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ORIGINAL PAPER

A comprehensive guide to genetic variants and post-translational modifications of cardiac troponin C

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Abstract

Familial cardiomyopathy is an inherited disease that affects the structure and function of heart muscle and has an extreme range of phenotypes. Among the millions of affected individuals, patients with hypertrophic (HCM), dilated (DCM), or left ventricular non-compaction (LVNC) cardiomyopathy can experience morphologic changes of the heart which lead to sudden death in the most detrimental cases. *TNNC1*, the gene that codes for cardiac troponin C (cTnC), is a sarcomere gene associated with cardiomyopathies in which probands exhibit young age of presentation and high death, transplant or ventricular fibrillation events relative to *TNNT2* and *TNNI3* probands. Using GnomAD, ClinVar, UniProt and PhosphoSitePlus databases and published literature, an extensive list to date of identified genetic variants in *TNNC1* and post-translational modifications (PTMs) in cTnC was compiled. Additionally, a recent cryo–EM structure of the cardiac thin filament regulatory unit was used to localize each functionally studied amino acid variant and each PTM (acetylation, glycation, s-nitrosylation, phosphorylation) in the structure of cTnC. *TNNC1* has a large number of variants (> 100) relative to other genes of the same transcript size. Surprisingly, the mapped variant amino acids and PTMs are distributed throughout the cTnC structure. While many cardiomyopathy-associated variants are localized in α-helical regions of cTnC, this was not statistically significant χ^2 ($p = 0.72$). Exploring the variants in *TNNC1* and PTMs of cTnC in the contexts of cardiomyopathy association, physiological modulation and potential non-canonical roles provides insights into the normal function of cTnC along with the many facets of *TNNC1* as a cardiomyopathic gene.

Keywords Heart · *TNNC1* · Cardiac troponin C (cTnC) · Cardiomyopathy · Genetic variant · Post-translational modification

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Introduction

Cardiomyopathy is a group of acquired or hereditary diseases characterized by structural and functional abnormalities of heart muscle. These anomalies can be associated with a large number of variants located in sarcomeric protein-encoding genes (Garfinkel et al. 2018; Landstrom et al. 2008; Tadros et al. 2020; van der Velden and Stienen 2019; Yotti et al. 2019). Through genetic testing, the presence of these variants can be discovered in patients who have a suspected inherited cardiovascular disease and/or patients who have family members presenting with a pathogenic variant (Musunuru et al. 2020). Severity of clinical outcomes varies widely, and common effects of these cardiomyopathies include heart failure and/or fatal arrhythmia (Semsarian et al. 2015). Generally, incidence of cardiomyopathy in the general population has been estimated to be at least 1:500 (Maron and Maron 2013) and possibly as high as 1:200

(Semsarian et al. 2015), and the percentage of cardiomyopathy increased by almost 27% during 10 years from 2005 to 2015 (Vos et al. 2016).

There are three major types of cardiomyopathy: dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), and restrictive cardiomyopathy (RCM). Characterized by dilated left ventricle and depressed contractility, with normal or decreased ventricular wall thickness (Fig. 1), DCM is considered the most common type, with an estimated prevalence of 1 per 2500 up to 1 per 250–400 individuals, but the vast majority is ischemic and not genetic (Hershberger et al. 2013; Jefferies and Towbin 2010; Towbin et al. 2006; Weintraub et al. 2017). Although DCM-acquired causes include infectious, environmental, and systematic factors, 25–35% of DCM cases are due to familial genetic variants (Luk et al. 2009; Maron et al. 2006). The seriousness of manifestations may vary from being asymptomatic to having acute heart failure (Choudhry et al. 2019). Ventricular dilation elevates end-diastolic pressure, which in turn contributes to progressive ventricular dilation, responsible for systolic dysfunction (Reichart et al. 2019). Severity of structural and functional abnormalities exhibited along with DCM progression further results in heart failure and, subsequently, may eventually necessitate heart transplant (Hershberger et al. 2013; Kirk et al. 2009; Weintraub et al. 2017).

HCM is the most common inherited heart disease (Maron et al. 2012), of which the estimated prevalence may be as high as 1:200 (Semsarian et al. 2015). According to both European Society of Cardiology (ESC) and American Heart Association (AHA) guidelines, HCM is typically characterized by changes in the shape of the heart such as abnormally increased wall thickness (Fig. 1) (Elliott et al. 2014; Gersh et al. 2011a, 2011b), which causes decrease of ventricular volume as well as end-diastolic pressure, yet systolic function is normal or even increased. Similar to DCM, individuals presenting with HCM can be asymptomatic (Brieler et al. 2017). It is reported as the most common cause of sudden death in the young, including athletes (Maron et al. 2006). During the past decades, multiple variants of genes expressing sarcomere proteins were detected, proving that HCM is a genetically heterogeneous disease (Wolf 2019). Thus, family history is always important for the diagnosis of HCM.

In contrast to DCM and HCM, RCM is much more rare and accounts for 5% of all cardiomyopathy cases (Brown et al. 2020). RCM can be characterized by increased myocardial stiffness which causes reduced ventricular filling and leads to diastolic dysfunction (Muchtar et al. 2017). Patients with RCM may exhibit clinical manifestations ranging from mild to severe, where the patient may need an implantable cardioverter defibrillator and ultimately a heart transplant due to eminent risk of sudden death (Webber et al. 2012; Wilkinson et al. 2010; Wittekind et al. 2019). The causes of RCM can be inherited or acquired, but most cases are acquired (Muchtar et al. 2017). Most RCM cases are due to infiltration or storage of abnormal substances in myocardium or fibrotic injury, and three leading causes include amyloidosis, cardiac sarcoidosis, and hemochromatosis (Costabel et al. 2017).

In addition to the three major types of cardiomyopathy described above, there are some uncommon types of cardiomyopathy. One example is left ventricular non-compaction cardiomyopathy (LVNC), which is recognized as a primary cardiomyopathy (Maron et al. 2006). It is characterized by left ventricular trabeculation, deep intertrabecular recesses and non-compacted myocardium (Jenni et al. 2007). LVNC results from failure of compaction of loose myocardial meshwork during fetal development (Samsa et al. 2013). Singh and Patel (2020) estimated the prevalence of LVNC to be between 0.05% and 0.24%, with 22% to 38% of patients showing biventricular involvement. Patients with LVNC can present with a variety of manifestations including

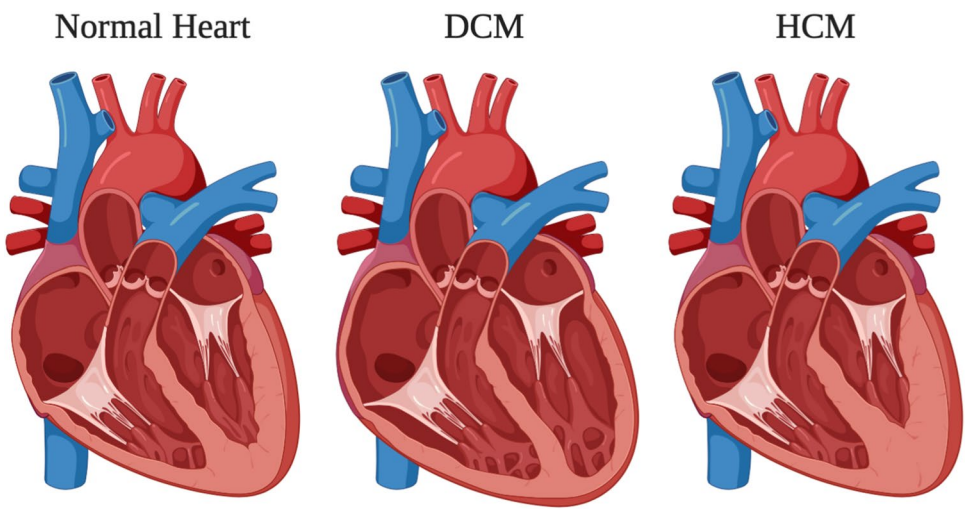

Fig. 1 Coronal section diagrams of the human heart in different pathological conditions. Hearts with: normal morphology (left); DCM (middle) with dilated ventricles; and HCM (right) with hypertrophic myocardium, interventricular septal thickening, and decreased left ventricular volume. Images generated with BioRender.com

arrhythmias, thromboembolism and even heart failure (Ikeda et al. 2015).

TNNC1 is among the genes associated with sarcomeric cardiomyopathy (Marques and de Oliveira 2016; Tadros et al. 2020; Willott et al. 2010; Yotti et al. 2019). *TNNC1* variants are found to be enriched in DCM (Mazzarotto et al. 2020) and also have association with HCM (Ingles et al. 2019; Pua et al. 2020). According to a comprehensive statistical analysis by Tadros et al. (2020), *TNNC1*-positive cardiomyopathy patients have a relatively severe prognosis and early onset in comparison with the other two subunits of Tn. However, as a rare-variants associated gene, *TNNC1* has limited diagnostic value, so patients do not have access to such genomic diagnosis. Additionally, some patients with genetic variants are now more likely to pass those variants on to the next generation because of elongated lifespan, since mortality has been reduced due to improvements in disease management (Richards and Garg 2010). Thus, *TNNC1* variants may not be as rare as initially believed. Because of these advancements and multi-generational access, it is suggested that genetic testing be done on the family member with the highest severity of phenotype in order to confidently identify pathogenic variant(s) (Musunuru et al. 2020). Studying rare variants in *TNNC1* helps to understand the underlying molecular/cellular mechanisms, and ultimately should pave the way for developing novel therapies to target the contractile apparatus, which may provide opportunities to broadly treat cardiac dysfunction irrespective of etiology. Companies such as Myokardia and Cytokinetics are developing treatments that target the sarcomere, implicating such therapies as feasible. Tadros et al. (2020) analyzed variants of the three troponin subunits to identify disease-associated hotspots. Their analysis showed that *TNNC1*-positive probands had younger ages of diagnosis and poorer clinical outcomes compared to genes for other cardiac troponin subunits, but no portion of the cardiac troponin C (cTnC) sequence reached statistical significance to be identified as a distinct hotspot.

Several advances have occurred since the study period included in the analysis by Tadros et al. (2020). First, initial structures of the vertebrate cardiac thin filament with and without Ca^{2+} have been determined by cryo–EM (Oda et al. 2020; Yamada et al. 2020). Second, additional pathogenic variants in *TNNC1* have been identified and characterized (e.g., Johnston et al. 2019; Landim-Vieira et al. 2020a). And third, additional variants in *TNNC1* have appeared in genomic databases. The purpose of this review is to summarize variants in *TNNC1* including those that were identified after the cutoff date for the dataset examined by Tadros et al. (2020), and to suggest structural regions of cTnC that should be considered for further analysis. Using the Yamada et al. (2020) cryo–EM structure (Fig. 2), we sought to compile and visualize amino acids affected by *TNNC1* variants, and also cTnC residues that can be altered by post-translational modifications in the context of the thin filament, and discuss the underlying molecular and cellular mechanisms.

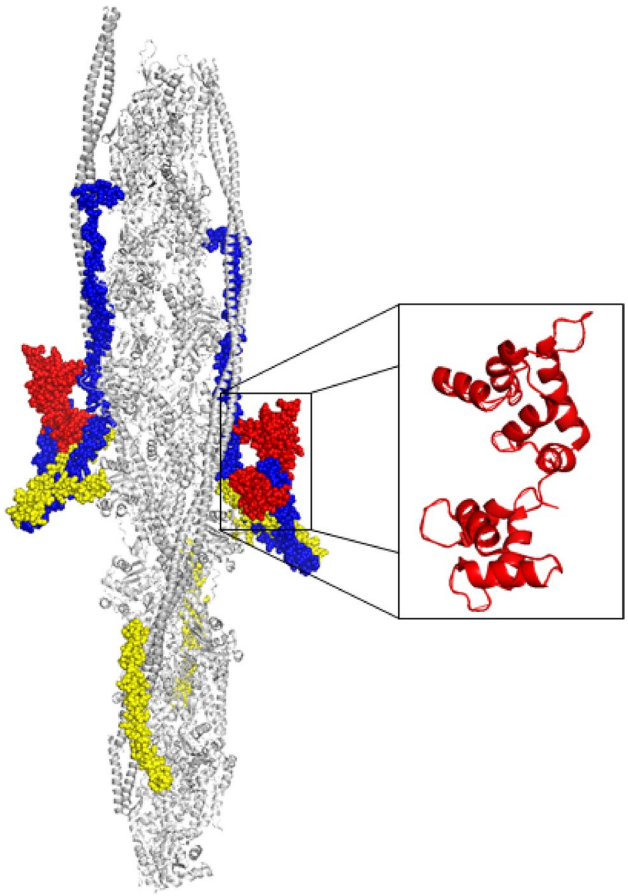

Fig. 2 Representation of a regulatory unit from the cardiac thin filament in the Ca^{2+}-free state, based on cryo–EM structure PDB 6KN7 (Yamada et al. 2020). The troponin complex (spacefill) is composed of three subunits: troponin C (cTnC, red), troponin I (cTnI, blue) and troponin T (cTnT, yellow). Tropomyosin and actin are exhibited as ribbons (gray). The top of the structure is oriented toward the pointed (minus) end, and the bottom is oriented toward the barbed (plus) end and Z-disk. Troponin C (red) is enlarged on the right. (Color figure online)

Structure and function of cTnC

Troponin C (TnC) is the expression product of either of two genes in the human genome, *TNNC1* and *TNNC2*, which are located on chromosomes 3 and 20, respectively, and that code for cTnC and fast skeletal troponin C (sTnC), respectively. *TNNC1* consists of six exons and five introns, and encodes TnC that is expressed not only in cardiomyocytes (cTnC—the primary focus of this review) but also in slow skeletal muscle (Katrukha 2013). cTnC is comprised of 161 amino acids (Roher et al. 1986) that fold into

a dumbbell-shape with two globular domains. The two domains of TnC are connected by a central segment (D–E linker) (Sia et al. 1997; Slupsky and Sykes 1995). In crystal structures of the troponin core domain, cTnC displays a flexible, intrinsically disordered D–E linker, while sTnC presents a rigid, well-ordered α-helical linker (Takeda et al. 2003; Vinogradova et al. 2005). The two globular domains of TnC are referred to by their NH_2- and COOH-terminal locations in the primary sequence. Beyond location, the two domains are distinguished by their structural and functional roles in the thin filament. cTnC exhibits predominantly α-helical content. In addition to the first α-helix (N–helix), TnC has two α-helix-containing EF-hand divalent cation-binding sites in each of the two globular domains, with different affinities and selectivity for Ca^{2+} versus Mg^{2+}.

Vertebrate striated muscle contraction is triggered by Ca^{2+} binding to the NH_2-terminal domain of TnC (Gordon et al. 2000). Both NH_2-terminal domain EF-hands (sites I and II) in sTnC bind Ca^{2+} during a cytoplasmic Ca^{2+}-transient to activate contraction. In contrast, site I of cTnC is inactive as a result of evolutionary selection. Therefore, cTnC has only site II for Ca^{2+} regulation of contraction. In both cTnC and sTnC, Ca^{2+} binding to the regulatory NH_2-terminal domain leads to conformational changes—initially in TnC, followed by conformational changes within troponin, and then the thin filament—that are ultimately responsible for removal of inhibition of actomyosin interactions (Oda et al. 2020; Yamada et al. 2020). Specifically, as cytosolic Ca^{2+} levels increase, Ca^{2+} binding to sites I and II of the NH_2-terminal domain of sTnC reveals a hydrophobic pocket in the NH_2-terminal domain (Slupsky and Sykes 1995) while in contrast, Ca^{2+} binding to site II of cTnC's NH_2-terminal domain primes the NH_2-terminal domain for opening, but without fully exposing the corresponding hydrophobic pocket (Sia et al. 1997). In both sTnC and cTnC, the α-helical switch peptide of TnI's COOH-terminal domain ultimately binds the hydrophobic pocket of TnC's NH_2-terminal domain in the presence of Ca^{2+}. Consequently, the C-terminal mobile domain of TnI is pulled away from actin, allowing tropomyosin to shift across the actin surface (Yamada et al. 2020), exposing myosin-binding sites on actin subunits and enabling the formation of cross-bridges when myosin motor domains bind.

TnC sites III and IV are located in the COOH-terminal domain and are generally thought not to participate directly in activation of contraction. Compared with NH_2-terminal site II in cTnC and sites I and II in sTnC, sites III and IV in both cTnC and sTnC bind Ca^{2+} with higher affinity but less selectivity because Mg^{2+} can also bind under physiological conditions. Divalent cation binding at sites III and IV stabilizes binding of an α-helical segment within TnI's NH_2-terminal portion at TnC's COOH-terminal domain; in other words, in both cardiac and skeletal troponin, TnI and TnC bind each other in an antiparallel, or head-to-tail fashion (Takeda et al. 2003; Vinogradova et al. 2005). In the relaxed state (e.g., low Ca^{2+} levels during cardiac diastole), sites III and IV are thought to be occupied primarily by Mg^{2+} (Potter and Gergely 1975) although it is not certain what fraction of these COOH-terminal sites might be occupied by Ca^{2+}, especially in cTnC when heart rate changes (Badr et al. 2016; Fuchs and Grabarek 2011).

As described above, cTnC plays a critical role in the conformation changes that occur on the thin filament in order to regulate the transition between on and off states of the regulatory units and, consequently, cardiac muscle contraction. A very useful model of Ca^{2+}-regulation of striated muscle contraction is the three-state model proposed by McKillop and Geeves (1993). According to this model, the thin filament is not simply switched on and off. Instead, there is a dynamic equilibrium of three states: blocked (B), closed (C), and open (M) states. During diastole, the cardiac thin filament is thought to be in mainly in the blocked state. Upon Ca^{2+} binding to site II, a series of thin filament conformational changes result in the formation of a weak contact between thin and thick filament (closed state). This interaction moves tropomyosin towards the groove of the F-actin and fully allows myosin to strongly interact with actin and generate force (open state). Once cytosolic Ca^{2+} levels decrease as Ca^{2+} is taken up by the sarcoplasmic reticulum, a new series of conformational changes happen at the thin filament returning it back to a closed and blocked state. While the McKillop and Geeves (1993) model was formulated from biochemical data, its significance was enhanced by being generally consistent with structural data (Pirani et al. 2005; Poole et al. 2006; Risi et al. 2017). Variants in cTnC can alter the dynamics of troponin subunit interactions. For example, cTnC-G159D has been reported to disrupt cTnI-cTnC interaction, preventing a normal shift in the open/closed cTnC conformation and resulting in blunted phopho-cTnI desensitization (Biesiadecki et al. 2007).

Since the recognition of *TNNC1* as a cardiomyopathy-associated gene, numerous functional studies have been published exhibiting consistent patterns of Ca^{2+} sensitization and desensitization for HCM and DCM, respectively (summarized in Tadros et al. 2020). However, the mechanism(s) by which the variants throughout cTnC can disturb Ca^{2+} binding to site II is unknown. That is because there are disparate cases, where some variants located at or near site II could directly affect Ca^{2+} binding (e.g., Miszalski-Jamka et al. 2017), while other variants located in the COOH-terminal domain, i.e., distant from site II, can also disturb Ca^{2+} binding to the NH_2-terminal domain allosterically (e.g., Landim-Vieira et al. 2020a; Miszalski-Jamka et al. 2017; Pinto et al. 2009, 2011; Ploski et al. 2016). Such altered NH_2-domain response observed in the presence of D145E has been shown to prevent Ca^{2+} binding to site IV and to

increase Ca^{2+} binding to site II allosterically (Swindle and Tikunova 2010). Interestingly, even though D141A has also been reported to abolish Ca^{2+} binding to site IV, no significant effect on myofilament Ca^{2+} sensitivity was observed (Negele et al. 1992).

Influencing myofilament Ca^{2+} sensitivity is not only a consequence of amino acid variants. TnC-targeting small molecules which can bind to either the NH_2- or COOH-terminal domains have been reported to disturb Ca^{2+} binding to site II. In these cases, Ca^{2+} sensitization can be achieved by direct binding of the molecule to the NH_2-terminal domain or by COOH-terminal binding which can cause an allosteric NH_2-terminal response. As an example of a Ca^{2+} sensitizer molecule, bepridil has been reported to act similarly to variants located in the NH_2-terminal domain of cTnC. This NH_2-terminal domain-binding molecule (Wang et al. 2002) was shown to influence myofilament Ca^{2+} sensitivity in addition to affecting both the number and kinetics of actomyosin cross-bridges (Gonzalez-Martinez et al. 2018). On the other hand, a Ca^{2+} sensitizer drug MCI-154 that binds to the COOH-terminal domain of cTnC elicits an allosteric NH_2-domain response by increasing Ca^{2+} binding to site II (Li et al. 2018). The COOH-terminal domain of cTnC has been also described as a target for desensitizing molecules. For example, (-)-epigallocatechin-3-gallate (EGCg) has been reported to bind to the COOH-terminal domain as a Ca^{2+} desensitizer with positive results on reversing diastolic dysfunction detected in mouse models of cardiomyopathy (Friedrich et al. 2016; Warren et al. 2015).

Genetic variation in human *TNNC1*

Cardiomyopathy associated variants in Ca^{2+}-regulatory proteins of the cardiac thin filament—including all three subunits of cardiac troponin—have been identified but are rare in comparison with the number of variants in thick filament proteins (Tardiff 2011; Willott et al. 2010; Yotti et al. 2019). This relative rarity of pathogenic variants in cardiac troponin is generally thought to be related to the high degree of evolutionary conservation of sequences, particularly for cTnC, and may derive from the severe outcomes at an early age (i.e., before reproduction) resulting in genetic variants not being passed on to new generations.

Sites that produce genetic variations in human *TNNC1* can be easily mapped using a cryo–EM structure of the cardiac thin filament (PDB 6KN7; Yamada et al. 2020) in which troponin is based on an X-ray crystal structure PDB 4Y99 (Takeda et al. 2003). The functionally identified variants here are A8V, L29Q, A31S, C84Y, Q122AfsX30, E134D, N144D, D145E (HCM/RCM); I4M, Y5H, G34S, Q50R, E59D, D75Y, E94V, M103I, D132N, I144D, D145E, I48V, G159D (DCM); and D62N, M81I, E94A, R102C (LVNC) (Table 1, Figs. 3, 4 and 5). Regardless of the type of cardiomyopathy, there is no distinct pattern as to where these variants are located in cTnC considering that the variants identified are found in either the NH_2-terminal or COOH-terminal. Beyond these variants, there are many others which have not been characterized as having cardiomyopathy associations (Table 1). With this being said, there is a distinct possibility of false negatives in Table 1 with regard to disease casualty, as the majority of variants have not been experimentally studied in detail. Using the American College of Medical Genetics and Genomics (ACMG) criteria, these variants are able to be categorized as pathogenic or putative (Richards et al. 2015). The pathogenic variants included in this analysis are A8V, A31S, A31T, M47I, P54H, C84Y, D88N, L97Q, D149G, G159D (Table 1). Variants that are pathogenic but have no clinically classified cardiomyopathies within this list are A31T, M47I, P54H, L97Q, D149G (Table 1).

TNNC1 is a gene that has relatively many variants (115) compared to other genes with its same transcript size (483 bases that code for amino acids) (Watkins et al. 2019). Multiple variants associated with one amino acid were counted as distinct. For example, the Aspartic acid on the second residue has two variants, D2N, and D2G, so this is counted as two variants not one (Table 1). Of the 161 amino acids in the primary sequence of cTnC, there are 77 (47.8%) with at least one identified variant (Table 1). Among these 77 affected residues, variants at 42 residues (26.1%) have been identified as significantly impacting the normal physiology (structure and function) of the heart, culminating in cardiomyopathy-related phenotypes. Not all of these variants have been functionally studied although there is evidence of disease association in patients (Table 1). Further categorization of variants associated with HCM/RCM, DCM, HCM/DCM, and LVNC are 3.7%, 7.5%, 15.5%, 2.5%, respectively. This analysis does not take into account the variant's level of pathogenicity. A conclusion can be drawn that there are more HCM/DCM-inducing variants in cTnC than other cardiomyopathy phenotypes. By structurally observing the location of the functionally studied variants in cTnC, 15 variants are located within α-helical regions and 10 are located on the linkers, within both NH_2- and COOH-terminal domains (Figs. 3, 4 and 5). Microsoft Excel's χ^2 function was used to statistically examine this observation. Even though this trend suggests that cardiomyopathy inducing variants might be more likely to be located within α-helical regions, this is a clear reflection of the proportion of cTnC structure comprised of α-helices $\chi^2\,(1, N=27)=0.124$, $p=0.72$.

Table 1 Location of amino acid variants in cTnC due to variants in *TNNC1*, regardless of their clinical significance

Variant	Cardio-myopathy association (if specified)	Source				
		Literature reference (Pubmed April 2020)	GnomAD (w/ version provided)	GnomAD MAF	ClinVar[D149] (w/ classification, if present)	Commercial submitter
D2N	DCM, HCM		No		Uncertain Significance	Yes
D2G			No		Uncertain Significance	Yes
I4M	DCM	(Johnston et al. 2019)	No		No	No
Y5H[a]	DCM	(Pinto et al. 2011)	No		No	No
Y5X			Yes (v 3)	6.97993E-06	No	No
K6Q	DCM	(Mazzarotto et al. 2020)[+]	No		No	No
A8V	HCM, RCM[b]	(Landstrom et al. 2008; Pinto et al. 2009; Ploski et al. 2016)	Yes (v 2.1.1) Yes (v 3)	4.80908E-06 6.98529E-06	Conflicting interpretations of pathogenicity	Yes
V9I	DCM		No		Uncertain Significance	Yes
V9G	HCM, DCM		No		Uncertain Significance	Yes
T13K			Yes (v 3)	6.97817E-06	No	No
K21T			Yes (v 2.1.1)	4.01126E-06	No	No
D25N			No		Uncertain Significance	Yes
L29Q	HCM	(Dweck et al. 2008; Hoffmann et al. 2001; Schmidt-mann et al. 2005)	Yes (v 2.1.1) Yes (v 3)	3.98213E-06 6.97778E-06	No	No
A31S	HCM, RCM	(Parvatiyar et al. 2012; Vasilescu et al. 2018)	No		Pathogenic	No
A31T			Yes (v 2.1.1) Yes (v 3)	3.18654E-05 6.97817E-06	Pathogenic	Yes
E32K	DCM, HCM		No		Uncertain Significance	Yes
D33N			No		Uncertain Significance	Yes
G34S	DCM	(Budde et al. 2019)				
M47R	DCM, HCM		No		Uncertain Significance	Yes
M47I			No		Likely Pathogenic	Yes
Q50R	DCM	(van Spaendonck-Zwarts et al. 2010)	No		No	No
N51K			No		Uncertain Significance	Yes
P52R	DCM, HCM		No		Uncertain Significance	Yes
T53A	DCM, HCM		No		Uncertain Significance	Yes
P54H			No		Likely pathogenic	Yes, with 1 academic lab submitter
E55RfsTer6			Yes (v 2.1.1)	3.97886E-06	No	No
E59D[c]	DCM	(Dweck et al. 2010; Lim et al. 2008)	No		No	No

Table 1 (continued)

Variant	Cardio-myopathy association (if specified)	Source				
		Literature reference (Pubmed April 2020)	GnomAD (w/ version provided)	GnomAD MAF	ClinVar[D149] (w/ classification, if present)	Commercial submitter
M60I			Yes (v 2.1.1) Yes (v 3)	3.18593E-05 6.97798E-06	No	No
D62N	LVNC	(Miszalski-Jamka et al. 2017)	No		Uncertain Significance	Yes
D62E	DCM, HCM		No		Uncertain Significance	Yes
E63D	DCM, HCM		No		Uncertain Significance	Yes
E63X			Yes (v 2.1.1) Yes (v 3)	3.18573E-05 6.97652E-06	No	No
V64A			No		Uncertain Significance	Yes
D65G			Yes (v 3)	6.97915E-06	No	No
G68S			Yes (v 2.1.1)	3.98337E-06	No	No
S69R	DCM, HCM		No		Uncertain Significance	Yes
G70S			Yes (v 2.1.1) Yes (v 3)	1.59137E-05 6.98041E-06	No	No
F74C			Yes (v 3)	6.98149E-06	No	No
D75Y[d]	DCM	(Dweck et al. 2010; Lim et al. 2008)	No		No	No
D75E			Yes (v 2.1.1)	7.95456E-06	No	No
M80K			Yes (v 3)	6.98275E-06	No	No
M81I	LVNC	(Takasaki et al. 2018)	Yes (v 2.1.1)	2.3861E-05	No	No
M81L	Uncertain Significance (possible DCM)	(Carnevale et al. 2020)	No		No	No
M81T			Yes (v 2.1.1) Yes (v 3)	7.95336E-06 1.39677E-05	Uncertain Significance	Yes
R83Q			Yes (v 2.1.1) Yes (v 3)	7.95336E-06 6.98227E-06	No	No
R83W	DCM	(Mazzarotto et al. 2020)*	No		No	No
C84Y	HCM	(Landstrom et al. 2008; Pinto et al. 2009)	No		Pathogenic	No
D88N	DCM, HCM		No		Conflicting interpretations of pathogenicity	Yes
D88Y			No		Uncertain Significance	Yes
G91R	DCM, HCM		No		Uncertain Significance	Yes
G91E			Yes (v 2.1.1)	3.97674E-06	No	No
K92R			Yes (v 2.1.1)	7.95361E-06	No	No
E94A	LVNC	(Takasaki et al. 2018)	No		No	No
E94Q			No		Uncertain Significance	Yes

Table 1 (continued)

Variant	Cardio-myopathy association (if specified)	Source				
		Literature reference (Pubmed April 2020)	GnomAD (w/ version provided)	GnomAD MAF	ClinVar[D149] (w/ classification, if present)	Commercial submitter
E94V	DCM	(Franaszczyk et al. 2020)	No		No	No
L97Q			No		Likely pathogenic	Yes
D99N			Yes (v 2.1.1)	3.97731E-06	No	No
D99V			Yes (v 2.1.1)	3.97728E-06	No	No
L100I			Yes (v 2.1.1)	7.9545E-06	No	No
R102C	LVNC, DCM	(Takasaki et al. 2018) (Mazzarotto et al. 2020)+	Yes (v 2.1.1) Yes (v 3)	1.19325E-05 6.98178E-06	Uncertain Significance	Yes
R102L			Yes (v 2.1.1)	7.95532E-06	Uncertain Significance	Yes
R102H			Yes (v 2.1.1) Yes (v 3)	1.39636E-05 7.95532E-06	No	No
M103I	DCM	(Pinto et al. 2011)	No		No	No
M103L			Yes (v 2.1.1) Yes (v 3)	3.97769E-06 6.98041E-06	No	No
D105H			Yes (v 3)	6.97963E-06	No	No
K106Q			Yes (v 2.1.1)	3.97785E-06	No	No
K106NfsTer21			Yes (v 2.1.1)	1.9917E-05	No	No
N107S			Yes (v 2.1.1)	3.18756E-05	No	No
G110C			No		Uncertain Significance	Yes
I112F			Yes (v 2.1.1)	3.98159E-06	No	No
I112M			Yes (v 2.1.1)	3.98194E-06	No	No
D113N	DCM, HCM		Yes (v 2.1.1) Yes (v 3)	7.9635E-06 1.39653E-05	Uncertain Significance	Yes
D113E			Yes (v 3)	6.97915E-06	No	No
D113TfsTer5			Yes (v 2.1.1)	3.98178E-06	No	No
D115E			Yes (v 2.1.1)	7.96254E-06	No	No
D115del			Yes (v 3)	6.97788E-06	No	No
E116D	DCM, HCM		No		Uncertain Significance	Yes
I119V			No		Uncertain Significance	Yes
I119T	DCM, HCM		Yes (v 2.1.1) Yes (v 3)	3.98096E-06 6.98188E-06	Uncertain Significance	Yes
M120I			Yes (v 2.1.1)	3.98115E-06	No	No
Q122AfsX30[e]	HCM	(Chung et al. 2011)	No		No	No
E126K			Yes (v 2.1.1) Yes (v 3)	3.98175E-06 6.98022E-06	Uncertain Significance	Yes
E126Q			Yes (v 2.1.1)	3.98175E-06	No	No
I128T	DCM, HCM		No		Uncertain Significance	Yes
T129M	DCM	(Mazzarotto et al. 2020)*	Yes (v 2.1.1) Yes (v 3)	3.98279E-06 6.98129E-06	Uncertain Significance	Yes
D131E			Yes (v 2.1.1) Yes (v 3)	3.98346E-06 6.98158E-06	Uncertain Significance	Yes

Table 1 (continued)

Variant	Cardio-myopathy association (if specified)	Source				
		Literature reference (Pubmed April 2020)	GnomAD (w/ version provided)	GnomAD MAF	ClinVar[D149] (w/ classification, if present)	Commercial submitter
D132N[f]	DCM[g]	(Landim-Vieira et al. 2020a) (Mazzarotto et al. 2020)*	No		Uncertain Significance	Yes
D132H			Yes (v 2.1.1)	3.98333E-06	No	No
D132del	DCM, HCM		No		Uncertain Significance	Yes
I133V	DCM, HCM		Yes (v 2.1.1) Yes (v 3)	3.9833E-06 6.97778E-06	Uncertain Significance	Yes
E134D	HCM	(Landstrom et al. 2008; Pinto et al. 2009)	No		Uncertain Significance	Yes
E134K	DCM, HCM		No		Uncertain Significance	Yes
E135K			Yes (v 2.1.1)	3.98362E-06	No	No
M137T			Yes (v 2.1.1)	3.98384E-06	No	No
G140E	DCM, HCM		Yes (v 2.1.1) Yes (v 3)	1.59398E-05 6.97895E-06	Uncertain Significance	Yes
G140R			Yes (v 2.1.1)	1.19541E-05	No	No
K142N	DCM, HCM		Yes (v 2.1.1)	3.98549E-06	Uncertain Significance	Yes
N144D	DCM, HCM	(Lu et al. 2018)	Yes (v 3)	1.39597E-05	Uncertain Significance	Yes
N144S			Yes (v 2.1.1) Yes (v 3)	1.99287E-05 6.98227E-06	Uncertain Significance	Yes
N144del	DCM, HCM		No		Uncertain Significance	Yes
D145E	HCM, DCM[h], RCM[i], Left Ventricle Hypertra beculation	(Landim-Vieira et al. 2020a; Miszalski-Jamka et al. 2017; Pinto et al. 2009, 2011; Ploski et al. 2016; Swindle and Tikunova 2010)	Yes (v 2.1.1) Yes (v 3)	3.98651E-06 7.67556E-05	Uncertain Significance	Yes
D145N	DCM, HCM		Yes (v 2.1.1)	1.19591E-05	Uncertain Significance	Yes
G146S			Yes (v 2.1.1) Yes (v 3)	1.4173E-05 2.09418E-05	No	No
R147C			Yes (v 2.1.1) Yes (v 3)	1.19602E-05 1.39593E-05	No	No
R147H			Yes (v 2.1.1)	3.9873E-06	No	No
I148V	DCM	(Pinto et al. 2011) (Mazzarotto et al. 2020)*	Yes (v 2.1.1) Yes (v 3)	7.97327E-06 1.39612E-05	Uncertain Significance	Yes
D149N	DCM	(Mazzarotto et al. 2020)*	No		Uncertain Significance	Yes
D149G			No		Likely pathogenic	Yes
D149Y			Yes (v 2.1.1) Yes (v 3)	7.08843E-06 6.98002E-06	No	No
D151V	DCM, HCM	(Mazzarotto et al. 2020)*	No		Uncertain Significance	Yes

Table 1 (continued)

Variant	Cardio-myopathy association (if specified)	Source				
		Literature reference (Pubmed April 2020)	GnomAD (w/ version provided)	GnomAD MAF	ClinVar[D149] (w/ classification, if present)	Commercial submitter
M157L	DCM, HCM		Yes (v 2.1.1) Yes (v 3)	3.98295E-06 6.9849E-06	Uncertain Significance	Yes
K158T	DCM, HCM		No		Uncertain Significance	Yes
K158N			Yes (v 2.1.1)	3.98371E-06	No	No
G159D	DCM	(Mogensen et al. 2004)	No		Likely pathogenic	Yes
G159S			Yes (v 2.1.1)	3.98384E-06	No	No
V160M			Yes (v 2.1.1)	3.98403E-06	No	No

All variants noted in GnomAD are considered rare by the MAF cutoff of less than 1E-04 and classified using ClinVar for their clinical significance. Databases (https://www.uniprot.org/, https://www.ncbi.nlm.nih.gov/clinvar/, https://gnomad.broadinstitute.org/) queried April 2020

[*]excluded from mentioned study by quality control tool

[+]passed study quality control

[a]cTnC,Y5H variant proband also had a myosin heavy chain variant MYH7,R1054C (per Tadros et al. 2020)

[b]One RCM proband was compound heterozygous with both cTnC,A8V and cTnC,D145E variants (Ploski et al. 2016)

[c]Compound heterozygous proband cTnC(E59D,D75Y); tested separately in vitro, cTnC,E59D data are consistent with it being benign on its own, while cTnC,D75Y on its own recapitulates the disease phenotype (Dweck et al. 2010; Lim et al. 2008)

[d]cf. ante

[e]Frameshift mutation (c.363dupG or p.Gln122AlafsX30) (Chung et al. 2011)

[f]In one family, cardiomyopathy was associated with compound heterozygous variants cTnC,D132N and cTnC,D145E, but not the individual variants on their own (Landim-Vieira et al. 2020a)

[g]cf. ante

[h]cf. ante. Also note that one cTnC,D145E proband with DCM also had a variant in MyBP-C,P910C (per Tadros et al. 2020)

[i]One RCM proband was compound heterozygous with both cTnC,A8V and cTnC,D145E variants (Ploski et al. 2016)

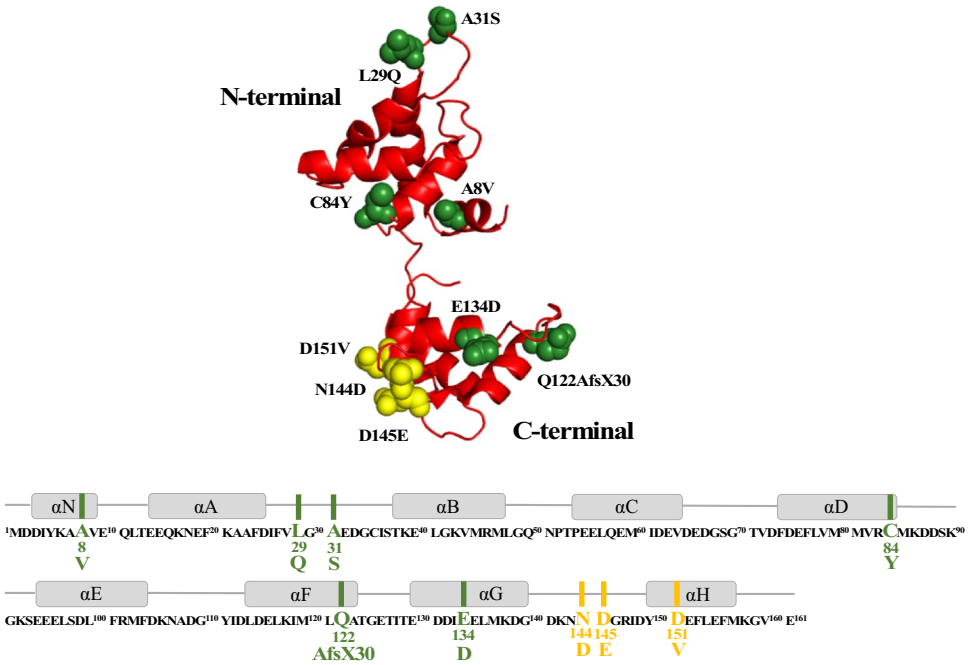

Fig. 3 (Top) PyMOL 3D structure of cTnC (red) showing amino acid variants (spheres) in cTnC (ribbon) that have been published in peer-reviewed journals and been associated with either HCM/RCM (green) or HCM/DCM (yellow) cardiomyopathies. From PDB 6KN7 (Yamada et al. 2020) as illustrated in Fig. 1. (Bottom) Representation of cTnC primary sequence and secondary structure regions with variants highlighted with their respective colors

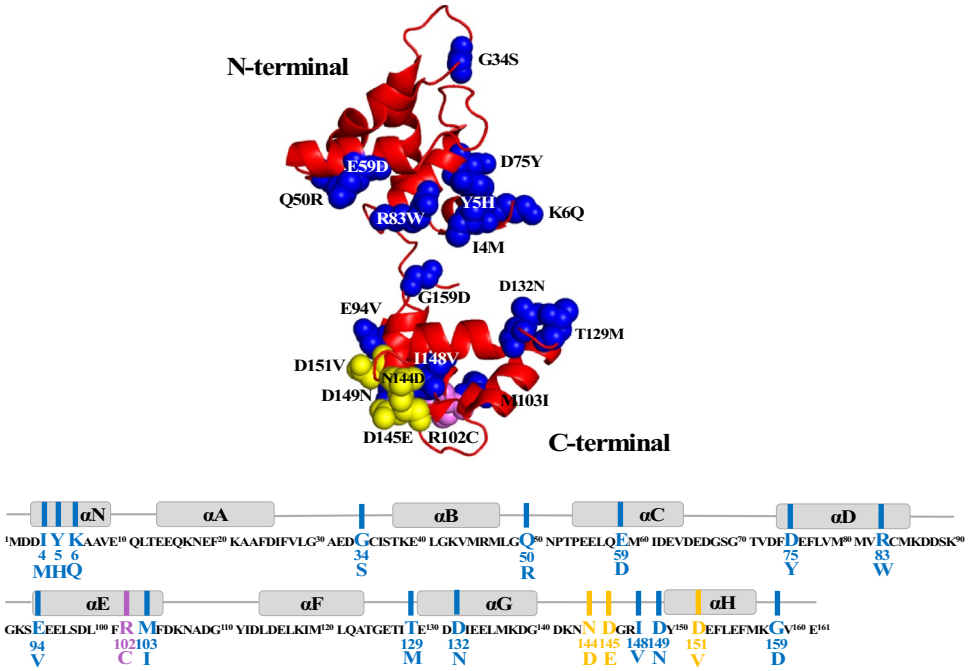

Fig. 4 (Top) PyMOL 3D structure of cTnC (red) showing amino acid variants (spheres) in cTnC (ribbon) that have been published in peer-reviewed journals and been associated with either DCM (blue), HCM/DCM (yellow), or LVNC/DCM (violet) cardiomyopathies. From PDB 6KN7 (Yamada et al. 2020) as illustrated in Fig. 1. (Bottom) Representation of cTnC primary sequence and secondary structure regions with variants highlighted with their respective colors

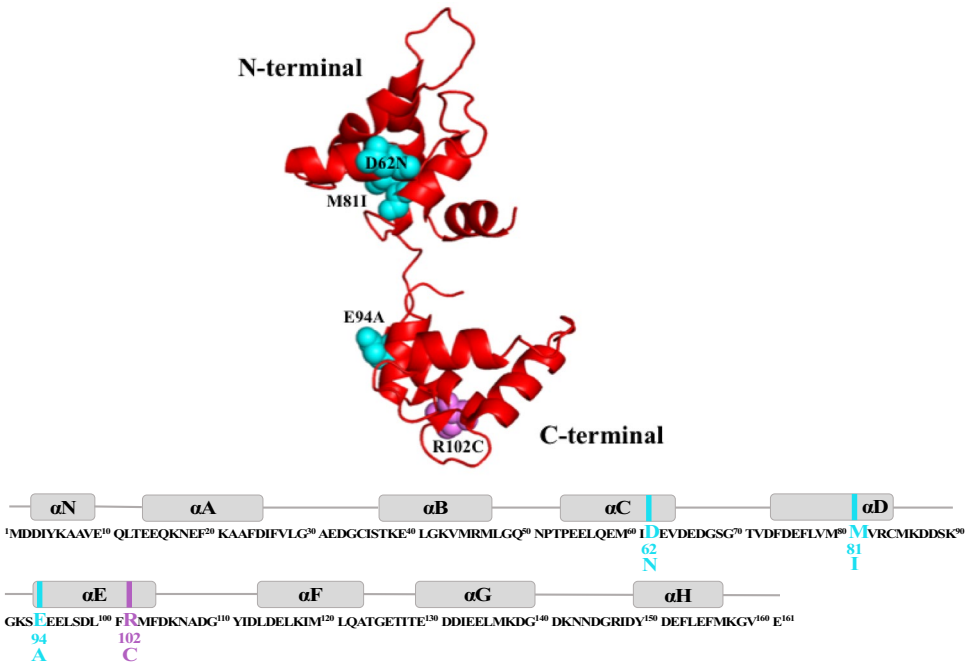

Fig. 5 (Top) PyMOL 3D structure of cTnC showing amino acid variants (spheres) in cTnC (ribbon) that have been published in peer-reviewed journals and been associated with either LVNC (cyan) or LVNC/DCM (violet) cardiomyopathies. From PDB 6KN7 (Yamada et al. 2020) as illustrated in Fig. 1. (Bottom) Representation of cTnC primary sequence and secondary structure regions with variants highlighted in cyan

Mechanisms of *TNNC1* cardiomyopathies

Post-translational modifications (PTMs) in cTnC

A post-translational modification (PTM) in the context of proteins occurs when functional groups get temporarily or permanently incorporated into consensus regions via covalent mechanisms as enzymatic and non-enzymatic processes that are fundamental to protein functionality (Harmel and Fiedler 2018; Müller 2018). Through biological systems, PTMs have been reported to be extensively important in maturation of synthesized proteins, signal transduction, and metabolism (Duan and Walther 2015). PTMs are known to cause a variety of outcomes *in vitro* and in vivo on a physiological level (Wang et al. 2014).

The PTMs included in this study include acetylation, glycation, S-nitrosylation, and phosphorylation (Table 2,

Table 2 Locations of post-translational modifications (PTMs; acetylation, glycation, s-nitrosylation and phosphorylation) of cTnC

Amino acid	PTM	Animal	Source(s)
M1	N-acetylmethionine	Human	(Roher et al. 1986); UniProt; NCBI Reference Sequence: NP_003271.1
K6	Glycation (CML = Nε-carboxymethyllysine)	Human	(Janssens et al. 2018)
K17	Lysine Acetylation	Rat	(Lundby et al. 2012)
C35	S-nitrosylation	Mouse	(Figueiredo-Freitas et al. 2015)
K39	Glycation (CML = Nε-carboxymethyllysine)	Human	(Janssens et al. 2018)
K43	Lysine Acetylation	Rat	(Lundby et al. 2012)
C84	S-nitrosylation	Mouse	(Irie et al. 2015)
S89	Phosphorylation	Rat	(Schlecht et al. 2014)
K92	Lysine Acetylation	Rat	(Lundby et al. 2012)
S98	Phosphorylation	Mouse (Human by similarity)	(Huttlin et al. 2010); UniProt, NCBI Reference Sequence: NP_003271.1

Databases (PhosphoSitePlus v 6.5.8: https://www.phosphosite.org/, UniProt: https://www.uniprot.org/) queried April–May 2020

Fig. 6). Acetylation of residues in cTnC is important for striated muscle contraction, as over 80% of proteins in striated muscle that are part of the contractile machinery are acetylated (Lundby et al. 2012). This lysine-targeted PTM is carried out by histone deacetylases (HDACs) and acetyltransferases (HATs), and typically involves the addition of an acetyl group onto specific sidechains within specific sequences (Table 2) (Gupta et al. 2008). This modification is responsible for increasing Ca^{2+} affinity to cTnC at low-affinity sites and high-affinity sites, by 3.5-fold, and two-fold, respectively (Grabarek et al. 1995). The acetylated M1 residue in native protein is not included in Fig. 5 as the sequence of cTnC in the Yamada et al. (2020) structure begins at D2; this does not eliminate the possibility of the existence of a modification in vivo. Residues K17, K43, and K92 in cTnC are also reported as acetylated (Table 2, Fig. 6). The irreversible glycation PTM, also known as advanced glycation end-product (AGE) modification, is thought to primarily affect cardiac proteins that are located within cells that have a high turnover rate and is observed in vitro when exposed to excess hexose sugar (Janssens et al. 2018). Physiological effects include altering the rapid cyclic dynamic movements of the troponin complex which in turn affect the association/dissociation kinetics of Ca^{2+} in cTnC. Thus the presence of a glycated amino acid residue can contribute to changes in myofilament

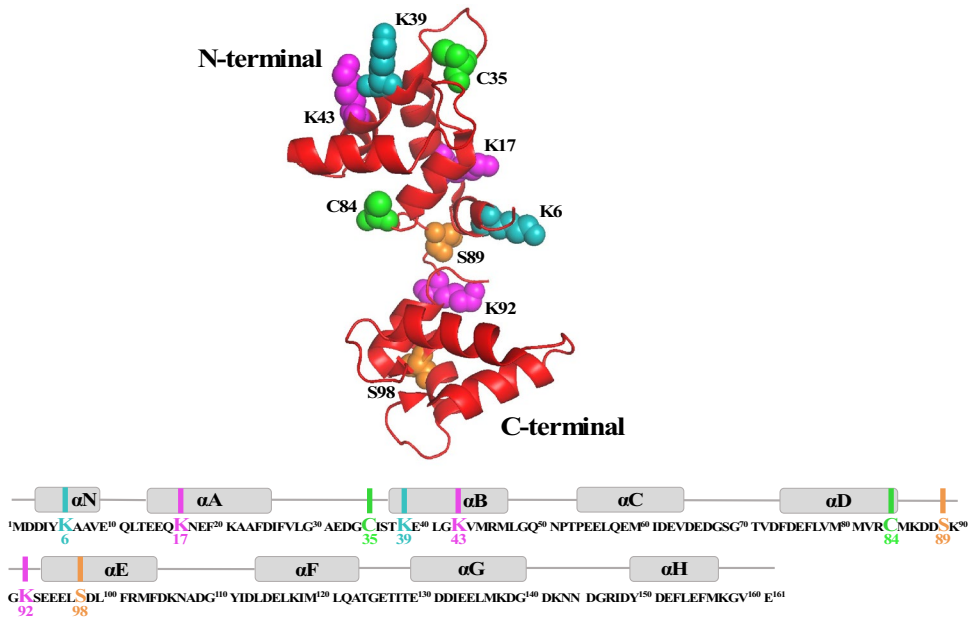

Fig. 6 (Top) PyMOL 3D structure of cTnC (red) showing post-translationally modified amino acids (spheres) in cTnC (ribbon). Acetylation is magenta, S-nitrosylation is green, phosphorylation is orange, and glycation is teal. From PDB 6KN7 (Yamada et al. 2020) as illustrated in Fig. 1. (Bottom) Representation of post-translationally modified amino acids in cTnC primary sequence and secondary structure regions with amino acids highlighted with their respective colors. PTMs were identified using UniProt, PhosphoSitePlus, and published literature (Table 1)

Ca^{2+} sensitivity and underlie heart failure and/or diabetic cardiomyopathy (Janssens et al. 2018). Residues K6 and K39 in cTnC are found to be glycated (Table 2, Fig. 6). The S-nitrosylation is completed when nitric oxide (NO) adds to thiols, and in turn alters myofilament contractility within the muscle cell. This process is accelerated under normal conditions when stress is present (Figueiredo-Freitas et al. 2015). Under physiological conditions, S-nitrosylation of cTnC desensitizes myofilaments to Ca^{2+} and reduces cross-bridge turnover (Figueiredo-Freitas et al. 2015). This modification therefore leads to reduced cardiac muscle contractility, posing physiological threats to affected individuals. The two Cys residues in cTnC, C35 and C84, are found to undergo S-nitrosylation PTM (Table 2, Fig. 6).

Another PTM with an important role in Ca^{2+} homeostasis through coordination with other PTMs is phosphorylation (Irie et al. 2015). Phosphorylation involves the introduction of a phosphate (PO_4) group to an amino acid sidechain (typically Ser, Thr or Tyr), in turn affecting the protein's conformational properties, function and interactions with other molecules (Ardito et al. 2017). Phosphorylation sites are often located in disordered regions of proteins throughout biological systems. Of the 15 Ser, Thr and Tyr residues in human cTnC, two Ser residues—S89 and S98—have been identified as targets for phosphorylation (Table 2, Fig. 6) (Huttlin et al. 2010). Decreased phosphopeptide count in heart tissue was shown to be correlated with differences in signaling and tissue heterogeneity (Huttlin et al. 2010). While relatively little is known about the physiological effects of cTnC phosphorylation, cTnI phosphorylation has received much more attention, initially because of its relevance to autonomic regulation of cardiac function (Solaro et al. 1976). Phosphorylation of cTnI at Ser-42 and Ser-44 may affect its interaction with cTnC and alter Ca^{2+} activation (Kobayashi and Solaro 2005). This alteration takes place because the modified reside in cTnI interacts with Glu-10 in cTnC, which is in the N–helix of the regulatory NH_2-terminal domain of cTnC (Kobayashi and Solaro 2005). Similarly, phosphorylation of cTnI residues Ser-23 and Ser-24 cTnI is important in regulating cardiac muscle contractility, largely through modulation of Ca^{2+} binding by cTnC (Biesiadecki et al. 2007). Although phosphorylation has been extensively studied, Ca^{2+}-dependent allosteric changes in the thin filament by phosphorylation of TnI are not fully understood (Dong et al. 2007). Furthermore, PTMs directly involving residues in cTnC can impact the regulation of Ca^{2+} binding (Grabarek et al. 1995). It will be important to continue to establish under what physiological conditions the residues in cTnC identified in Table 2 become modified and to what extent (i.e., what fraction of cTnC molecules are modified at that residue), and also what other PTMs may occur in cTnC.

Co-localization of amino acid variants and PTMs

The S-nitrosylated residue C84 in cTnC (Table 2) may also be altered by an HCM-inducing C84Y variant (Table 1) (Figueiredo-Freitas et al. 2015; Landstrom et al. 2008). The C84Y variant involves replacing a cysteine at position 84 with a tyrosine. Not only does the removal of Cys preclude the possibility of S-nitrosylation at that site, but the introduction of Tyr to the amino acid sequence introduces a new possibility for PTM. Tyrosine is commonly modified by sulfation (Yang et al. 2015) and phosphorylation (Harney et al. 2005). Considering that S-nitrosylation is known to decrease myofilament Ca^{2+} sensitivity, the replacement of C84 with Tyr increases Ca^{2+} affinity of isolated cTnC as well as Ca^{2+} sensitivity of myofilament function in the intact sarcomere (Pinto et al. 2009).

The significance of Cys residues in TnC extends beyond the naturally occurring phenomena listed in Tables 1 and 2. Grabarek et al. (1990) introduced a disulfide into sTnC via site-directed mutagenesis that inactivated the Ca^{2+}-regulatory nature of TnC. This disulfide bridge was located between the B–C linker and helix D of the central helix (Grabarek et al. 1990). In contrast, disulfide bridge formation between the native Cys residues (C35 and C84) in cTnC results in a constitutively activated cTnC (referred to as aTnC) that does not require Ca^{2+} for activation of force generation, unloaded shortening, or rapid kinetics of isometric tension redevelopment (Chase et al. 1994a, 1994b; Hannon et al. 1993; Martyn et al. 1994). This intermolecular disulfide bridge is thought to produce conformational changes that mimic some aspects of Ca^{2+}-binding; distortion of the structure of the NH_2-terminal domain would likely reduce dynamics of cTnC, could alter site II in a manner that is similar to the structure cTnC in the Ca^{2+}-bound state, and probably induces opening of the hydrophobic patch for cTnI-binding. Site-specific variants to the Cys-84 residue in cTnC (Table 1) would decrease or eliminate the likelihood of PTMs at that and nearby residues, and in turn lead to changes in the structure or function of cTnC. Characterizing PTMs in the pathogenic, HCM-inducing variant C84Y has provided, and will certainly continue to provide insights into how PTMs act on a molecular level to influence phenotypes.

The acetylated residue K92 (Table 2) also experiences a K92R variant (Table 1). The introduction of an Arg amino acid to the sequence is conservative in the sense that both Lys and Arg have basic sidechains, but it brings forward the possibility of new PTMs. Commonly, PTMs associated with Arg are classified as non-enzymatic and enzymatic. Non-enzymatic PTMs are carbonylation and advanced glycation end products (AGEs) (Slade et al. 2014). Enzymatic PTM's of Arg residues are citrullination, methylation, phosphorylation, ADP-ribosylation (Slade et al. 2014). K92 lies in the middle of the flexible DE-linker that connects the NH_2- and

COOH-terminal domains of cTnC, not far from residue C84 (Takeda et al. 2003; Yamada et al. 2020). This region is important for Ca^{2+} dependent regulation of cardiac troponin (Manning et al. 2012). Knowing this, removal or modification of a residue in this region may alter its regulatory properties. Additionally, the introduction of amino acid variants into the sequence of cTnC as discussed in this review may have the possibility to alter the likelihood of PTMs at the affected amino acid or nearby residues. This does not remove the possibility of PTMs, as PTMs are necessary for function and species complexity (Darling and Uversky 2018). At the time of writing, there are no reports on the pathogenicity of the K92R variant (Table 1). Therefore, this variant could be further clinically studied to further investigate the physiological significance of PTM at K92 (Table 2) and to determine the disease relevance of both PTMs and the variant.

Haploinsufficiency vs. dominant negative mechanism

All of the pathogenic variants in *TNNC1* identified to-date encode single or double amino acid substitutions. Therefore, mutant cTnC is expected to incorporate normally into the sarcomere, thereby causing disease via a dominant negative ('poison peptide') mechanism (Gomes and Potter 2004). This contrasts with what is observed for cardiomyopathic variants in the genes encoding myosin-binding protein C and titin, which typically produce truncating variants and elicit disease via haploinsufficiency (Yotti et al. 2019). Upon incorporation into the contractile apparatus, cTnC mutants trigger contractile dysfunction due to altered interactions with cTnT, cTnI or Ca^{2+} binding affinity, or a combination thereof. Although numerous *TNNC1* variants have been reported to be associated with cardiomyopathy (Table 1), only several have been extensively characterized structurally and functionally. The DCM-associated cTnC-G159D variant has been suggested to impair interactions with full-length cTnT and enhance interactions with full-length cTnI, as determined indirectly by a mammalian two-hybrid assay (Mogensen et al. 2004). The cTnC-G159D variant was also previously shown to decrease myofilament Ca^{2+} sensitivity and blunt the lusitropic effect of protein kinase A-mediated phosphorylation of cTnI at the N-terminal serine residues (Biesiadecki et al. 2007). The first study to directly demonstrate that cTnT binds to cTnC showed that the intrinsically disordered carboxy-terminus of cTnT directly participates in Ca^{2+}-dependent interactions with the regulatory NH_2-domain of cTnC, and that the binding affinity between these two subunits is increased in the presence of a DCM-associated variant in cTnC (I4M; Table 1) (Johnston et al. 2019). Additional DCM-associated cTnC variants (Y5H, M103I, and I148V) have been shown to decrease Ca^{2+} sensitivity of isometric tension generation (Pinto et al. 2011). It should be noted that the proband carrying the Y5H variant also had a genetic variant encoding a R1045C substitution in the β-myosin heavy chain (Pinto et al. 2011). Therefore, the relative contributions of these variants to the pathophysiology observed in this proband remain uncertain, and this lessons applies to other probands, some of which are compound heterozygous (Landim-Vieira et al. 2020a; Lim et al. 2008; Ploski et al. 2016), with variants in both cTnC and another cardiomyopathy gene (Table 1).

Altered interactions with TnI and/or TnT, or perhaps tropomyosin/actin

In contrast to DCM variants, HCM variants in cTnC tend to increase myofilament Ca^{2+} sensitivity. For example, the HCM-associated cTnC-A8V variant significantly increases Ca^{2+} sensitivity of isometric tension via direct enhancement of Ca^{2+}-binding to site II and increased binding to full-length cTnI (Gonzalez-Martinez et al. 2018; Kawai et al. 2017; Martins et al. 2015; Zot et al. 2016). Another HCM-associated variant in cTnC that leads to markedly increased myofilament Ca^{2+} sensitivity is C84Y (Landstrom et al. 2008). The double, compound heterozygous cTnC variant (E59D/D75Y) associated with DCM (Lim et al. 2008) has also been shown to decrease myofilament Ca^{2+} sensitivity and reduce strong actin-myosin binding (Dweck et al. 2010). Interestingly, the HCM-associated cTnC-D145E variant has been shown to alter Ca^{2+} binding in the C-domain of cTnC (Marques et al. 2017; Swindle and Tikunova 2010), which could alter interactions with the N-terminal region of cTnI and/or C-terminal region of cTnT. Some cTnC variants can also impact actomyosin adenosine triphosphatase (ATPase) activity (e.g., Veltri et al. 2017). For example, it has been reported that the HCM-associated cTnC-A31S variant increases Ca^{2+}-activated actomyosin ATPase activity in addition to increasing Ca^{2+} sensitivity of isometric force (Parvatiyar et al. 2012). Taken together, *TNNC1*-encoded cardiomyopathic variants generally cause myofilament dysfunction via disruption of Ca^{2+}-binding and/or altered interactions with other troponin subunits.

It is now evident, however, that the pathogenicity of *TNNC1* variants is not always explained by defects in sarcomere contractility. For example, the functional consequences of *TNNC1*-encoded compound heterozygous missense variants (D132N and D145E) that were identified in two siblings with early-onset DCM (Landim-Vieira et al. 2020a). These variants were inherited, one from each parent. Strikingly, we found no significant differences in the contractile parameters of permeabilized cardiac muscle preparations reconstituted with 50% D145E/50% D132N cTnC variants compared with WT (Landim-Vieira et al. 2020a). This observation raised the intriguing possibility that pathogenic

variants in the *TNNC1* alleles may cause cardiomyopathy through mechanisms beyond myofilament dysfunction.

Potential non-canonical (mitochondrial/nuclear) roles of cTnC

In a seminal study in 2009 on cardiomyocyte renewal in humans by Bergmann and colleagues from the Frisén lab, *TNNT2* and *TNNI3* were coincidentally identified for the first time as being directly associated with isolated cardiomyocyte nuclei (Bergmann et al. 2009). By immunofluorescence microscopy and immunoblotting, both subunits were unambiguously shown to be associated with human cardiomyocyte nuclei that were purified from human cardiac ventricular tissue and then sorted by immunolabeling and flow-cytometry (Bergmann et al. 2009). In a follow up study, Asumda and Chase (2012) identified all three subunits of cardiac troponin (cTnI, cTnT and cTnC) along with Tm and nuclear actin within the nuclei of cultured neonatal rat ventricular cardiomyocytes by immunofluorescence confocal microscopy. Evidence from this report also showed that nuclear expression of the myofilament proteins occurs early during cardiomyogenic differentiation of rat, multipotent, bone marrow-derived mesenchymal stem cells (Asumda and Chase 2012). Further, bioinformatics predictions were used to show that cTnI and cTnT contain putative nuclear localization signals (NLS) in both of the aforementioned studies (Asumda and Chase 2012; Bergmann et al. 2009). Some pathogenic variants may introduce or nullify a NLS, therefore potentially altering nucleocytoplasmic shuttling (Chase et al. 2013). Interestingly, using the approach previously applied to cTnC WT (Chase et al. 2013), cTnC variant V64A (Table 1) is predicted to localize to the cardiomyocyte nucleus to a much greater extent than WT or any other cTnC variant that affects a single amino acid. It remains to be tested experimentally, however, whether cTnC-V64A is in fact concentrated in cardiomyocyte nuclei.

Although the precise roles of nuclear-localized myofilament proteins in cardiomyocytes remain largely unclear, cancer cells might be able to provide some insight. Certain cancer cell lines and human tumor tissues display overexpression of troponin at the gene and protein levels (Johnston et al. 2018). Bioinformatic pathway analyses suggest that troponin subunits may play a role in the organization of chromatin, DNA repair processes, RNA metabolism, and gene transcription (Johnston et al. 2018). These results point to the intriguing possibility that myofilament proteins might have dual roles in cardiomyocytes: to regulate muscle contraction and participation in Ca^{2+}-dependent signaling in the nucleus, which could indeed have profound implications for understanding the molecular pathophysiological mechanisms of cardiomyopathies caused by thin filament variants.

In 2015, Wu and colleagues provided compelling evidence for the possibility that a cardiomyopathic variant in a troponin subunit has a regulatory role in the nuclear compartment of cardiomyocytes (Wu et al. 2015). Using induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) derived from human patients harboring a DCM-linked sarcomeric variant (cTnT-Arg173Trp), they found increased nuclear localization of cTnTArg173Trp compared to iPSC-CMs derived from non-DCM relatives (Wu et al. 2015). To ascertain the biological significance of nuclear-localized *TNNT2*, co-immunoprecipitation followed by mass-spectrometry was used to identify potential interacting proteins (Wu et al. 2015). Interestingly, they found *TNNT2* was bound, either directly or indirectly, to histone H3 and histone demethylases, among other interacting partners (Wu et al. 2015). After further correlating these observations with changes in chromatin marks, the authors proposed that *TNNT2* participates in the epigenetic regulation of phosphodiesterase (PDE) gene expression, which was altered as a result of the cTnT-Arg173Trp DCM variant (Wu et al. 2015). A more recent study also revealed that a RCM variant in cTnI might directly interact with histone deacetylase 1 and repress gene expression of phosphodiesterase 4D in mutant cardiomyocytes (Zhao et al. 2020a; Zhao et al. 2020b). These results are particularly captivating because they not only transform the way we view the pathogenesis of inherited cardiomyopathies, but also how we view myofilament proteins from a fundamental, basic science perspective. Because other thin myofilament proteins have been identified within cardiomyocyte nuclei, it would seem likely that this phenomenon is not exclusive to these specific variants in *TNNT2* and *TNNI3* or even the WT sequences. In addition to thick filament components *MYH7* and *MYBPC3*, pathogenic genetic variants may also occur in thin filament genes for *TPM1*, *TNNC1*, *TNNI3* and *TNNT2* which are linked to cardiomyopathy. The two variants demonstrated to alter gene expression raise the possibility that, in addition to disrupting the biomechanical properties of the sarcomere, they and other variants could also perturb the architecture of chromatin, the architecture of the entire nucleus, or even the not-well-understood elevation of ploidy and nuclearity of cardiomyocytes (Landim-Vieira et al. 2020b).

Future directions

Cardiac TnC is a highly conserved protein from an evolutionary viewpoint. Thus investigating the locations of the surprisingly large number of variants in the cardiomyopathic gene *TNNC1* and PTMs of cTnC is crucial to further understand the underlying mechanisms of heart disease progression. Through extensive analyses, this review categorizes all of the currently documented variants in cTnC which may

or may not cause cardiomyopathies. Additionally, compiling residues altered by amino acid variants and/or PTMs in cTnC allows us to localize the affected residues in the 3D structure. It will be important to assess the functional significance of cTnC PTMs, as their functional roles are relatively undiscussed in the literature. Further work should be done to investigate variants by characterizing human iPSC-CMs engineered with established pathogenic variants in *TNNC1* HCM/RCM (A8V) and DCM (I4M). There are pathogenic variants A31T, M47I, P54H, L97Q, and D149G that do not have any known cardiomyopathy associated (Table 1). These variants need to be investigated to further warrant a possible cardiomyopathy association. Studying structural dynamics (HDX) and protein–protein interactions (e.g., by crosslinking) of cTnC in the thin filament (native or reconstituted) should also be completed along with high-resolution structural analyses (NMR and cryo–EM). Finally, further probing non-canonical roles of cTnC from a basic science perspective and also from a translational perspective would provide a better understanding of these sarcomeric proteins beyond muscle contraction.

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