electron. In an EPR experiment, the paramagnetic material is placed in a strong static magnetic field and exposed to microwave-frequency radiation in the gigahertz (GHz) range. The interaction of the applied microwave frequency with the unpaired electron results in an EPR absorption spectrum. The presence of a paramagnetic species in a protein is therefore prerequisite for EPR measurements. However, Tn and its associated thin filament proteins have no net electronic spin and are therefore EPR silent.

Previously, EPR was mostly limited to metalloproteins with inherent paramagnetic centers but could also be applied to proteins which possessed a single naturally occurring cysteine residue to which a paramagnetic spin label could be selectively attached. A resurgence of protein EPR spectroscopy occurred in the late 1980s with the advent of simple molecular biology tools that could be used to introduce cysteine residues at desired locations for the attachment of a spin label (Altenbach et al. 1989; Altenbach et al. 1990). Termed site-directed spin labeling (SDSL) (Hubbell et al. 1998; Hubbell

et al. 2013), this approach results in minimal structural and functional perturbation to most proteins and has therefore been a particularly powerful approach for investigating the assembly and dynamics of large multi-protein complexes such as membrane proteins (Bordignon and Bleicken 2018) and the Tn thin filament system, the focus of this review.

In order to perform SDSL, all native cysteine residues should first be replaced by serine or alanine residues. The choice of serine or alanine depends on the role the native cysteine is playing in the protein. In general, if cysteines are on the surface of the protein, a serine is usually a well-tolerated substitution. However, if replacing a partially buried cysteine with a serine leads to destabilization of the protein, then an alanine substitution should be examined. Once the cys-less background has been generated, a new cysteine residue is then introduced at the desired site for spin labeling (Fig. 3). Fortunately, the Tn subunits have only a few native cysteine residues, and the removal of all native cysteines from Tn has been shown to not impair the function of the protein (Dong et al. 1996; Cordina et al. 2012). The covalent attachment of a spin label to Tn can then be achieved through the formation of a disulfide bond with a sulfhydryl-specific nitroxide spin label (Fig. 3b). Commonly used nitroxide labels include MTSL ((1-oxyl-2,2,5,5-tetramethylpyrrolidine-3-methyl) methanethiosulfonate), MSL (4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy), and InVSL (2-[(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methenyl]indan-1,3-dione) (Fig. 3a), which are approximately the size of a tryptophan residue. If functionally important cysteines are present in a target protein, unnatural amino acids can be engineered through which a nitroxide probe can react or the nitroxide group is directly incorporated into the amino acid (McNulty et al. 2000; Kim et al. 2013; Evans and Millhauser 2015). To date, the engineering of unnatural amino acids has not yet been adopted for examining the structure of Tn as the replacement of the native cysteines, and introduction of new cysteine residues at desired locations have all been well tolerated.

The EPR signal from the introduced nitroxide spin label is highly sensitive to the applied magnetic field and also its local environment. The generated EPR lineshape typically depends on the orientation of the spin label group relative to the applied magnetic field, as well as the rate that the spin label is able to re-orientate itself on the EPR timescale (nanoseconds for X-band). The unpaired electron in the $p-\pi$ orbital of the N–O bond of the nitroxide spin label also interacts with the nitrogen nucleus, splitting the EPR signal. In general, for freely tumbling nitroxide radicals in solution, the resulting EPR spectrum shows three sharp, narrowly spaced spectral lines with similar intensities and widths. As the motion of a spin label slows, such as due to local restrictions, the spectral lines broaden and the splitting between the peaks increases. As a result, local protein structure can be revealed by monitoring

Fig. 3 Site-directed spin labeling. **a** Common nitroxide probes used in Tn studies include MTSL, MSL (Li et al. 1997), InVSL (Li et al. 1997), and the bifunctional spin label—BSL (Chatani et al. 2005). The nitroxide group is enclosed in a pyrrole ring which contains a double bond to limit the flexibility of the label. The methyl substituents adjacent to the pyrrole ring stabilize the radical through sterically hindering attack. **b** Covalent

attachment of MTSL to the protein via a disulfide bond to a cysteine sidechain. **c** The nitroxide group is tethered to sites on either TnC (gray) or TnI (green) via a flexible linker arm (PDB: 1J1D). The boxed insets show the number of rotamers for each of the attached spin label as calculated with MMM (Jeschke 2018). The spin-labeled rotamers show that MTSL is attached to residues on Tn with different local environments

the mobility of the spin label but also the hydrophobicity of the spin label to the surrounding solution (McHaourab et al. 1996). If performed in conjunction with scanning through a series of consecutively placed spin-labeled constructs in a polypeptide chain, also known as “cysteine-scanning,” then secondary structural information can be revealed through the EPR trends in label mobility or solvent accessibility. That is, periodic changes in these parameters with a value of “2” allow the secondary structure assignment of a β strand. A trend value of “3.6” is assigned as an α -helix. However, without a doubt, the most powerful application of SDSL is the ability to

measure long-range distances within proteins and protein complexes, up to distances as long as 80 Å.

EPR distance measurements require the strategic placement of two spin labels at desired sites. The strength of the dipolar interaction between the two spin labels then provides the distance information that can be used for building or testing structural models, or for tracking of protein dynamics and conformational changes. In general, there are three techniques for obtaining distance information between two interacting spin labels: (i) *exchange* EPR (4–8 Å) due to short through-bond interacting pathways (Miick et al. 1992); (ii) *continuous*

wave (CW) static and dynamic dipolar EPR (8–25 Å) (Rabenstein and Shin 1995; McHaourab et al. 1997); and (iii) *pulsed* EPR methods including double-quantum coherence (DQC) (Borbat et al. 2002) and double electron-electron resonance (DEER) (Milov et al. 1981; Fajer et al. 2007). For the first two methods, distances are extracted from lineshape broadening and are therefore limited to short-range interactions of ~4 to 25 Å. On the other hand, pulsed EPR methods can detect interacting spins at longer distances when the dipole-dipole interaction weakens and line broadening effects becomes difficult to detect. Distances from as short as ~17 Å and up to ~80 Å have been reported using pulsed EPR methods (Fajer et al. 2007). Lastly, an NMR-based approach that can be used in conjunction with SDSL for obtaining intermediate-range distances is paramagnetic relaxation enhancement (PRE)-NMR. Unlike CW and pulsed EPR methods, the major advantage of the PRE-NMR approach is that it only requires the introduction of a single paramagnetic species. Multiple distance constraints of up to ~30 Å can then be measured due to dipolar interactions between the nuclear spins of the protein residues and the unpaired electron of the spin label (Battiste and Wagner 2000). The PRE approach is reviewed further in Clore and Iwahara (2009).

Applications of SDSL-EPR to understanding troponin structure and function

While X-ray structures and several NMR structures are available for parts of the Tn complex, we still do not have a complete structure of the intact Tn complex; let alone a structure within the context of the thin filament. Further, while we have good models of the proposed structural changes triggered by Ca^{2+} binding, the models do not provide an understanding of the dynamic events that are also likely to modulate the Tn regulatory role. Many questions therefore exist which are not easily addressed by X-ray crystallography or by using solution NMR. These include: (i) the response of TnC to Ca^{2+} binding and its interaction with the TnI switch and inhibitory peptide regions; (ii) the conformation of the TnC central linker; (iii) the identity and movement of the TnI mobile domain and its interaction with the thin filament; (iv) the orientation of Tn within thin filaments during the contractile cycle; and (v) the effect of disease-causing mutations. Here, we discuss the contribution of SDSL in addressing of several of these questions, and we summarize several EPR and NMR measurements that we believe have best contributed to the building of our current structural model of the Tn complex. Where appropriate, we have been careful to emphasize the differences in the structure and regulatory behavior between the two striated muscle Tn isoforms, cardiac and skeletal, as revealed by the SDSL approach.

Mobility and accessibility

Mobility of a spin label can report on Ca^{2+} -induced structural changes in TnC and TnI

As Ca^{2+} binding triggers muscle activation, tracking the conformational changes induced across any component of Tn, through to the thin filament, is clearly central for understanding muscle regulation. This can be done across all levels of hierarchy, from the isolated TnC subunit through to whole reconstituted muscle fibers, via the simple incorporation of a single spin label at a strategic location on TnC (Li and Fajer 1994; Szczesna and Fajer 1995; Li et al. 1997; Li and Fajer 1998; Nakamura et al. 2005; Ueki et al. 2005; Aihara et al. 2006; Potluri et al. 2017; Potluri et al. 2019). However, not every targeted site will exhibit sensitivity to Ca^{2+} binding as the EPR spectrum is dependent on the local environment surrounding the labeled residue. For example, the continuous wave (CW) EPR spectrum from the attachment of the MTSL spin label to either of the cardiac TnC native cysteines (C35 and C84) displayed different EPR lineshape responses upon Ca^{2+} binding. The spin label attached to C84 was found to be most sensitive to Ca^{2+} binding (Fig. 4), both in isolated TnC and in reconstituted muscle fibers, and not C35 (Nakamura et al. 2005). TnC84 is located on a site between TnC and TnI whereas TnC35 is in the vicinity of the inactive Ca^{2+} -binding site (site I) of the cardiac isoform. The C84 spin-labeled residue is therefore considered to be at an optimal position to monitor for structural changes across TnC in response to Ca^{2+} -binding events and has been used by others (Ueki et al. 2005), including our group (Potluri et al. 2017; Potluri et al. 2019), to achieve this. It is also a useful target residue for examining perturbations of the Ca^{2+} -binding response due to point mutations linked to hypertrophic cardiomyopathy such as L29Q (Fig. 4) (Potluri et al. 2019).

A second example of a suitable Tn residue that can be selectively targeted to report on Ca^{2+} -induced structural changes is the spin-labeled TnI133 residue of the skeletal TnI isoform (Aihara et al. 2006). TnI133 is located near the TnI switch segment region (skeletal TnI residues 116 to 131). In the low Ca^{2+} state, the CW EPR spectral lineshape was sharp with a corresponding correlation time (τ) of ~2 ns. The peaks of the EPR spectrum became broader, and the τ increased to ~5 ns, under conditions of high Ca^{2+} . These changes in the mobility of the spin label attached to TnI133 were interpreted as dissociation of the switch region of TnI from TnC under conditions of low Ca^{2+} . The flexible switch region was then described to move towards the thin filament to interact with the surface of actin. This proposed release movement of the switch region is consistent with the X-ray structures and remains a key feature of our current molecular model of muscle regulation (Fig. 2).

Fig. 4 Ca^{2+} -triggered changes in the EPR mobility of MTSL attached to TnC84 (left) and in the presence of cardiomyopathy mutation L29Q (right). Ribbon structure of cardiac TnC (PDB: 1AJ4) showing Ca^{2+} (red) bound to the N-lobe and the location of the L29Q hypertrophic cardiomyopathy mutation (green). The arrows indicate the mobile component on the CW-EPR (X-band) spectra. The spin label at residue C84

increases in mobility in response to Ca^{2+} binding. This response is enhanced in the presence of the L29Q mutation, suggesting structural perturbation of the N-lobe of TnC due to the disease-causing mutation (adapted from Potluri et al. (2019) with permission from American Chemical Society)

Orientation of TnC and TnI in thin filaments and muscle fibers

While the EPR spectrum is sensitive to changes in the mobility of the spin label, the EPR lineshape also depends on the orientation between the magnetic field and the spin label. If the spin label can be immobilized to the protein to generate a stereospecific attachment, this can then be used to facilitate the measurement of the orientation of the labeled protein domain in an aligned system. A stereospecific attachment can be best achieved by labeling with a biofunctional spin label such as BSL or a spin label which is directly incorporated in by means of a peptide bond such as TOAC (Fig. 3). This approach has indeed been used to study the orientation of Tn within muscle fibers where the splitting of the spectral peaks provides a measure of the average angle of the protein domain with respect to the aligned fiber. As before, the width of the EPR signal provides a measure of orientational disorder where a high degree of disorder is reflected by a broad resonance peak.

The coupling of structural changes in the skeletal Tn isoform, induced by Ca^{2+} binding and the binding and cycling of myosin heads to the thin filaments, was extensively studied by the Fajer group who took advantage of the ability to target the single native cysteine residue of both TnC (C98) and TnI (C133) with spin labels (Li et al. 1997; Li and Fajer 1998). This approach was also successful due to their development of methodology to exchange the spin-labeled TnC, or TnI, into thin filaments and muscle fibers (Li and Fajer 1994; Szczesna and Fajer 1995). In these early studies, both the orientation and motion of TnC, with respect to the muscle fiber axis, was found to be modulated by the binding of Ca^{2+} and also by cross-bridge attachment (Li et al. 1997). However, in contrast to the results using labeled TnC, this same study reported that the global motion of TnI in muscle fibers, labeled at TnI133, was not strongly affected (Li et al. 1997). A follow-up study

from the same group also showed the coupling of the motion of TnC to the various myosin intermediate states of the ATPase cycle (Li and Fajer 1998). An example of changes in the EPR spectrum collected in skeletal muscle fibers for a spin label attached to TnC98 are shown in Fig. 5. Together, these studies were some of the earliest evidence that structural changes occurring within TnC were intimately linked with the regulation of muscle contraction. Moving forward, the adoption of spin labels with other chemistries, such as bifunctionalized spin labels, should also be well suited for future angular analysis of an orientated sample, provided a double disulfide linkage to Tn can be achieved (Chatani et al. 2005).

The structure of the inhibitory region of TnI by cysteine scanning

While the attachment of a spin label to a single site can be used to monitor conformational changes and help define the conformation of protein domains in aligned systems, cysteine-scanning can be used to assign secondary structure and also identify tertiary and quaternary contacts in large complexes. Prior to the availability of the crystal structure of the cardiac Tn core, we used the cysteine scanning approach to reveal the structure and position of the inhibitory region of cardiac TnI within the Tn complex. The inhibitory region is important as, alone, it is capable of inhibiting force generation in muscle as shown by a synthetic peptide representative of this region (Syska et al. 1976; Tao et al. 1990). At the time, little was known about the structure of the inhibitory region, although two structural models were available. Both models showed the TnI inhibitory region in complex with TnC, but with vastly contrasting secondary structure assignments: an α -helix model and a β -hairpin model (Vassilyev et al. 1998; Tung et al.

Fig. 5 Conformation of TnC in aligned muscle fibers. An EPR spectrum provides information on the disorder and orientation of a rigidly attached spin-labeled domain in an aligned system, such as a muscle fiber. The splitting and width of the EPR signal provides a measure of the orientation and disorder of the protein domain. In this example, EPR spectra are shown for TnC labeled with MSL at C98 in muscle fibers. The arrows denote the orientation of the spin label with respect to the fiber under

conditions of: (i) relaxation, (ii) rigor, (iii) with bound Ca^{2+} in the absence of acto-myosin cross-bridges, and (iv) with bound Ca^{2+} in the presence of rigor cross-bridges (from left to right). β_0 is the average tilt angle of the spin label with respect to the fiber axis and $\Delta\beta$ is the half-width of the Gaussian distribution centered on β_0 (adapted from Li and Fajer 1994 with permission from American Chemical Society)

2000). To resolve this discrepancy, 17 residues of TnI (cardiac residues 129–145) were, in turn, mutated to cysteine residues and labeled with MTSL. Trends in the mobility and accessibility of the 17 spin-labeled constructs upon interaction with TnC and TnT were then obtained (Fig. 6).

From the accessibility measurements using the water soluble quencher NiEDDA (Fig. 6b), we could assign a defined region of secondary structure of α -helix across residues 129 to 137, followed by a disordered region towards the C-terminal end of the inhibitory region (residues 138–145). A distinct fingerprint of the interaction of the TnI inhibitory region with TnT and TnC was also revealed through the mapping of the changes in mobility of the 17 spin-labeled constructs (Fig. 6a, c). Our proposed structure and positioning of the TnI inhibitory region within the Tn complex, as assigned by these cysteine scanning experiments, was later verified by the X-ray structure of the core of the Tn complex (PDB: 1J1D) (Takeda et al. 2003).

Cysteine scanning the disease prone TnI mobile domain in thin filaments

Using a similar approach, our group have also performed cysteine scanning through the cardiac TnI mobile domain (MD, residues 175 to 206) to elucidate the structure of this region and its interaction with other proteins in the muscle thin filaments (Cooke et al. 2010). The MD was not resolved in the X-ray structures but is expected to be an important player in the Ca^{2+} -dependent regulation through its proposed role as a

secondary actin-binding domain (Fig. 2). NMR studies of an isolated peptide representing this region have described the MD as a highly dynamic domain with, at most, some nascent secondary structure present in solution (Murakami et al. 2005). However, through monitoring of trends in our EPR mobility measurements, we identified two helical structural elements across the MD (res. 175–179 and 192–202) and further confirmed this using CW-EPR distance measurements. Pulsed EPR measurements (DEER) were also used to measure the interspin distance across the length of this domain (between residues 178 and 206) and suggested that the MD exists in an extended conformation. Both CW and DEER distance measurements are discussed later in this review. Interestingly, the construction of an elongated extended helical model from our EPR results showed that residues of the MD associated with genetic cardiomyopathy disorders are clustered on the interacting surface between the MD and the thin filament (model unpublished).

Interspin distance measurements to track conformational changes and build structures

Both intra- and inter-subunit short- and long-range distance constraints are useful to track conformational changes, build structures, or distinguish between competing structural models. As introduced in the “Site-directed spin labeling of troponin” section, there are several EPR methods that can be used to measure the interspin distance between two interacting spin-labeled sites, including CW-EPR and pulsed

Fig. 6 Cysteine scanning mobility and accessibility measurements reveal the structure and footprint of the inhibitory region of TnI in the Tn complex. **a** A subset of CW-EPR spectral lineshapes from residues 130–138 of the TnI inhibitory region in the cardiac ternary complex (TnC/TnI/TnT). The mobility of the spin label is interpreted by full lineshape analysis to obtain rotational correlation times (τ). **b** The accessibility trend (red dots) of spin-labeled residues (TnI 130–138) to NiEDDA provides a profile consistent with an α -helix model (gray line).

methods (DEER). For CW-EPR, the dipolar coupling between two spin labels results in line broadening effects on the EPR spectrum. The strength of the broadening of the EPR signal is related to the spin-spin distance. The mean interspin distance can be extracted from the broadened spectrum via a variety of methods, including convolution of the unbroadened spectrum of the single label with the corresponding dipolar double-labeled spectrum (Fajer et al. 2007). Some CW analysis methods can also provide a description of the distance distribution between the two spins (Potluri et al. 2017). However, the upper distance limit for measuring dipolar coupling by the broadening of the CW spectrum is approximately 2.0–2.5 nm, hence this approach is not well suited for measuring large-scale conformational changes (Banham et al. 2008). Fortunately, the upper limit of CW-EPR overlaps with the lower distance limit of the pulsed DEER method (~ 2.0 nm) which is better suited for measuring longer interspin distances (up to 8.0 nm). It is also best if both approaches are used in parallel (Potluri et al. 2017).

c Mapping of the difference between the τ of the spin label measured in the binary (TnC/TnI) and ternary (with TnT) complexes reveals the “footprint” for the interaction of the TnI inhibitory region with the TnT subunit. The changes in τ are color mapped onto the TnC-TnI binary structure (PDB: 1EW7); where gray represents little difference change and yellow represents a 200-fold increase in the τ of the spin label (adapted from Brown et al. (2002))

Continuous wave EPR measurements within troponin

For troponin, CW-dipolar line broadening methods have been used to measure both short-range intra- and inter-subunit interspin distances, to identify or validate secondary structure elements, to position Tn elements with respect to the complex, and to track small conformational changes (Brown et al. 2002; Ueki et al. 2005; Cooke et al. 2010; Potluri et al. 2017). These experiments have been particularly important for understanding the roles of key regions of TnI which could not be provided by the X-ray crystal structures, including the TnI inhibitory region of the cardiac isoform (Brown et al. 2002; Takeda et al. 2003). As mentioned in the previous section, support for the assignment of the structure of the inhibitory region of TnI was obtained through CW-EPR measurements. An α -helical structural model was confirmed across TnI residues 130 to 138 through the measurement of four intradomain interspin pairs (135/139, 146/150, 141/146, 142/146) (Brown et al. 2002). In general, a design strategy of including double mutant pairings of $(i, i + 3)$ and $(i, i + 4)$, where i represents the first-labeled

cysteine residue and the integer indicates the positioning of the second-labeled residue upstream from i , can be particularly helpful when needing to distinguish between helical or β -strand structural elements through the observation of a pattern of proximities (Cooke and Brown 2011).

CW interdomain interspin distances have also been used to assess the opening of the regulatory N-lobe of the cardiac isoform of TnC upon binding of Ca^{2+} in the Tn binary complex (Ueki et al. 2005). In this study, the authors introduced spin labels onto residues C42 and C58, located on helices B and C, respectively, and measured the CW distance to a spin label located on helix D (C84). Ca^{2+} binding was shown to move the C helix to an almost fully open conformation, similar to that observed for the skeletal isoform by NMR. This result was in contrast to the reporting of the unexpected absence of an open-state conformation of the cardiac TnC N-lobe by NMR solution studies (Sia et al. 1997). Interestingly, the authors proposed from this study that the full opening of the N-lobe of the cardiac isoform required not only Ca^{2+} binding, but also the interaction of TnC with TnI (Sia et al. 1997).

We have also used CW-dipolar EPR to confirm the geometric interaction and subsequent release of the cardiac TnI switch region with the hydrophobic pocket of the N-lobe of TnC (Fig. 7) (Potluri et al. 2017). In this recent study, the interspin distances between TnI151 to TnC35 or TnC84 both showed short distances. While the interspin pair of TnI151 to TnC35 was close to the limit of the CW method, the distance between TnI151 to TnC84 was short (9–10 Å) with a narrow distance population (3–8 Å). Together, both these distances indicated the close interaction of the TnI switch region with the TnC N-lobe, as predicted by the X-ray crystal structure under Ca^{2+} saturating conditions. However, DEER was essential to fully describe the release of the switch region upon the removal of Ca^{2+} (Potluri et al. 2017), and will be discussed later.

Pulsed EPR measurements within troponin

Pulsed EPR methods, such as DEER and DQC, for measuring long-range interspin distances has come of age over the last two decades. The most common of these methods, due to the availability of commercial instrumentation, is the 4-pulse DEER experiment (also known as PELDOR—pulse electron-electron double resonance). First introduced in 2000, after the development of dead-time free pulses (Milov et al. 2000), pulsed methods are useful for extracting distances from 1.5 to up to ~8 nm. The method also provides a detailed description of the distribution of the measured distance or, when multiple distances are present, distance populations. In brief, DEER is able to reveal distances information through the application of a series of pulses that produce a spin echo which is then modulated by the frequency of the dipolar interaction (Fig. 8a). There are several excellent and

comprehensive reviews of the DEER-pulsed technique which discuss the strength and limitations of DEER as a spectroscopic ruler and the reader is directed to these for further information on performing DEER measurements and extracting the interspin distance information (Fajer et al. 2007, Jeschke 2012). As for all of the EPR measurements discussed so far, the DEER method is not limited by the size of the protein or protein complex. This property has therefore resulted in DEER being widely and successfully used for detecting conformational changes within the intact Tn complex and also in reconstituted thin filament environments.

The first demonstration of the use of DEER with the Tn complex was performed by our group in 2002 (Brown et al. 2002). As discussed earlier, interspin distances were measured within the Tn complex to position the cardiac TnI inhibitory region with respect to TnC. In total, 7 interdomain distances were designed to help distinguish between the α -helical and β -hairpin structural models. All the CW and DEER distances from these spin-labeled pairs suggested that the distances between the inhibitory region and the central linker of TnC were substantially further apart than either models at that time predicted. For example, and as reported by spin labels positioned on TnC94 and TnI142, the closest the two domains came near each other was 30 Å. Today's models still reflect the absence of any specific strong interactions of the inhibitory region of TnI with TnC. However, some suggest that this key region of TnI may interact with other regions of Tn; potentially the cardiac-specific N-terminal extension region (residues 1–40) (Howarth et al. 2007).

DEER has also been used to characterize conformational changes within the Tn complex upon Ca^{2+} binding. The research group of Arata et al performed a follow-up study to their 2005 CW-EPR study (Nakamura et al. 2005) and used pulsed EPR to follow the conformational changes across the N-domain of TnC (Ueki et al. 2005). DEER provided the improved characterization of the interspin distances across TnC that were previously found to be close to the upper limit of the CW technique (between 20 and 25 Å). The DEER experiments showed that the distances in the Ca^{2+} -free state between spin labels attached to TnC35 and TnC84 of cardiac TnC were 26.0 Å and 27.2 Å in the isolated Tn complex and muscle fibers, respectively. This distance decreased to 23.2 Å upon the addition of Ca^{2+} for the muscle fiber sample but no significant change was reported for the isolated Tn complex. This study was the first to report successful pulsed distance measurements within whole muscle fibers and re-iterated that an open to close transition of the N-domain of TnC was also a feature of the cardiac isoform, and not just the skeletal isoform (Slupsky and Sykes 1995).

The Arata group has also used pulsed EPR to follow the release of the TnI switch region from the N-lobe of TnC in the skeletal isoform (Fig. 8). Interspin distances were measured between TnI133 on the regulatory TnI switch region

Fig. 7 CW-EPR dipolar broadening to measure short interspin distances within Tn. **a** Location of MTSL-labeled residues in the Tn ternary complex (PDB: 1J1E). Interspin distances were measured by CW-EPR between TnI151/TnC35 and TnI151/TnC84. **b** CW-EPR spectra for double spin-labeled Tn ternary samples of TnC35/TnI151 (upper) and TnC84/TnI151 (lower). The gray spectrum represents the single spin-labeled sample as a reference spectrum, the red dashed line represents the

double-labeled spectrum, and the blue line shows the best fit to the double-labeled spectrum. The greater the degree of lineshape broadening, when comparing the double to the single-labeled spectrum, the shorter the interspin distance. **c** The Gaussian distribution of interspin distances shown in **b** (adapted from Potluri et al. (2017) with permission from Elsevier)

to three sites on TnC (TnC44, TnC61, and TnC98) (Fig. 8b). This was done in the ternary Tn complex and also in reconstituted thin filaments (Aihara et al. 2010). In the skeletal X-ray structure (Vinogradova et al. 2005), the switch region of TnI binds to the N-domain of TnC under conditions of high Ca^{2+} (PDB: 1YTZ). The DEER distances in the high Ca^{2+} state were consistent with the X-ray structure and showed narrow distribution widths ($\sim 3\text{--}12$ Å), supporting the close interaction of the switch region with TnC (Fig. 8c). Upon removal of Ca^{2+} , the pulsed EPR distances between the switch region and the three labeled TnC sites all increased, as well as the corresponding width of distance distributions between the spin labels (to 20–80 Å). A model of the conformational changes of the TnI regulatory region was proposed by the authors to reflect these longer and broader distance populations that dominate in the Ca^{2+} -free state. The model postulated that the N-lobe of TnC releases the switch region in the absence of Ca^{2+} ; the switch then *swings* thermally in Brownian motion, which is consistent with their earlier study (Aihara et al. 2006). The switch region is then able to fluctuate between binding weakly to a site on the actin thin filament and the N-lobe of TnC. The flexibility inferred by these EPR pulsed experiments for the N-lobe of skeletal TnC is compatible with NMR studies which report the tumbling of N-lobe relative to C-lobe and actin surface (Blumenschein et al. 2005; Sun et al. 2006).

Similar to this study on the skeletal isoform, our group have also recently performed CW and DEER experiments to characterize the release of the switch region from TnC in the cardiac isoform (Potluri et al. 2017). The magnitude of the distance of release of the switch region from the N-lobe was significantly less than what was reported for the skeletal isoform (Aihara et al. 2010). In the presence of saturating Ca^{2+} , the majority of the cardiac interspin distances between the switch region (TnI151) and TnC (TnC84) were short (9–10 Å) with a very narrow distance distribution width (3–8 Å). This result is consistent with a close interaction of the switch within the hydrophobic pocket of the N-lobe of TnC. However, additional spin populations at a longer average distance (25–30 Å) and a broader distance distribution width (14–29 Å) were also detected in the Ca^{2+} -saturating state suggesting the interaction is not absolute. Interestingly, the removal of Ca^{2+} did not result in a large-scale increase in either distance, as was observed for the skeletal isoform; rather, we observed a shift in the population of interacting spins between the two distance populations. Noticeably, the removal of Ca^{2+} led to an increase in the proportion of the spins ascribed to the longer distances, suggesting that the TnI switch region likely remains close to TnC at all times and is therefore primed to interact upon the influx of Ca^{2+} .

In addition to the studies already discussed, there are other studies which have also reported an increase in structural heterogeneity of Tn upon the release of Ca^{2+} for both the cardiac

Fig. 8 DEER spectroscopy on Tn reveals the release of the switch region of TnI. **a** Schematic overview of the 4-pulse DEER experiment. The DEER pulse method reveals distance information by producing a spin echo that is modulated by the frequency of the dipolar interaction. The observe frequency (ν) excites a subpopulation of spins in the sample with a sequence of three pulses, usually with length orders of 16, 32, and 32 ns, to induce a spin echo. After the second pulse in the observe sequence, the pump ν excites a second subpopulation to induce coupling with first-spin population. The pump pulse moves within the dipolar evolution time window to modulate the intensity of the refocused echo (highlighted in red). **b** Interspin distance pairs to track the release of the TnI switch peptide from the TnC N-domain in skeletal muscle thin filaments. **c** Representative DEER traces of interspin pairs TnC44/TnI133 (upper) and TnC61/TnI133 (lower) in the presence (red) and absence (blue) of Ca^{2+} . The corresponding distance distribution profiles for each of the traces are shown on the right (adapted from Aihara et al. (2010) with permission from American Society for Biochemistry & Molecular Biology)

and skeletal isoforms (Abe et al. 2018), as well as Tropomyosin (Risi et al. 2017). Structural heterogeneity in Tn can be attributed to either an increase in disorder of the TnC subunit via the flexible central linker connecting the two domains or increased fluctuations in the TnI switch region due

to its release. The hypothesis that the central helix “melts” or is more disordered in the Ca^{2+} -free state is supported by the X-ray structures of the skeletal Tn isoform (Vinogradova et al. 2005). Lastly, these two reports examining the release of the TnI switch also reinforce the importance of independently

Fig. 9 The PRE-NMR distance-dependent effect. **a** A ^{15}N -TROSY or HSQC NMR experiment is collected on a spin-labeled paramagnetic sample (red). The sample is then reduced to its diamagnetic equivalent (blue). The paramagnetic relaxation enhancement (T_2) causes a distance-dependent ($T_2 \propto 1/r^6$) peak broadening which can be observed by overlaying the spectra. The PRE peak broadening effects are illustrated for three residues at positions A, B, and C on the structure of cardiac TnC (PDB: 1AJ4). A reduction in peak intensity is observed for residues

within a ~ 25 Å radius of the spin label (residue B). At distances < 10 Å, the PRE effect is very strong and peaks are broadened beyond detection (residue C). At distances beyond 25–30 Å, no change in peak width is detectable (residue A). **b** Shows an example of the complete PRE-NMR long-range distance coverage of the N-lobe of TnC using 4 spin-labeled sites (reproduced from Cordina et al. (2013) with permission from American Chemical Society)

deciphering the regulatory mechanisms of Tn for each striated muscle isoform. While there are many similarities observed in large-scale structural changes upon activation, the dynamics and structural heterogeneity of each striated isoform may play a greater role in fine tuning the control of muscle regulation than previously thought. DEER analysis is therefore an important tool to contribute to this understanding as not only can it provide long-range distance information in large intact complexes, but more importantly, it provides distance distribution and population information of all contributing structural forms in solution. Another complementary powerful approach for obtaining multiple long-range distance constraints and dynamic information using spin labeling, which can be used to model the structural changes and conformational space, is PRE-NMR. This approach is discussed next.

Paramagnetic relaxation enhancement NMR—expanding the SDSL toolbox for Tn

In traditional NMR, transverse relaxation occurs due to dipolar interactions between nuclear spins, such as protons. Since the magnetic moment of nuclei is small, these dipolar interactions, which are extracted from interproton nuclear Overhauser effects (NOEs), are typically weak and occur over very short ranges (< 5 Å). In contrast, the magnetic moment of an unpaired electron, such as from an introduced spin label, is ~ 600 times larger than the nuclear moment of a proton and

therefore can cause an increase in the relaxation rates of nearby spin nuclei (Clore and Iwahara 2009). This is known as the paramagnetic relaxation enhancement or “PRE” effect. In the NMR spectrum, the PRE effect is observed as broadening of the NMR resonance peaks (Fig. 9a). The interaction is distant dependent and the effect can be detected at distances of up to 25–30 Å away from the paramagnetic center. The magnitude of the PRE-broadening effect provides a measure of the transverse relaxation rate which is obtained by comparing the peak linewidths from an NMR spectrum obtained in the presence of the spin label to a spectrum acquired in the absence of the paramagnetic species (or the diamagnetic reference). The calculated PRE rates (T_2) are then converted to distances (r) using a modified form of the Solomon-Bloembergen equation (Liang et al. 2006; Volkov et al. 2006).

The placement of a single EPR spin label at a desired location on isotopically labeled ^{15}N -protein is the only prerequisite to perform PRE-NMR. Then, 2D ^{15}N -HSQC (heteronuclear single quantum coherence) spectra of the paramagnetic and diamagnetic sample are collected for PRE analysis. Unlike the EPR methods described earlier, the PRE-NMR approach is still somewhat limited by the size of the complex than can be examined. In the case of larger proteins (> 20 kDa), where a slowing in the tumbling rate can cause the NMR signal to de-phase rapidly, ^{15}N -TROSY (transverse relaxation optimized spectroscopy) experiments should be performed. To date, we have successfully performed PRE-NMR

Fig. 10 PRE-NMR shows that Ca²⁺-binding shifts the population of the cardiac N-lobe of TnC to an open state conformation. **a** Representative PRE-NMR spectra for cardiac TnC labeled at C48. The paramagnetic ¹⁵N-HSQC spectra are shown in maroon and the diamagnetic in black. NMR spectra are collected for a Ca²⁺ titration series. **b** Representative PRE profile (Γ_2) of C48 construct measured under Ca²⁺ bound (filled circles) and free (empty circles) conditions; and for comparison, back-calculated Γ_2 values obtained from PDB: 1AJ4 are shown as a gray line. Inset structure shows the position of the spin-labeled C48 residue. Movement towards the spin label upon Ca²⁺ binding is shown in blue

and away in red on both the Γ_2 profiles and the TnC structure. Interestingly, the distance mapping on helix A shows movement consistent with rotation of this helix. **c** Comparison of available NMR structures of the regulatory N-lobe of cardiac TnC. From left to right: Ca²⁺-free apo closed state (PDB: 1SPY), Ca²⁺-bound holo “primed” state (PDB: 1AP4), and TnI switch region with bound Ca²⁺ or “open” state (PDB: 1MXL). Ca²⁺ and switch binding opens the TnC N-lobe by increasing the A–B interhelical angle by ~40° and through extension of helix B (reproduced from Cordina et al. (2013) with permission from American Chemical Society)

on both the isolated TnC subunit and the binary complex of Tn (TnC-TnI, ~43 kDa). With both of these samples, PRE enabled the mapping of long-range intradomain, interdomain, and inter-subunit interactions (Cordina et al. 2012; Cordina et al. 2013; Cordina et al. 2014; Potluri et al. 2019). Through the strategic placement of a small number of spin labels, the mapping of entire domains of TnC (~10 Da) can be rapidly achieved (Fig. 9b).

Interdomain orientation of cardiac troponin C reveals a compact state

While there have been many NMR studies on Tn, these have been largely limited to isolated TnC domains or TnC in complex with representative peptide fragments of key function regions of TnI. While these studies have provided us with atomic resolution details for both the regulatory and structural

domains of TnC, a lack of NOEs between the two structural elements has hindered our understanding of the relative orientation between the two domains. We therefore first sought to use PRE-NMR to evaluate the interdomain orientation of cardiac TnC through the placement of spin labels at 4 positions on TnC (TnC35, 84, 94, 136) (Cordina et al. 2012). Ensemble fitting of the long-range interdomain PRE distances revealed that the cardiac TnC isoform preferentially adopts a collapsed and relatively compact conformation in solution. While flexible, the defined range of relative domain orientations selected agreed well with the conformations of TnC observed in the cardiac X-ray crystal structure (Takeda et al. 2003). This flexibility and collapsed conformation of TnC may be important to help position the N-domain, and thus the TnI switch, in close proximity to the thin filament at all times throughout the regulatory cycle.

The Ca^{2+} -triggered shift between closed and open conformations of the N-lobe of cardiac TnC

Building on our characterization of the interdomain dynamics of TnC by PRE, we then engineered the placement of spin labels at two further sites (C12 and C48) in the regulatory N-lobe to assess the Ca^{2+} -triggered structural changes across this domain in full-length TnC (Cordina et al. 2013). Although EPR studies had suggested that both open and closed structural states occur for the Ca^{2+} -saturated cardiac TnC isoform (Nakamura et al. 2005; Ueki et al. 2005), a true open state for the Ca^{2+} -saturated form had not been observed by NMR. From our four spin-labeled sites (C12, C35, C48, and C84), a total of approximately 400 long-range distance constraints were obtained (Fig. 9B) which were then used to model the magnitude and direction of movement of the N-domain helices upon Ca^{2+} binding. An example of the Ca^{2+} -induced

changes in the NMR spectra from the TnC C48-labeled construct is shown in Fig. 10. While these conformational changes agreed well with an open state structure, similar to the open form that is observed when the inhibitory region of TnI is bound, the movement towards an open state was not absolute. That is, a significant population (~70%) of the closed form of TnC remained present in the presence of saturating Ca^{2+} conditions. We concluded that Ca^{2+} binding to cardiac TnC creates a dynamic equilibrium between the closed and open structural states to prime the N-lobe for interaction with the TnI switch peptide. In the same study, we also examined the effect of a cardiomyopathy point mutation (A8V) on this dynamic equilibrium between the closed and open forms. The PRE data showed that the Ca^{2+} -triggered equilibrium for the N-domain is indeed perturbed by the A8V mutation. A more “open” N-domain conformation was found in both the Ca^{2+} -bound and Ca^{2+} -free states.

Ca^{2+} -induced PRE distance changes reveal the possessive nature of cardiac TnC for its regulatory switch

Lastly, we have recently pushed the size limits of the PRE method by examining the interaction between the TnI and TnC cardiac subunits in the intact binary complex (~43 kDa). SDSL was used to position spin labels in several functionally important regions of TnI, including the N-region (TnI57), the inhibitory region (TnI143), and two sites on the switch region (TnI151 and TnI159) (Cordina et al. 2014). Due to the large size of the binary complex, ^{15}N -TROSY experiments were utilized in this study. Analysis of the PRE-derived distance constraints from these sites showed that the spin-labeled N-region of TnI remains tightly bound to the structural C-lobe throughout the Ca^{2+} regulatory cycle, as observed in the X-ray structures of the cardiac Tn core (PDB: 1J1D)

Fig. 11 The “molecular movie” of cardiac Tn. **a** The structural model shows the central linker of TnC to be highly flexible with a bent configuration that keeps it in close proximity to the actin thin filament and also the switch (SP) and inhibitory (IR) peptide regions of TnI, at all times. In conditions of low Ca^{2+} , the N-lobe of TnC is predominantly in a closed conformation. **b** Upon binding of Ca^{2+} to site II of TnC, an N-lobe population of ~27% can be detected in an open state (Cordina et al. 2013). **c** The N-lobe of the regulatory domain is now primed to interact with the SP of TnI via conformational selection. The close proximity of TnI to TnC in the cardiac complex is favorable for increasing the frequency of

collisions between the N-lobe of cTnC and the regulatory switch region, counterbalancing the reduction in collision probability that results from the incomplete opening of the N-lobe of TnC that is unique to the cardiac isoform. **d** Binding of the switch region to TnC releases both the IR and the mobile domain (MD) of actin from the thin filament. The IR region has a helical content, which is stabilized through interactions with TnT; and a highly disordered section to allow the SP to interact with the hydrophobic pocket of the N-lobe. Image adapted from Vinogradova et al. (2005), copyright (2005) National Academy of Sciences

(Takeda et al. 2003). Changes in distances measured in the absence and presence of calcium suggest a model of regulation by cardiac Tn where the switch region is closely bound within the hydrophobic N-lobe of cTnC in the presence of Ca^{2+} . Upon removal of Ca^{2+} , the inhibitory switch region displayed a higher degree of conformational freedom, although it continues to remain in close vicinity to the central linker of TnC at all times. Our PRE distance data also showed that only a small movement (~ 10 Å) of the switch regulatory region of TnI occurs upon the removal of Ca^{2+} . The high flexibility of this region, and close interaction of both the switch and inhibitory regions with TnC, is in agreement with earlier EPR findings (Brown et al. 2002; Potluri et al. 2017).

Conclusions—the SDSL toolkit for building a structural model of troponin

SDSL, used in combination with the spectroscopic techniques of EPR and NMR, has provided us with a complementary set of tools for examining the structure and dynamics of Tn. We can now use these spectroscopic tools to build upon the X-ray structures and work towards developing a complete structural model or “molecular movie” of the troponin complex (Fig. 11). From the many SDSL-EPR and PRE-NMR experiments discussed in this review, we are now able to better correlate structural changes with function through the simple selective targeting of important, and often disease prone, regions of Tn with spin labels. As described, the measurements of the mobility, accessibility, and orientation from a single attached spin label on Tn can be used to map structural changes in Tn, from the level of an isolated protein subunit, right through to whole intact muscle filaments. EPR and PRE methods can also be combined to provide mid- (< 25 Å) to long-range (~ 4 – 80 Å) distance restraints for structure determination, and more importantly, to follow the large conformational changes associated with biological activity across all levels of structural hierarchy.

In conclusion, the SDSL method has been instrumental in providing important dynamic information that shows that X-ray and NMR structures of isolated components of Tn are not necessarily the same as the structures within their native environment. Further, these SDSL tools have also revealed several differences in both structure and modes of regulation between the two Tn-striated muscle isoforms with the story of the cardiac isoform appearing to be more complicated than its skeletal counterpart. Specifically, the cardiac Tn isoform is also controlled or regulated by a secondary mechanism—phosphorylation. The role of phosphorylation in cardiac muscle regulation is of significance because altered levels of Tn phosphorylation are often found associated with heart disease and the failing heart (Bodor et al. 1997; Wijnker et al. 2014). Unfortunately, there is still very little known about the manner

through which phosphorylation “fine-tunes” the Ca^{2+} regulation of cardiac muscle but this remains a key focus for many groups, including ours.

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Compliance with ethical standards

Conflict of interest Ehsan Kachooei declares that he has no conflict of interest. Nicole M. Cordina declares that she has no conflict of interest. Louise J. Brown declares that she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

- Abe J, Ueki S, Yamauchi S, Arata T, Ohba Y (2018) Double quantum coherence EPR reveals the structure–function relationships of the cardiac troponin C–troponin I complex regulated by Ca^{2+} ions and a phosphomimetic. *Appl Magn Reson* 49:893–910
- Aihara T, Ueki S, Nakamura M, Arata T (2006) Calcium-dependent movement of troponin I between troponin C and actin as revealed by spin-labeling EPR. *Biochem Biophys Res Commun* 340:462–468
- Aihara T, Nakamura M, Ueki S, Hara H, Miki M, Arata T (2010) Switch action of troponin on muscle thin filament as revealed by spin labeling and pulsed EPR. *J Biol Chem* 285:10671–10677
- Altenbach C, Flitsch SL, Khorana HG, Hubbell WL (1989) Structural studies on transmembrane proteins. 2. Spin labeling of bacteriorhodopsin mutants at unique cysteines. *Biochemistry* 28:7806–7812
- Altenbach C, Marti T, Khorana HG, Hubbell WL (1990) Transmembrane protein structure: spin labeling of bacteriorhodopsin mutants. *Science* 248:1088–1092
- Arata T, Aihara T, Ueda K, Nakamura M, Ueki S (2007) Calcium structural transition of troponin in the complexes, on the thin filament, and in muscle fibres, as studied by site-directed spin-labelling EPR. *Adv Exp Med Biol* 592:125–135
- Banham JE, Baker CM, Ceola S, Day IJ, Grant GH, Groenen EJ, Rodgers CT, Jeschke G, Timmel CR (2008) Distance measurements in the borderline region of applicability of CW EPR and DEER: a model study on a homologous series of spin-labelled peptides. *J Magn Reson* 191:202–218
- Battiste JL, Wagner G (2000) Utilization of site-directed spin labeling and high-resolution heteronuclear nuclear magnetic resonance for global fold determination of large proteins with limited nuclear Overhauser effect data. *Biochemistry* 39:5355–5365
- Behrmann E, Muller M, Penczek PA, Mannherz HG, Manstein DJ, Raunser S (2012) Structure of the rigor actin-tropomyosin-myosin complex. *Cell* 150:327–338
- Blumenschein TM, Stone DB, Fletterick RJ, Mendelson RA, Sykes BD (2005) Calcium-dependent changes in the flexibility of the regulatory domain of troponin C in the troponin complex. *J Biol Chem* 280:21924–21932
- Bodor GS, Oakeley AE, Allen PD, Crimmins DL, Ladenson JH, Anderson PA (1997) Troponin I phosphorylation in the normal and failing adult human heart. *Circulation* 96:1495–1500
- Borbat PP, McHaourab HS, Freed JH (2002) Protein structure determination using long-distance constraints from double-quantum coherence ESR: study of T4 lysozyme. *J Am Chem Soc* 124:5304–5314

- Bordignon E, Bleicken S (2018) New limits of sensitivity of site-directed spin labeling electron paramagnetic resonance for membrane proteins. *Biochim Biophys Acta Biomembr* 1860:841–853
- Brown LJ, Sale KL, Hills R, Rouviere C, Song L, Zhang X, Fajer PG (2002) Structure of the inhibitory region of troponin by site directed spin labeling electron paramagnetic resonance. *Proc Natl Acad Sci U S A* 99:12765–12770
- Chatani S, Nakamura M, Akahane H, Kohyama N, Taki M, Arata T, Yamamoto Y (2005) Synthesis of C2-chiral bifunctionalised spin labels and their application to troponin C. *Chem Commun (Camb)* 14:1880–1882
- Clore GM, Iwahara J (2009) Theory, practice, and applications of paramagnetic relaxation enhancement for the characterization of transient low-population states of biological macromolecules and their complexes. *Chem Rev* 109:4108–4139
- Cooke JA, Brown LJ (2011) Distance measurements by continuous wave EPR spectroscopy to monitor protein folding. In: Hill AF, Barnham KJ, Bottomley SP, Cappai R (eds) *Protein folding, Misfolding, and disease: methods and protocols*. Humana Press, Totowa, NJ, pp 73–96
- Cooke JA, Chamoun J, Howell MW, Curmi PMG, Fajer PG, Brown LJ (2010) Structure and dynamics of the mobile domain of troponin I by SDSL-EPR. *Biophysical J* 98:148a
- Cordina NM, Liew CK, Gell DA, Fajer PG, Mackay JP, Brown LJ (2012) Interdomain orientation of cardiac troponin C characterized by paramagnetic relaxation enhancement NMR reveals a compact state. *Protein Sci* 21:1376–1387
- Cordina NM, Liew CK, Gell DA, Fajer PG, Mackay JP, Brown LJ (2013) Effects of calcium binding and the hypertrophic cardiomyopathy A8V mutation on the dynamic equilibrium between closed and open conformations of the regulatory N-domain of isolated cardiac troponin C. *Biochemistry* 52:1950–1962
- Cordina NM, Liew CK, Potluri PR, Curmi PM, Fajer PG, Logan TM, Mackay JP, Brown LJ (2014) Ca²⁺-induced PRE-NMR changes in the troponin complex reveal the possessive nature of the cardiac isoform for its regulatory switch. *PLoS One* 9:e112976
- Dong W, Rosenfeld SS, Wang CK, Gordon AM, Cheung HC (1996) Kinetic studies of calcium binding to the regulatory site of troponin C from cardiac muscle. *J Biol Chem* 271:688–694
- Dong WJ, Xing J, Robinson JM, Cheung HC (2001) Ca²⁺ induces an extended conformation of the inhibitory region of troponin I in cardiac muscle troponin. *J Mol Biol* 314:51–61
- Evans EG, Millhauser GL (2015) Genetic incorporation of the unnatural amino acid p-acetyl phenylalanine into proteins for site-directed spin labeling. *Methods Enzymol* 563:503–527
- Fajer PG, Brown LJ, Song L (2007) Practical pulsed dipolar ESR (DEER). In: Hemminga MA, Berliner L (eds) *ESR spectroscopy in membrane biophysics*. Springer US, New York, pp 95–128
- Findlay WA, Sonnichsen FD, Sykes BD (1994) Solution structure of the TR1C fragment of skeletal muscle troponin-C. *J Biol Chem* 269:6773–6778
- Gagne SM, Tsuda S, Li MX, Smillie LB, Sykes BD (1995) Structures of the troponin C regulatory domains in the apo and calcium-saturated states. *Nat Struct Biol* 2:784–789
- Gagne SM, Li MX, McKay RT, Sykes BD (1998) The NMR angle on troponin C. *Biochem Cell Biol* 76:302–312
- Gomes AV, Potter JD (2004) Molecular and cellular aspects of troponin cardiomyopathies. *Ann N Y Acad Sci* 1015:214–224
- Gordon AM, Homsher E, Regnier M (2000) Regulation of contraction in striated muscle. *Physiol Rev* 80:853–924
- Herzberg O, James MN (1985) Structure of the calcium regulatory muscle protein troponin-C at 2.8 Å resolution. *Nature* 313:653–659
- Howarth JW, Meller J, Solaro RJ, Trewella J, Rosevear PR (2007) Phosphorylation-dependent conformational transition of the cardiac specific N-extension of troponin I in cardiac troponin. *J Mol Biol* 373:706–722
- Hubbell WL, Gross A, Langen R, Lietzow MA (1998) Recent advances in site-directed spin labeling of proteins. *Curr Opin Struct Biol* 8:649–656
- Hubbell WL, Lopez CJ, Altenbach C, Yang Z (2013) Technological advances in site-directed spin labeling of proteins. *Curr Opin Struct Biol* 23:725–733
- Jeschke G (2012) DEER distance measurements on proteins. *Annu Rev Phys Chem* 63:419–446
- Jeschke G (2018) MMM: a toolbox for integrative structure modeling. *Protein Sci* 27:76–85
- Kim CH, Axup JY, Schultz PG (2013) Protein conjugation with genetically encoded unnatural amino acids. *Curr Opin Chem Biol* 17:412–419
- Layland J, Solaro RJ, Shah AM (2005) Regulation of cardiac contractile function by troponin I phosphorylation. *Cardiovasc Res* 66:12–21
- Li HC, Fajer PG (1994) Orientational changes of troponin C associated with thin filament activation. *Biochemistry* 33:14324–14332
- Li HC, Fajer PG (1998) Structural coupling of troponin C and actomyosin in muscle fibers. *Biochemistry* 37:6628–6635
- Li HC, Hideg K, Fajer PG (1997) The mobility of troponin C and troponin I in muscle. *J Mol Recognit* 10:194–201
- Li KL, Rieck D, Solaro RJ, Dong W (2014) In situ time-resolved FRET reveals effects of sarcomere length on cardiac thin-filament activation. *Biophys J* 107:682–693
- Liang B, Bushweller JH, Tamm LK (2006) Site-directed parallel spin-labeling and paramagnetic relaxation enhancement in structure determination of membrane proteins by solution NMR spectroscopy. *J Am Chem Soc* 128:4389–4397
- McHaourab HS, Lietzow MA, Hideg K, Hubbell WL (1996) Motion of spin-labeled side chains in T4 lysozyme. Correlation with protein structure and dynamics. *Biochemistry* 35:7692–7704
- McHaourab HS, Oh KJ, Fang CJ, Hubbell WL (1997) Conformation of T4 lysozyme in solution. Hinge-bending motion and the substrate-induced conformational transition studied by site-directed spin labeling. *Biochemistry* 36:307–316
- McNulty JC, Silapje JL, Carnevali M, Farrar CT, Griffin RG, Formaggio F, Crisma M, Toniolo C, Millhauser GL (2000) Electron spin resonance of TOAC labeled peptides: folding transitions and high frequency spectroscopy. *Biopolymers* 55:479–485
- Miick SM, Martinez GV, Fiori WR, Todd AP, Millhauser GL (1992) Short alanine-based peptides may form 3₁₀-helices and not alpha-helices in aqueous solution. *Nature* 359:653–655
- Milov AD, Salikhov KM, Shirov M (1981) Application of ELDOR in electron spin echo for paramagnetic center spin distributions in solids. *Fiz Tverd Tela* 23:975–982
- Milov AD, Mar'yasov AG, Samoilova RI, Tsvetkov YD, Raap J, Monaco V, Formaggio F, Crisma M, Toniolo C (2000) Pulsed electron double resonance of spin-labeled peptides: data on the peptide-chain secondary structure. *Dokl Biochem* 370:8–11
- Murakami K, Yumoto F, Ohki SY, Yasunaga T, Tanokura M, Wakabayashi T (2005) Structural basis for Ca²⁺-regulated muscle relaxation at interaction sites of troponin with actin and tropomyosin. *J Mol Biol* 352:178–201
- Nakamura M, Ueki S, Hara H, Arata T (2005) Calcium structural transition of human cardiac troponin C in reconstituted muscle fibres as studied by site-directed spin labelling. *J Mol Biol* 348:127–137
- Narita A, Yasunaga T, Ishikawa T, Mayanagi K, Wakabayashi T (2001) Ca²⁺-induced switching of troponin and tropomyosin on actin filaments as revealed by electron cryo-microscopy. *J Mol Biol* 308:241–261
- Potluri PR, Chamoun J, Cooke JA, Badr M, Guse JA, Rayes R, Cordina NM, McCamey D, Fajer PG, Brown LJ (2017) The concerted movement of the switch region of troponin I in cardiac muscle thin filaments as tracked by conventional and pulsed (DEER) EPR. *J Struct Biol* 200:376–387

- Potluri PR, Cordina NM, Kachooei E, Brown LJ (2019) Characterization of the L29Q hypertrophic cardiomyopathy mutation in cardiac troponin C by paramagnetic relaxation enhancement nuclear magnetic resonance. *Biochemistry* 58:908–917
- Rabenstein MD, Shin YK (1995) Determination of the distance between two spin labels attached to a macromolecule. *Proc Natl Acad Sci U S A* 92:8239–8243
- Risi C, Eisner J, Belknap B, Heeley DH, White HD, Schroder GF, Galkin VE (2017) Ca^{2+} -induced movement of tropomyosin on native cardiac thin filaments revealed by cryoelectron microscopy. *Proc Natl Acad Sci U S A* 114:6782–6787
- Sia SK, Li MX, Spyropoulos L, Gagne SM, Liu W, Putkey JA, Sykes BD (1997) Structure of cardiac muscle troponin C unexpectedly reveals a closed regulatory domain. *J Biol Chem* 272:18216–18221
- Slupsky CM, Sykes BD (1995) NMR solution structure of calcium-saturated skeletal muscle troponin C. *Biochemistry* 34:15953–15964
- Solaro RJ, Henze M, Kobayashi T (2013) Integration of troponin I phosphorylation with cardiac regulatory networks. *Circ Res* 112:355–366
- Sun YB, Brandmeier B, Irving M (2006) Structural changes in troponin in response to Ca^{2+} and myosin binding to thin filaments during activation of skeletal muscle. *Proc Natl Acad Sci U S A* 103:17771–17776
- Sundaralingam M, Bergstrom R, Strasburg G, Rao ST, Roychowdhury P, Greaser M, Wang BC (1985) Molecular structure of troponin C from chicken skeletal muscle at 3-angstrom resolution. *Science* 227:945–948
- Sykes BD (2003) Pulling the calcium trigger. *Nat Struct Biol* 10:588–589
- Syska H, Wilkinson JM, Grand RJ, Perry SV (1976) The relationship between biological activity and primary structure of troponin I from white skeletal muscle of the rabbit. *Biochem J* 153:375–387
- Szczesna D, Fajer PG (1995) The tropomyosin domain is flexible and disordered in reconstituted thin filaments. *Biochemistry* 34:3614–3620
- Takeda S, Yamashita A, Maeda K, Maeda Y (2003) Structure of the core domain of human cardiac troponin in the Ca^{2+} -saturated form. *Nature* 424:35–41
- Tao T, Gong BJ, Leavis PC (1990) Calcium-induced movement of troponin-I relative to actin in skeletal muscle thin filaments. *Science* 247:1339–1341
- Tsuda S, Miura A, Gagne SM, Spyropoulos L, Sykes BD (1999) Low-temperature-induced structural changes in the apo regulatory domain of skeletal muscle troponin C. *Biochemistry* 38:5693–5700
- Tung CS, Wall ME, Gallagher SC, Trewella J (2000) A model of troponin-I in complex with troponin-C using hybrid experimental data: the inhibitory region is a beta-hairpin. *Protein Sci* 9:1312–1326
- Ueki S, Nakamura M, Komori T, Arata T (2005) Site-directed spin labeling electron paramagnetic resonance study of the calcium-induced structural transition in the N-domain of human cardiac troponin C complexed with troponin I. *Biochemistry* 44:411–416
- Vassilyev DG, Takeda S, Wakatsuki S, Maeda K, Maeda Y (1998) Crystal structure of troponin C in complex with troponin I fragment at 2.3-Å resolution. *Proc Natl Acad Sci U S A* 95:4847–4852
- Vibert P, Craig R, Lehman W (1997) Steric-model for activation of muscle thin filaments. *J Mol Biol* 266:8–14
- Vinogradova MV, Stone DB, Malanina GG, Karatzaferi C, Cooke R, Mendelson RA, Fletterick RJ (2005) Ca^{2+} -regulated structural changes in troponin. *Proc Natl Acad Sci U S A* 102:5038–5043
- Volkov AN, Worrall JA, Holtzmann E, Ubbink M (2006) Solution structure and dynamics of the complex between cytochrome c and cytochrome c peroxidase determined by paramagnetic NMR. *Proc Natl Acad Sci U S A* 103:18945–18950
- Wijnker PJ, Murphy AM, Stienen GJ, van der Velden J (2014) Troponin I phosphorylation in human myocardium in health and disease. *Neth Heart J* 22:463–469
- Willott RH, Gomes AV, Chang AN, Parvatiyar MS, Pinto JR, Potter JD (2010) Mutations in troponin that cause HCM, DCM AND RCM: what can we learn about thin filament function? *J Mol Cell Cardiol* 48:882–892

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