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ARTICLE

Cardiomyopathic troponin mutations predominantly occur at its interface with actin and tropomyosin

Larry S. Tobacman[1] and Anthony Cammarato[2]

Reversible Ca^{2+} binding to troponin is the primary on-off switch of the contractile apparatus of striated muscles, including the heart. Dominant missense mutations in human cardiac troponin genes are among the causes of hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy. Structural understanding of troponin action has recently advanced considerably via electron microscopy and molecular dynamics studies of the thin filament. As a result, it is now possible to examine cardiomyopathy-inducing troponin mutations in thin-filament structural context, and from that to seek new insight into pathogenesis and into the troponin regulatory mechanism. We compiled from consortium reports a representative set of troponin mutation sites whose pathogenicity was determined using standardized clinical genetics criteria. Another set of sites, apparently tolerant of amino acid substitutions, was compiled from the gnomAD v2 database. Pathogenic substitutions occurred predominantly in the areas of troponin that contact actin or tropomyosin, including, but not limited to, two regions of newly proposed structure and long-known implication in cardiomyopathy: the C-terminal third of troponin I and a part of the troponin T N terminus. The pathogenic mutations were located in troponin regions that prevent contraction under low Ca^{2+} concentration conditions. These regions contribute to Ca^{2+}-regulated steric hindrance of myosin by the combined effects of troponin and tropomyosin. Loss-of-function mutations within these parts of troponin result in loss of inhibition, consistent with the hypercontractile phenotype characteristic of HCM. Notably, pathogenic mutations are absent in our dataset from the Ca^{2+}-binding, activation-producing troponin C (TnC) N-lobe, which controls contraction by a multi-faceted mechanism. Apparently benign mutations are also diminished in the TnC N-lobe, suggesting mutations are poorly tolerated in that critical domain.

Introduction

Hypertrophic cardiomyopathy (HCM) predominantly results from pathogenic mutation in the cardiac contractile apparatus: a thick-filament or a thin-filament protein. The first such gene linkages were described several decades ago (Watkins et al., 1995b; Watkins et al., 1995a; Thierfelder et al., 1994; Kimura et al., 1997; Geisterfer-Lowrance et al., 1990; Schwartz et al., 1995; Nakajima-Taniguchi et al., 1995; Forissier et al., 1996). Ever since, investigators have had sustained interest in how the mutations cause dysfunction and disease. The dual purpose of such work is to gain pathophysiological insight that may be of practical use and physiological insight that may be of fundamental value for understanding cardiac contraction and its regulation. In particular, both pathophysiologic and physiological knowledge can result from the study of dominant missense mutations, which are the characteristic mutation type (except for myosin binding protein-C, which typically is truncated).

In January 2020, the structural biology of troponin advanced greatly with the publication of a cryo-EM study of the regulated thin filament (Yamada et al., 2020). The structure of troponin as bound to the actin filament was derived, in both the presence and absence of Ca^{2+}. Although the results may be challenged or revised in time, independent cryo-EM or computational work (Doran et al., 2020; Oda et al., 2020; Pavadai et al., 2020a; Pavadai et al., 2020b; Burbaum et al., 2020 *Preprint*; Wang et al., 2020 *Preprint*) is already extending or confirming many aspects, such as the orientation of the troponin core domain. These recent developments are transformative for structural understanding of the primary on-off regulatory switch in cardiac and other striated muscles: reversible Ca^{2+} binding to troponin. Cumulatively, the structural biology reports have established a new starting point for understanding and investigation (Tobacman, 2021).

[1]Departments of Medicine and of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL; [2]Departments of Medicine and of Physiology, Johns Hopkins University, Baltimore, MD.

Correspondence to Larry S. Tobacman: LST@uic.edu

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One result of this progress is that disease-linked mutations can be considered in a structural context that previously was unknown. This is true for individual mutations: their detailed mechanistic effects and varied phenotypes can be studied even more fruitfully in the future than has been the case until now. Additionally, one can now consider troponin mutations broadly for their pattern, if any, in relation to thin-filament structure. The Yamada et al. (2020) cryo-EM study revealed that troponin is highly extended. It contacts almost every actin in the thin filament, either directly or via close interaction with actin-bound tropomyosin. Troponin sits right on the filament surface, highly stretched out, to a striking extent. A primary exception to this is the bulk of the troponin core domain, which until the recent results was the only part of troponin for which there was a widely accepted atomic model. Most of the core domain is now seen to lie at a distance from the actin-tropomyosin surface.

With the above context in mind, the current paper examines the locations on the thin filament of a representative set of disease-linked troponin mutations. For this, pathogenic or very likely pathogenic mutation sites were collected from those identified in published, well-curated cardiomyopathy consortium reports (Walsh et al., 2017; Ho et al., 2018; Alfares et al., 2015). The results indicate that pathogenicity occurs predominantly in the areas of troponin that contact actin or tropomyosin, i.e., troponin's interface with the thin filament. These areas include, but are not limited to, two regions long identified as hotspots for HCM mutations: the C-terminal third of cardiac troponin I (TnI) and a helical region within the N-terminal half of cardiac troponin T (TnT). These two regions are directly involved in troponin's function as the off switch for muscle contraction. Outside of troponin's interface with the thin filament, i.e., in the bulk of the troponin core domain, there is relative paucity of disease-linked mutations in troponin: pathogenic mutations occur, but pathogenic amino acid positions occur more sparsely. The mechanistic implications of this pattern are considered below.

The present report also examines the structural locations of sites where mutations are apparently tolerated. These were identified from the public gnomAD v2 database (Karczewski et al., 2020) of alleles and allele frequencies derived from exome sequence data from 123,136 individuals and whole-genome sequencing from 15,496 individuals. The data include alleles and allele frequencies found in a population that is not enriched for disease but from which one cannot exclude the possibility of individuals with disease. All troponin amino acid substitution sites encoded by one or more missense alleles were noted, and then residues were excluded if the clinical consortia had reported at that site any substitution that might be pathogenic, i.e., confirmed pathogenic, likely pathogenic, or variant of uncertain significance (VUS). The resulting list of mutation sites is relatively tolerant to mutation: as a group, the sites are much less likely to have pathogenic mutations than the set of sites in which pathogenesis was confirmed or judged probable. The apparently tolerant sites had a different distribution than the pathogenic sites. The distribution was much wider, but again not random. In particular, the cardiac troponin C (TnC) N-lobe, where no pathogenic mutations occurred, also had the lowest density of apparently tolerated mutation sites, suggesting that the heart is poorly accommodating to altered structure in this critical part of troponin. Thus, structural biology, human genomics, and clinical genetic data may be combined to seek insight into disease-causing troponin dysfunction, and into the thin-filament regulatory mechanism itself.

Materials and methods

Data sources

Investigators have published data on sarcomeric gene missense mutations believed to cause cardiomyopathy for almost three decades. However, it was not until comparatively recently that the prevalence of incidental mutations was appreciated via genome sequencing of many individuals, and it was only in 2015 that the American College of Medical Genetics and Genomics promulgated current standardized criteria for judging pathogenicity of sequence variants (Richards et al., 2015). Fortunately, standardized sets of alleged cardiomyopathy mutations have been evaluated since then, using those criteria. These were published in large international studies compiled by partly overlapping groups of cardiovascular investigators (Walsh et al., 2017; Ho et al., 2018; Alfares et al., 2015). For the present study, supplemental materials associated with those papers were downloaded, and the data for troponin were extracted and analyzed in sum.

For comparison to the above dataset, incidentally identified missense alleles can be examined for cardiac troponin genes via the public data in gnomAD v2 (Karczewski et al., 2020), which is derived from whole-genome and whole-exome sequencing of humans around the world. For the present study, alleles and allele frequencies were downloaded for *TNNI3*, *TNNT2*, and *TNNC1*, the cardiac troponin subunit genes. The great preponderance of the gnomAD alleles are not pathogenic, so the dataset can be contrasted with the missense alleles that the consortia found to be disease-linked. However, one cannot exclude the possibility of individuals with disease within the gnomAD data.

From the above data, the location of each residue in troponin was classified as one of four types (Table S1). Pathogenic locations were sites with at least one substitution designated to be confirmed pathogenic or likely pathogenic (estimated as 90% probability of pathogenicity) by either consortium. VUS locations were residues where no amino acid substitution had been designated pathogenic or likely pathogenic and at least one mutation had been designated VUS. Locations characterized as apparently tolerant of mutation had at least one substitution detected in gnomAD (i.e., by whole-genome or whole-exome sequencing) and no mutations that were characterized clinically as pathogenic, likely pathogenic, or VUS. The remaining, uncharacterized residues made up 60% of troponin.

In addition, each residue was characterized relative to the very revealing, recently reported cryo-EM study of the cardiac thin filament (Yamada et al., 2020). In that structural biology report, 469 of troponin's 659 residues were placed into an atomic model of the thin filament. For the present study, each troponin residue was noted for whether it was identified in the cryo-EM–derived atomic model of the thin-filament low

Ca^{2+} apo-state, PDB accession no. 6kn8, as well as for its location within defined structural regions of troponin.

Statistical analyses

Multinomial distributions were used to assess whether mutation-type distributions were as expected if random, or if they involved local densities that were either higher or lower than readily explained by random distribution. For this, structural regions of troponin were assessed, such as subunits or other areas that were well defined by troponin's structure as bound to the thin filament. The subject under study, in other words, was mutation site density within troponin structures, rather than mathematical analysis of clustering anywhere within full protein sequences.

Let H be the number of troponin residues having at least one harmful mutation (confirmed pathogenic or likely pathogenic), and let K indicate the number of residues in troponin. Then, the number of distributions of H mutation residue sites among K sites of troponin is given by

$$N_H = \frac{K!}{(K\text{-}H)! \times H!}.$$

The subset of the above distributions for which an index number (f) of harmful mutation sites occur in the selected region such as a subunit (i.e., there are f harmful mutation sites in the region) is given by

$$N_{fH} = \left[\frac{S!}{(S\text{-}f)! \times f!}\right] \times \left\{\frac{(K-S)!}{[K\text{-}S\text{-}(H\text{-}f)]! \times (H\text{-}f)!}\right\},$$

where S is the number of residues in the troponin region selected for analysis of the density of harmful allele sites.

The probability of observing a distribution of character corresponding to NfH is

$$\Phi_{fH} = \frac{N_{fH}}{N_H}.$$

The calculated distributions of Φ_{fH} versus f showed expected Gaussian shapes, sums to unity, and maxima when $f = S \times H/K$. For assessment of statistical significance, P values were obtained as one-sided tail sums of Φ.

Online supplemental material

Table S1 delineates each troponin residue's characterization as one of three types with respect to mutation or as unclassified with respect to mutation effect. So-called harmful sites have at least one pathogenic or likely pathogenic missense substitution in the present dataset; VUS sites have at least one VUS missense substitution and no pathogenic or likely pathogenic substitutions in the dataset; and tolerated sites have at least one missense substitution in gnomAD v2 and no pathogenic, likely pathogenic, or VUS substitutions in the dataset.

Results

Location pattern of pathogenic mutations

Troponin genetic data within recent, broad cardiomyopathy consortia publications (Walsh et al., 2017; Ho et al., 2018; Alfares et al., 2015) were grouped together, and the well-curated pathogenicity of all missense alleles was noted. From this information, a set of locations within troponin was established, which are characteristic of where pathogenic substitutions tend to occur. The dataset is representative, rather than inclusive of all pathogenic sites within troponin. Important aims were to avoid both misattribution of pathogenicity and selection bias, by drawing from recent clinical consortia publications rather than parsing the cardiomyopathy literature. Also, the standardized criteria used by the investigators for the designation "likely pathogenic" are stringent, requiring a high probability of pathogenesis, estimated as 90% (Richards et al., 2015). Therefore, for the present study, alleles judged as either likely pathogenic or confirmed pathogenic were combined and termed pathogenic. Correspondingly, an amino acid site in troponin was designated as pathogenic if at least one substitution at that position had been judged confirmed pathogenic or likely pathogenic by one of the previous reports. Each of these prior studies reported HCM alleles. One of them also reported dilated cardiomyopathy (DCM) alleles.

In the combined data, there were 52 pathogenic sites in troponin. The emphasis of the current study is on pathogenesis generally rather than specific phenotype. However, the great majority of the identified pathogenic sites had an allele linked to HCM. Six allele sites (corresponding to TnT residues 131, 134, 139, and 205 and TnI residues 182 and 184) had a DCM attribution and no HCM attribution in this dataset. Mutations at all the other sites were tabulated by the publications as pathogenic for HCM, and the existence of alleles that can cause either phenotype has been questioned (Watkins et al., 2011). The distribution totals among troponin subunits and other structural areas are presented in Table 1, and additional detail is provided in Table S1. Overall, the 52 sites comprise 7.9% of the 659 residues in troponin. If one excludes the cardiac/slow skeletal muscle TnC gene (*TNNC1*), which is a very rare genetic cause of cardiomyopathy (Alfares et al., 2015), pathogenic alleles were present at 10.2% (51 of 498 residues) of the amino acid positions in cardiac troponin's other two subunits, TnI (*TNNI3*) and TnT (*TNNT2*). The pathogenic mutation site density was slightly more in TnI than in TnT (12.4% vs. 8.7%), but with no more variation than might occur randomly.

On the other hand, when one considers the distribution within subunits, the mutations were not random in location. This is particularly the case for the C-terminal third of TnI. In the recently published, cryo-EM–derived atomic model of the Ca^{2+}-free thin filament (Yamada et al., 2020), the C-terminal third of TnI has no globular portion and instead lies highly stretched out along actin, contacting three actin monomers. It contains almost all of the sites where pathogenic substitutions occur in TnI, a highly significant result ($P < 0.0001$). Specifically, the region contains 23 of the 26 pathogenic sites in TnI: HCM residues 141, 144, 145, 157, 162, 166, 170, 171, 176, 178, 183, 186, 190, 192, 196, 198, 199, 201, 203, 204, and 209, plus the DCM sites mentioned above. Investigators have long noted, ever since the first confirmation of TnI pathogenicity in HCM (Kimura et al., 1997), that the C-terminal third of TnI (including residues 137–147, which are inhibitory as an isolated peptide; Talbot

Table 1. **Distribution of a representative set of pathogenic missense substitution sites in troponin**

	Troponin subunit or subunit region	Size of region (aa)	Sites with pathogenic substitution	Density of sites with pathogenic substitution (%)	P vs. troponin	P vs. TnI-TnT	Sites without pathogenic substitution	Sites without pathogenic and with VUS substitution	Density of sites without pathogenic and with VUS substitution (%)	P vs. TnI-TnT
Troponin										
All	TnI, TnT, TnC	659	52	7.9			607	44	7.2	
All except TnC	TnI,TnT	498	51	10.2			447	41	9.2	
Unseen by cryo-EM	(see legend)	190	11	5.8		0.0064	179	12	6.7	NS
TnI										
All	1–210	210	26	12.4		NS	184	19	10.3	NS
N 2/3	1–136	136	3	2.2		0.0001	133	9	6.8	NS
C 1/3	137–210	74	23	31.1		<0.0001	51	10	19.6	NS
TnT										
All	1–288	288	25	8.7		NS	263	22	8.4	NS
Cryo-EM tail helix	87–150	64	10	15.6		NS	54	6	11.1	NS
TnC										
All	1–161	161	1	0.6	<0.0001		160	3	1.9	
N-lobe	1–85	85	0	0	0.0010		85	0	0	
C-lobe	86–161	76	1	1	0.012		75	3	4.0	

The left two columns delineate troponin regions analyzed. The middle columns concern pathogenic sites, defined in publications as amino acid residues with at least one confirmed pathogenic or likely pathogenic substitution (Walsh et al., 2017; Ho et al., 2018; Alfares et al., 2015). The right four columns concern troponin residues with no pathogenic missense mutations and at least one missense VUS (VUS substitution site) observed in cardiomyopathy subjects. P values for the observed densities were calculated as described in Materials and methods. NS indicates P > 0.05. Areas unseen by cryo-EM are TnI 1–40 and TnT residues 1–86, 151–198, and 273–288.

and Hodges, 1981) is a very frequent region for mutations. In the current analysis, it was possible to define the boundaries of the region according to troponin structure on the thin-filament: TnI residues 137–210 all lie extended along the filament in the relaxed-muscle, low-Ca^{2+}-concentration state. Notably, the current collection of mutation sites represents a full 31% pathogenic site density in this part of troponin. Across this region, there is great vulnerability to cardiomyopathy from single amino acid changes. The high mutation density makes mechanistic considerations important to consider: how do substitutions at any one of so many positions produce disease-inducing dysfunction? A likely partial explanation is altered Ca^{2+} sensitivity, which is a common finding in previous functional studies (Siddiqui et al., 2016; Köhler et al., 2003; Kobayashi and Solaro, 2006; Dvornikov et al., 2016; Cheng et al., 2015). However, this explanation then prompts another question: how do the various substitutions act to do this, and what common mechanisms are involved?

The mutation distribution within parts of TnT did not clearly differ from random expectation given the overall density within TnI-TnT. Current information regarding the structure of TnT on the thin filament indicates that it comprises at least five separate structural regions: a critical, troponin-anchoring helix (residues 87–150) that overlies the end-to-end connections between adjacent tropomyosins on the thin filament; TnT within the core domain (199–272); and three regions of unknown 3-D structure (the N terminus, residues 1–86, much of which is hypervariable in sequence; linker residues, residues 151–198, connecting the anchoring helix mentioned above to the troponin core domain; and a short C-terminal segment, 273–288). TnT's divided and partially undefined structure impairs statistical assessment of pathogenic density in its several structural regions. However, at least three pathogenic missense mutation sites were found in each of the five regions. Specifically, in addition to the DCM-causing mutation sites mentioned above, HCM was attributed to one or more nonsynonymous substitutions at TnT positions 66, 79, 82, 86, 92, 94, 97, 102, 110, 130, 141, 163, 174, 179, 195, 218, 268, 269, 278, 285, and 286.

The largest mutation density within TnT, 15.6%, was in the TnT 87–150 helix that anchors troponin on the thin filament (White et al., 1987; Hinkle et al., 1999; Gangadharan et al., 2017; Palm et al., 2001; Jin and Chong, 2010) and overlies the

end-to-end connections between adjacent tropomyosins (Yamada et al., 2020; White et al., 1987; Pavadai et al., 2020b; Murakami et al., 2008). Several HCM- or DCM-causing causing mutations have been studied in great detail after being discovered in this region and in the evolutionarily conserved preceding few residues in the TnT sequence (Watkins et al., 1995b; Tardiff, 2011; Mirza et al., 2005; Gollapudi et al., 2015; Manning et al., 2012; McConnell et al., 2017). From the current dataset, the anchoring helix has a greater pathogenic substitution site density than does TnI-TnT overall (10.2%), but this did not reach statistical significance (P = 0.10). One can detect borderline significance, but only by resorting to consideration within TnT only; the 15.6% pathogenic residue density in this helix is higher than the 8.7% density in TnT overall (P = 0.03). The anchoring helix was the only region of TnT that had a statistically significant density of pathogenic sites by any criterion.

One pathogenic mutation was identified in TnC, which is included here for completeness because it was delineated in the clinical dataset. Clinical groups do not typically screen for TnC mutations, because it has been so rare to prove pathogenicity in any kindred.

Pathogenic mutation site locations in the context of thin-filament structure

Going beyond primary sequence locations, Fig. 1 A illustrates the quaternary structural locations of the 41 pathogenic troponin sites that fall within the thin-filament atomic model. HCM residues are indicated in dark blue and DCM mutations in light blue. The prevalence of mutations in selected regions of TnI and TnT has long been noted, but until now it has not been possible to understand the pattern in the context of atomic structure. Now, from the figure, it can be appreciated that almost all of the mutation sites, 39 of 41, are in regions of troponin located at the thin-filament surface, in contact with actin and/or tropomyosin. Only two sites deviate from this strong tendency and instead are seen at significant radius. Therefore, the paucity of pathogenic mutations in TnC, and also in the core domain portion of TnI, are part of a larger pattern. The troponin regions relatively far from troponin's protein–protein interaction interface, such as the bulk of the troponin core domain and much of TnC, are comparatively rare as sites for pathogenic missense substitutions. Finally, to be clear, the pathogenic residues are characteristically in regions of troponin contacting actin or tropomyosin, rather than being characteristically in direct contact as individual residues. There are too many pathogenic mutation sites for them all to be in direct contact. Furthermore, the underlying EM data are not at resolution sufficient to identify side chains.

The structural locations of these same mutations can also be defined in the Ca^{2+}-saturated state of the thin filament (Yamada et al., 2020). In this condition (not depicted), proximity of the sites to the actin-tropomyosin surface persists for all of the TnT sites, and also for TnI sites 141, 144, and 145. Most of the other TnI sites are in parts (residues 167–210) of the subunit that detach from actin in the presence of Ca^{2+} and are either mobile or otherwise undetected. They are not included in the published atomic model of the Ca^{2+}-saturated state. Finally, three pathogenic mutation sites are within TnI 151–166, which switches from the actin surface and instead attaches to the Ca^{2+}-saturated TnC N-lobe. Thus, some pathogenic mutations in our representative set occur in a part of troponin that interfaces with actin in Ca^{2+}-free conditions, and in the presence of Ca^{2+}, becomes part of an intratroponin interaction that is central to the on-off switch for contraction.

Fig. 1 A necessarily omits the pathogenic substitution sites that occur in troponin regions that are of unknown structure and that are not detected in the cryo-EM study, i.e., within TnI 1–40 or TnT 1–86, 151–198, or 273–288. A significant portion of the 11 pathogenic sites in these regions nevertheless appear to lie close to troponin's interface with actin-tropomyosin. 4 of the 11 pathogenic sites closely precede TnT 87, which is located at the actin surface, and three other sites closely follow TnT 272, which is also on the actin surface (Yamada et al., 2020). On the other hand, the overall density of pathogenic sites within the entirety of the undetected regions is 5.8% (Table 1), about half the density in TnI-TnT overall (P = 0.0064).

Location pattern of variants of uncertain significance

Some alleles identified by the clinical consortia were designated VUS. Across disease-linked genes generally, i.e., not limited to troponin, the clinical prognosis of cardiomyopathy patients with VUS alleles is intermediate between that of subjects with pathogenic alleles and the better prognosis of subjects with benign alleles or no mutations (Ho et al., 2018). Therefore, in some HCM cases, the VUS mutations appear to be harmful. In the present dataset, among the 447 TnI and TnT residue sites remaining after excluding sites with a pathogenic mutation, there were 41 sites with VUS mutations. 19 were in TnI and 22 in TnT, for a combined site density of 9.2% in the two subunits. As shown in Table 1, their locations within the subunits were no different from random; no region had a statistically significant clustering or paucity of VUS substitutions. The same pattern was visible qualitatively within the atomic structure (Fig. 1 B); the 26 sites with at least one VUS mutation were widely distributed in the troponin subunits. Finally, for completeness, Table 1 includes three TnC VUS mutations noted by the clinical groups in TnC. This number of sites provides little insight because, as noted above, the evaluation of patients presenting with cardiomyopathy often does not include screening for mutations in TnC.

Location pattern of apparently benign variants present in the gnomAD database

To obtain a comparison that might provide additional insight into the distribution of pathogenic substitutions, the location of apparently benign mutations was investigated. One starting difference between this investigation, and the analysis above of pathogenic mutations, is in expectation. Investigators have always taken careful note of cardiomyopathic site location within troponin. There is a rich starting literature to provide context to the current observations. In contrast, there is no particular prior expectation for the distribution of sites with apparently benign mutations.

For comparison to the pathogenic sites, sites of missense substitutions were compiled that have no pathogenic or VUS

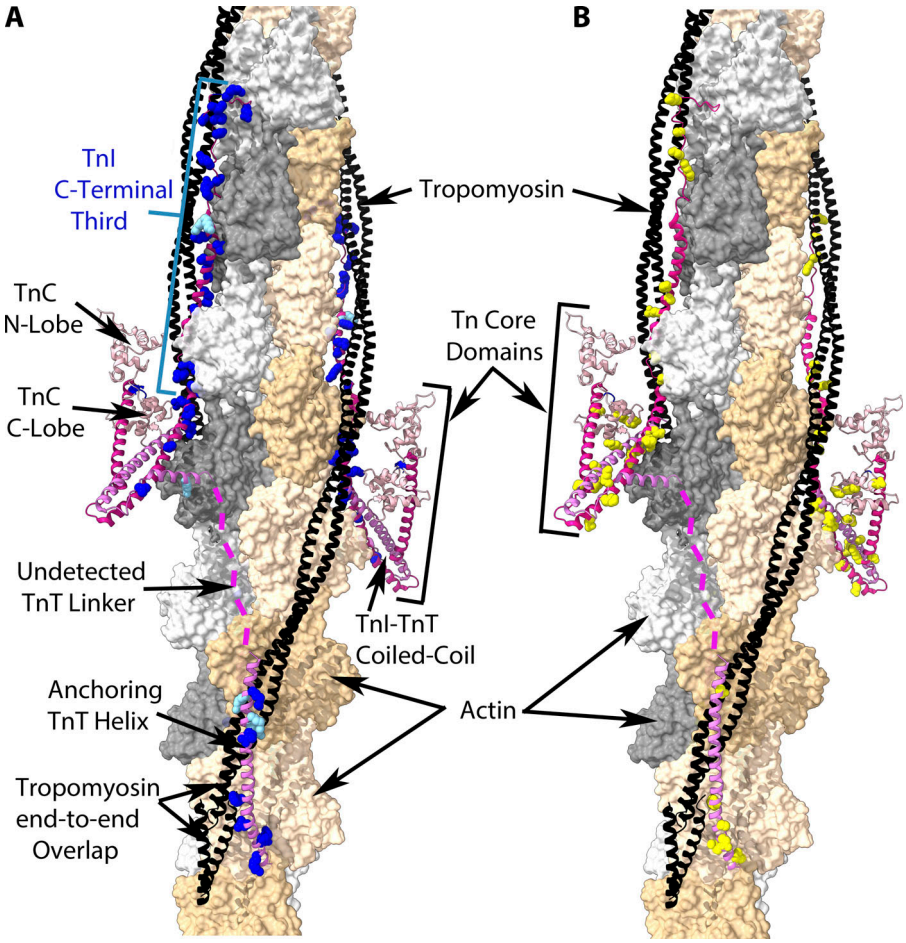

Figure 1. **Pathogenic missense mutation locations in the Ca^{2+}-free thin filament.** The atomic model of the regulated cardiac thin filament (PDB accession no. 6kn7; Yamada et al., 2020) is shown, under the low-Ca^{2+} condition of relaxed muscle. Troponin (Tn) and tropomyosin are illustrated in cartoon format, with troponin subunits in pale pink (TnC), purple (TnT), and magenta (TnI) and tropomyosin in black. Actin monomers are in surface format and neutral gray and beige tones. Troponin residues encoded by pathogenic missense mutations are indicated as blue spherical atoms. **(A)** Troponin sites with pathogenic HCM mutations in dark blue and sites of DCM-inducing substitutions in sky blue. Note the proximity of these sites to the actin-tropomyosin surface. **(B)** Troponin residues that are sites of variants of uncertain significance are indicated in yellow. They are more widely distributed within troponin, compared with A.

substitutions in the present dataset, but that do have mutations identified in the gnomAD database of >130,000 individuals. Although most such mutations were identified incidentally by whole-exome or -genome sequencing, there is no guarantee that all the alleles are benign. Nor is it possible to declare how many of the alleles might be pathogenic. However, it is the case that the frequency of pathogenic alleles is very much less than in the pathogenic dataset. The former is not enriched for disease; the latter is. In this manner, 171 sites were identified in troponin that, as a group, are relatively tolerant of mutations (Table 2). Of these, 57 were in TnI, 76 in TnT, and 38 in TnC. Correspondingly for the three subunits, these totals result in apparently tolerant site densities of 35%, 32%, and 23%, in the residues that remain after excluding sites with pathogenic or VUS mutations. Statistical analyses of site distribution included all three subunits, not just TnI and TnT (Table 2).

Of the apparently tolerated mutation sites, 103 are in parts of troponin detected in the thin-filament atomic model. They are widely distributed within it, as illustrated in Fig. 2 A (TnI and TnT mutations) and Fig. 2 B (TnC mutations). Unlike the pattern for pathogenic substitutions, there are apparently tolerant mutation sites close to the thin-filament surface, and also, an abundance of sites farther from the surface. Unexpectedly, the distribution was not random. In particular, the N-lobe of TnC, which plays an outsized role in the calcium regulation of contraction, had only nine sites with gnomAD mutations, a 9% density, compared with 30% in troponin overall ($P < 0.0001$). Other regions had somewhat fewer or more sites than expected, but the TnC N-lobe stands out because the deviation from expectation is so large. Apparently benign substitution sites are much decreased in this region: there were 70% fewer sites than expected based on random distribution of tolerated mutation sites within troponin.

No other part of any subunit had a deviation from the average gnomAD mutation density that was nearly as striking as was found in the TnC N-lobe. The densities varied widely from region to region, from a low of 17.1% to a high of 40.7%. Most of these values appear to reflect the effect of mutation exclusion from the TnC N-lobe, rather than true clustering. If one excludes the TnC N-lobe from consideration, the remaining troponin residues have an overall site density of 34%, and densities within the structural components differ from this only modestly (Table 2). Residues outside the atomic model, for example, had a gnomAD mutation density of 40.7%, not much different from 34%. One possible exception is the C-terminal third of TnI, the same area that had such a high density of pathogenic substitution sites. Seven pathogenic sites occur within the 41 residues of the TnI C terminus that had no pathogenic or VUS mutations. This corresponds to an apparently benign site density of 17%, which is half the density expected ($P = 0.036$).

Table 2. **Distribution within troponin of apparently tolerated mutation sites from gnomAD v2**

	Troponin subunit or subunit region	Sites without VUS or pathogenic substitution	Sites with gnomAD alleles only	Density of sites with gnomAD alleles only (%)	P vs. troponin (or P vs. troponin without TnC N-lobe)
Troponin					
All	TnI, TnT, TnC	563	171	30.4	
All except TnC	TnI, TnT	406	133	32.8	
All except TnC N-lobe	TnC86–161, TnI, TnT	478	162	33.9	
Unseen by Cryo-EM	(See legend)	167	68	40.7	0.0004 (0.014)
TnI					
All	1–210	165	57	34.5	NS (NS)
N 2/3	1–136	124	50	40.3	0.005 (NS)
C 1/3	137–210	41	7	17.1	0.036 (0.011)
TnT					
All	1–288	241	76	31.5	NS (NS)
Cryo-EM tail helix	87–150	48	11	22.9	NS (NS)
TnC					
All	1–161	157	38	24.2	0.029
N-lobe	1–85	85	9	10.6	<0.0001
C-lobe	86–161	72	29	40.3	0.036 (NS)

Distribution is shown for all 563 troponin residues that were not implicated in recent summaries of clinical experience as sites of any pathogenic or possibly pathogenic (VUS) missense substitutions, but that did contain at least one missense substitution in the gnomAD v2, whole-exome, whole-genome population data. Areas unseen by cryo-EM are the same as in Table 1. P values for the observed densities were calculated as described in Materials and methods. NS indicates P > 0.05.

Discussion

A principal finding of this report is that cardiomyopathy-inducing missense mutations in troponin usually occur in proximity to the protein–protein interface between troponin on one hand and actin-tropomyosin on the other. If the result is new, as this report contends, it is because only recently have advances in knowledge of the thin filament made such a finding possible. In January 2020, troponin's positioning on the thin filament was first established via direct structural determination (Yamada et al., 2020). This cryo-EM study of the cardiac thin filament achieved resolution sufficient to create atomic models including most of troponin, in both the presence and absence of Ca^{2+}. The structural findings were supported and extended throughout the year by excellent independent reports (Pavadai et al., 2020b; Pavadai et al., 2020a; Oda et al., 2020; Doran et al., 2020). At the resolution pertinent to the broad themes in our study, these other studies either agree with Yamada et al. (2020) or disagree only in the sense of omission (fewer parts of troponin were resolved).

Within the thin-filament structure, an unambiguous interface location is evident for protein sequence areas previously implicated as frequent sites of HCM-causing missense mutations. These include the C-terminal third of TnI, which binds to actin primarily and also to tropomyosin, and a portion of the TnT N-terminal tail region of troponin, which binds to tropomyosin primarily and also, apparently, to actin (Yamada et al., 2020). Additionally, the current pathogenic site dataset includes several sites that are in the troponin core domain, but that within the core domain share the feature of proximity to the actin filament. It should be emphasized that the pattern is trend rather than rule, even within the representative pathogenic dataset here studied, not to mention the larger universe of troponin-linked cardiomyopathy mutations. Two pathogenic sites located well off the actin-tropomyosin surface are part of the current dataset. Troponin amino acid substitutions that produce cardiomyopathy tend to occur near troponin's interface with actin or tropomyosin, but can occur elsewhere in troponin. There is equal potential mechanistic value to the study of mutations in TnT, TnI, and TnC that act more at a distance than directly (Tikunova et al., 2018; Cheng et al., 2015; Manning et al., 2012), or to the study of mutations close to the actin-tropomyosin interface. Despite this caveat, proximity is the clear pattern. For pathogenic sites within the atomic model collected in the current dataset, 95% (39 of 41) are in parts of troponin that contact actin and/or tropomyosin.

This strong pattern very likely has mechanistic significance. A rather obvious generality appears relevant: amino acid substitutions at interface locations can be pathogenic, when the protein–protein interactions are important. Correspondingly, troponin has an essential function in turning off muscle contraction in diastole, and the basis of this function is troponin's interactions with actin and tropomyosin. However, it is notable that almost all of the interface can be a cause of disease. That is, missense substitutions in almost any part of troponin's extremely extended protein–protein interface (Fig. 1 A) can be pathogenic for cardiomyopathy. This raises the question whether there is a common aspect to troponin's function, present widely along the interface, which might be important mechanistically for many missense mutations.

The recent structural biology findings reinforce the belief that troponin works by acting, in concert with tropomyosin, via a steric blocking mechanism. The proteins barricade part of the actin monomer surface from the strong myosin binding required for force production (Yamada et al., 2020; Vibert et al., 1997; Tobacman, 2021; McKillop and Geeves, 1993; Huxley, 1972; Doran et al., 2020). For myosin to produce force, troponin and tropomyosin must get out of the way. Until the recent work,

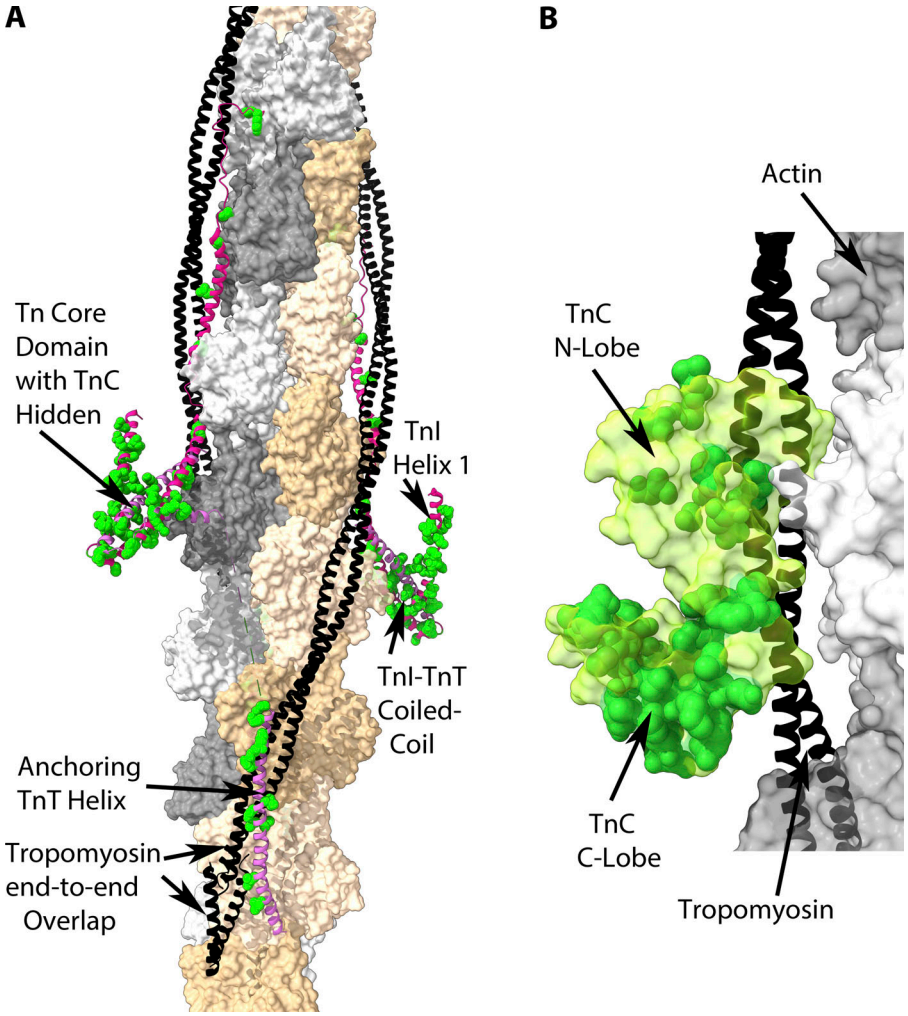

Figure 2. **Apparently tolerated missense mutation locations in the thin filament. (A)** The low-Ca^{2+} condition cardiac thin filament is shown, with the TnC subunit hidden to better view the other two subunits of the troponin core domain. Filament orientation is the same as in Fig. 1. Bright green spheres indicate TnI and TnI residues that are apparently tolerant of mutation. These residues have at least one amino acid substitution detected in gnomAD sequencing of >130,000 individuals, and no substitutions were judged pathogenic or VUS in the current dataset. They are widely distributed and not confined to the actin-tropomyosin surface. **(B)** In greater close-up, selected features of the Ca^{2+}-saturated state of the thin filament (from PDB accession no. 6kn8; Yamada et al., 2020), the structure that would be present in contracting muscle. TnC (translucent pale green) is shown, and both TnI and TnT are hidden. Note the relative paucity of bright green, apparently tolerated mutation sites in the N-lobe, relative to either the C-lobe of TnC or (in A) the remainder of troponin. Also note that the TnC N-lobe makes multiple close contacts: with actin (gray) and tropomyosin (black, seen behind the translucent TnC), as well as with TnI (not depicted) and with the Ca^{2+} ion that is the primary on-off switch for contraction.

only tropomyosin was understood to affect this steric hindrance directly. Now, it is understood structurally how troponin adds to the mechanism. In part this is direct, by troponin's own locations on actin, which sterically hinder strong myosin binding. The C-terminal portion of TnI, in particular, stretches along the thin filament and interferes with myosin only when Ca^{2+} is dissociated from TnC. A mutation that increases Ca^{2+} affinity will decrease troponin's inhibitory function under physiological conditions. Correspondingly, HCM-linked mutations in this region of TnI (as elsewhere) usually enhance Ca^{2+} sensitivity. Additionally, the troponin tail (TnT N terminus) has important inhibitory effects, (Tobacman et al., 2002; Maytum et al., 2002), and the recent structural results offer mechanistic insight: TnT helix 87–150 anchors the tropomyosin end-to-end overlap in one or two inhibitory orientations. This functional interpretation of the structural data has additional support, which comes from investigations of pathogenic substitutions. HCM-related TnT mutations in this region produce a defect in the ability of troponin to shut off actin-myosin function in the absence of Ca^{2+}, both in vitro and in vivo (Madan et al., 2020 and references therein).

When Ca^{2+} binds to troponin, alterations in troponin and tropomyosin diminish steric hindrance to myosin, but do not eliminate it. For myosin to produce force, tropomyosin must move further over the actin surface, via a dynamic process that is not yet understood. The pathogenic mutations in troponin may well influence the dynamic troponin-tropomyosin behavior that accompanies myosin cross-bridge function. In fact, many pathogenic mutations in both TnT and tropomyosin affect stability and dynamics, at least for the proteins as studied in isolation from the thin filament (Palm et al., 2001; Heller et al., 2003; Zheng et al., 2016). Regardless whether such altered dynamics are of frequent mechanistic importance in cardiomyopathy, the interface locations of the pathogenic mutation sites suggest alteration of troponin-tropomyosin's barricade against myosin. This concept ties together the mechanisms of thin-filament and thick-filament mutation pathogenesis (Alamo et al., 2017; Nag et al., 2017). Alteration in myosin function may well be the heart of the matter. Hence, therapeutic strategies targeting the motor may be broadly effective for treating cardiomyopathy stemming from thin- or thick-filament lesions.

Another finding in the current work is the structural location of the parts of troponin where pathogenic missense mutations are relatively absent. The troponin core domain's TnI-TnT coiled coil, most of which extends far off the actin surface, is an uncommon region for pathogenic mutations. The coiled coil does

not participate directly in troponin's inhibitory function. Similarly, the TnC C-lobe and the long TnI helix 1 that attaches to the C-lobe contain no pathogenic mutation sites in this representative set. Both regions lie mostly off the actin surface and have little direct role in the inhibitory action of troponin. The relative paucity of pathogenic sites is consistent with most random mutations having mild or absent effects on thin-filament regulation. On the other hand, exceptions to this pattern published previously, such as pathogenic mutations at TnC 159 and TnI 21, indicate focal areas of high mechanistic interest (Cheng et al., 2015; Mirza et al., 2005).

However, a different explanation is required for the absence of pathogenic sites in the TnC N-lobe. Reversible Ca^{2+} binding to the N-lobe is the primary on-off switch for cardiac contraction (Ebashi et al., 1967; Tobacman, 1996; Gordon et al., 2000). Nevertheless, the current dataset contains no pathogenic mutations in the N-lobe of TnC. Similarly, beyond the present dataset, the entirety of the cardiac TnC gene is very rarely a source of HCM mutations. This is doubly surprising, not only because this part of troponin has high functional importance, but also because altered Ca^{2+} affinity is characteristic in mechanistic investigation of HCM- and DCM-inducing TnI, TnT, and tropomyosin mutations (Szczesna et al., 2000; Robinson et al., 2007; Michele et al., 1999; Harada and Potter, 2004; Bing et al., 1997; Heller et al., 2003). It has been a mystery why human cardiomyopathy does not occur due to pathogenic mutations in the TnC N-lobe that alter Ca^{2+} binding affinity.

Ca^{2+} binding to the TnC N-lobe activates contraction. Furthermore, the Ca^{2+}-saturated N-lobe itself is activating, by mechanisms that were unknown until the cryo-EM study. In the Ca^{2+}-saturated state, the N-lobe comes into contact with actin and tropomyosin. In so doing, it displaces the TnI switch helix from actin and also promotes a tropomyosin pivot that diminishes steric hindrance of the myosin binding site (Fig. 2 B; Yamada et al., 2020; Tobacman, 2021; Pavadai et al., 2020a). This activation theme for the TnC N-lobe stands in contrast to the actions of most of troponin, which are to inhibit contraction, both directly and by effects on tropomyosin. The reverse pattern means that human missense loss-of-function mutations in the N-lobe and loss-of-function mutations elsewhere in troponin have opposite effects on the heart: diminution of contraction versus hypercontractility. One reason that HCM mutation sites are rare is because to produce the typical hypercontractile behavior, a mutation in the TnC N-lobe must produce a gain of function, such as enhanced Ca^{2+} affinity. Only the rare random mutation will produce such an effect.

One may also speculate why, at least at present, there are no known DCM-inducing loss-of-function alleles in the TnC N-lobe. One possibility is that missense mutations in the TnC N-lobe often cause a phenotype too severe for the characteristic cardiomyopathy clinical course: nearly normal cardiac development followed by years or many years of life. In support of this possibility, the TnC N-lobe contains a paucity of apparently benign missense alleles in the gnomAD database. Additional support comes from the recent structural biology results. In Fig. 2 B, TnC (translucent green) is shown in the Ca^{2+}-saturated state of the thin filament. Note the close contact of the N-lobe to both actin and tropomyosin. These newly revealed interactions (Yamada et al., 2020) are specific, occur only when Ca^{2+} is present, and affect both tropomyosin's orientation on actin and the inhibitory actions of TnI (the TnC N-lobe and the TnI switch peptide compete for the same binding site on actin; Yamada et al., 2020; Tobacman, 2021). These functionally important N-lobe actions are in addition to the N-lobe's Ca^{2+}-dependent attachment to the TnI switch helix, and to Ca^{2+} itself.

The above findings provide context to the fact that TnC is rarely a cause of cardiomyopathy. Both lobes of TnC lack pathogenic mutation sites, but perhaps not for the same reason. In the C-lobe, mutations may tend to have little consequence, as is the case for other parts of the troponin core domain that lie off the thin-filament surface. In the much more critical N-lobe of the subunit, only a structurally rare gain-of-function allele could cause HCM. Also, random mutations in the TnC N-lobe may often cause too much loss of function for viability. In other words, the TnC N-domain has an outsized role in regulation, and also, this role is in some ways opposite to that of the remainder of troponin. These features may explain the paucity of pathogenic missense substitutions in the TnC N-lobe, and this should be testable by future experiments.

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References

Alamo, L., J.S. Ware, A. Pinto, R.E. Gillilan, J.G. Seidman, C.E. Seidman, and R. Padrón. 2017. Effects of myosin variants on interacting-heads motif explain distinct hypertrophic and dilated cardiomyopathy phenotypes. *eLife*. 6:e24634. https://doi.org/10.7554/eLife.24634

Alfares, A.A., M.A. Kelly, G. McDermott, B.H. Funke, M.S. Lebo, S.B. Baxter, J. Shen, H.M. McLaughlin, E.H. Clark, L.J. Babb, et al. 2015. Results of clinical genetic testing of 2,912 probands with hypertrophic cardiomyopathy: expanded panels offer limited additional sensitivity. *Genet. Med.* 17:880–888. https://doi.org/10.1038/gim.2014.205

Bing, W., C.S. Redwood, I.F. Purcell, G. Esposito, H. Watkins, and S.B. Marston. 1997. Effects of two hypertrophic cardiomyopathy mutations in alpha-tropomyosin, Asp175Asn and Glu180Gly, on Ca2+ regulation of thin filament motility. *Biochem. Biophys. Res. Commun.* 236:760–764. https://doi.org/10.1006/bbrc.1997.7045

Burbaum, L., J. Schneider, S. Scholze, R.T. Böttcher, W. Baumeister, P. Schwille, J.M. Plitzko, and M. Jasnin. 2020. Molecular-scale visualization of sarcomere contraction within native cardiomyocytes. *bioRxiv*. https://doi.org/https://doi.org/10.1101/2020.09.09.288977 (Preprint posted September 9, 2020).

Cheng, Y., V. Rao, A.Y. Tu, S. Lindert, D. Wang, L. Oxenford, A.D. McCulloch, J.A. McCammon, and M. Regnier. 2015. Troponin I Mutations R146G and R21C Alter Cardiac Troponin Function, Contractile Properties, and

Modulation by Protein Kinase A (PKA)-mediated Phosphorylation. *J. Biol. Chem.* 290:27749–27766. https://doi.org/10.1074/jbc.M115.683045

Doran, M.H., E. Pavadai, M.J. Rynkiewicz, J. Walklate, E. Bullitt, J.R. Moore, M. Regnier, M.A. Geeves, and W. Lehman. 2020. Cryo-EM and Molecular Docking Shows Myosin Loop 4 Contacts Actin and Tropomyosin on Thin Filaments. *Biophys. J.* 119:821–830. https://doi.org/10.1016/j.bpj.2020.07.006

Dvornikov, A.V., N. Smolin, M. Zhang, J.L. Martin, S.L. Robia, and P.P. de Tombe. 2016. Restrictive Cardiomyopathy Troponin I R145W Mutation Does Not Perturb Myofilament Length-dependent Activation in Human Cardiac Sarcomeres. *J. Biol. Chem.* 291:21817–21828. https://doi.org/10.1074/jbc.M116.746172

Ebashi, S., F. Ebashi, and A. Kodama. 1967. Troponin as the Ca++-receptive protein in the contractile system. *J. Biochem.* 62:137–138. https://doi.org/10.1093/oxfordjournals.jbchem.a128628

Forissier, J.F., L. Carrier, H. Farza, G. Bonne, J. Bercovici, P. Richard, B. Hainque, P.J. Townsend, M.H. Yacoub, S. Fauré, et al. 1996. Codon 102 of the cardiac troponin T gene is a putative hot spot for mutations in familial hypertrophic cardiomyopathy. *Circulation.* 94:3069–3073. https://doi.org/10.1161/01.CIR.94.12.3069

Gangadharan, B., M.S. Sunitha, S. Mukherjee, R.R. Chowdhury, F. Haque, N. Sekar, R. Sowdhamini, J.A. Spudich, and J.A. Mercer. 2017. Molecular mechanisms and structural features of cardiomyopathy-causing troponin T mutants in the tropomyosin overlap region. *Proc. Natl. Acad. Sci. USA.* 114:11115–11120. https://doi.org/10.1073/pnas.1710354114

Geisterfer-Lowrance, A.A., S. Kass, G. Tanigawa, H.P. Vosberg, W. McKenna, C.E. Seidman, and J.G. Seidman. 1990. A molecular basis for familial hypertrophic cardiomyopathy: a beta cardiac myosin heavy chain gene missense mutation. *Cell.* 62:999–1006. https://doi.org/10.1016/0092-8674(90)90274-I

Gollapudi, S.K., J.C. Tardiff, and M. Chandra. 2015. The functional effect of dilated cardiomyopathy mutation (R144W) in mouse cardiac troponin T is differently affected by α- and β-myosin heavy chain isoforms. *Am. J. Physiol. Heart Circ. Physiol.* 308:H884–H893. https://doi.org/10.1152/ajpheart.00528.2014

Gordon, A.M., E. Homsher, and M. Regnier. 2000. Regulation of contraction in striated muscle. *Physiol. Rev.* 80:853–924. https://doi.org/10.1152/physrev.2000.80.2.853

Harada, K., and J.D. Potter. 2004. Familial hypertrophic cardiomyopathy mutations from different functional regions of troponin T result in different effects on the pH and Ca2+ sensitivity of cardiac muscle contraction. *J. Biol. Chem.* 279:14488–14495. https://doi.org/10.1074/jbc.M309355200

Heller, M.J., M. Nili, E. Homsher, and L.S. Tobacman. 2003. Cardiomyopathic tropomyosin mutations that increase thin filament Ca2+ sensitivity and tropomyosin N-domain flexibility. *J. Biol. Chem.* 278:41742–41748. https://doi.org/10.1074/jbc.M303408200

Hinkle, A., A. Goranson, C.A. Butters, and L.S. Tobacman. 1999. Roles for the troponin tail domain in thin filament assembly and regulation. A deletional study of cardiac troponin T. *J. Biol. Chem.* 274:7157–7164. https://doi.org/10.1074/jbc.274.11.7157

Ho, C.Y., S.M. Day, E.A. Ashley, M. Michels, A.C. Pereira, D. Jacoby, A.L. Cirino, J.C. Fox, N.K. Lakdawala, J.S. Ware, et al. 2018. Genotype and Lifetime Burden of Disease in Hypertrophic Cardiomyopathy: Insights from the Sarcomeric Human Cardiomyopathy Registry (SHaRe). *Circulation.* 138:1387–1398. https://doi.org/10.1161/CIRCULATIONAHA.117.033200

Huxley, H.E. 1972. Structural changes in the actin and myosin containing filaments during contraction. *Cold Spring Harb. Symp. Quant. Biol.* 37:361–376. https://doi.org/10.1101/SQB.1973.037.01.046

Jin, J.P., and S.M. Chong. 2010. Localization of the two tropomyosin-binding sites of troponin T. *Arch. Biochem. Biophys.* 500:144–150. https://doi.org/10.1016/j.abb.2010.06.001

Karczewski, K.J., L.C. Francioli, G. Tiao, B.B. Cummings, J. Alföldi, Q. Wang, R.L. Collins, K.M. Laricchia, A. Ganna, D.P. Birnbaum, et al. Genome Aggregation Database Consortium. 2020. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature.* 581:434–443. https://doi.org/10.1038/s41586-020-2308-7

Kimura, A., H. Harada, J.E. Park, H. Nishi, M. Satoh, M. Takahashi, S. Hiroi, T. Sasaoka, N. Ohbuchi, T. Nakamura, et al. 1997. Mutations in the cardiac troponin I gene associated with hypertrophic cardiomyopathy. *Nat. Genet.* 16:379–382. https://doi.org/10.1038/ng0897-379

Kobayashi, T., and R.J. Solaro. 2006. Increased Ca2+ affinity of cardiac thin filaments reconstituted with cardiomyopathy-related mutant cardiac troponin I. *J. Biol. Chem.* 281:13471–13477. https://doi.org/10.1074/jbc.M509561200

Köhler, J., Y. Chen, B. Brenner, A.M. Gordon, T. Kraft, D.A. Martyn, M. Regnier, A.J. Rivera, C.K. Wang, and P.B. Chase. 2003. Familial hypertrophic cardiomyopathy mutations in troponin I (K183D, G203S, K206Q) enhance filament sliding. *Physiol. Genomics.* 14:117–128. https://doi.org/10.1152/physiolgenomics.00101.2002

Madan, A., M.C. Viswanathan, K.C. Woulfe, W. Schmidt, A. Sidor, T. Liu, T.H. Nguyen, B. Trinh, C. Wilson, S. Madathil, et al. 2020. *TNNT2* mutations in the tropomyosin binding region of TNT1 disrupt its role in contractile inhibition and stimulate cardiac dysfunction. *Proc. Natl. Acad. Sci. USA.* 117:18822–18831. https://doi.org/10.1073/pnas.2001692117

Manning, E.P., J.C. Tardiff, and S.D. Schwartz. 2012. Molecular effects of familial hypertrophic cardiomyopathy-related mutations in the TNT1 domain of cTnT. *J. Mol. Biol.* 421:54–66. https://doi.org/10.1016/j.jmb.2012.05.008

Maytum, R., M.A. Geeves, and S.S. Lehrer. 2002. A modulatory role for the troponin T tail domain in thin filament regulation. *J. Biol. Chem.* 277:29774–29780. https://doi.org/10.1074/jbc.M201761200

McConnell, M., L. Tal Grinspan, M.R. Williams, M.L. Lynn, B.A. Schwartz, O.Z. Fass, S.D. Schwartz, and J.C. Tardiff. 2017. Clinically Divergent Mutation Effects on the Structure and Function of the Human Cardiac Tropomyosin Overlap. *Biochemistry.* 56:3403–3413. https://doi.org/10.1021/acs.biochem.7b00266

McKillop, D.F., and M.A. Geeves. 1993. Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. *Biophys. J.* 65:693–701. https://doi.org/10.1016/S0006-3495(93)81110-X

Michele, D.E., F.P. Albayya, and J.M. Metzger. 1999. Direct, convergent hypersensitivity of calcium-activated force generation produced by hypertrophic cardiomyopathy mutant alpha-tropomyosins in adult cardiac myocytes. *Nat. Med.* 5:1413–1417. https://doi.org/10.1038/70990

Mirza, M., S. Marston, R. Willott, C. Ashley, J. Mogensen, W. McKenna, P. Robinson, C. Redwood, and H. Watkins. 2005. Dilated cardiomyopathy mutations in three thin filament regulatory proteins result in a common functional phenotype. *J. Biol. Chem.* 280:28498–28506. https://doi.org/10.1074/jbc.M412281200

Murakami, K., M. Stewart, K. Nozawa, K. Tomii, N. Kudou, N. Igarashi, Y. Shirakihara, S. Wakatsuki, T. Yasunaga, and T. Wakabayashi. 2008. Structural basis for tropomyosin overlap in thin (actin) filaments and the generation of a molecular swivel by troponin-T. *Proc. Natl. Acad. Sci. USA.* 105:7200–7205. https://doi.org/10.1073/pnas.0801950105

Nag, S., D.V. Trivedi, S.S. Sarkar, A.S. Adhikari, M.S. Sunitha, S. Sutton, K.M. Ruppel, and J.A. Spudich. 2017. The myosin mesa and the basis of hypercontractility caused by hypertrophic cardiomyopathy mutations. *Nat. Struct. Mol. Biol.* 24:525–533. https://doi.org/10.1038/nsmb.3408

Nakajima-Taniguchi, C., H. Matsui, S. Nagata, T. Kishimoto, and K. Yamauchi-Takihara. 1995. Novel missense mutation in alpha-tropomyosin gene found in Japanese patients with hypertrophic cardiomyopathy. *J. Mol. Cell. Cardiol.* 27:2053–2058. https://doi.org/10.1016/0022-2828(95)90026-8

Oda, T., H. Yanagisawa, and T. Wakabayashi. 2020. Cryo-EM structures of cardiac thin filaments reveal the 3D architecture of troponin. *J. Struct. Biol.* 209:107450. https://doi.org/10.1016/j.jsb.2020.107450

Palm, T., S. Graboski, S.E. Hitchcock-DeGregori, and N.J. Greenfield. 2001. Disease-causing mutations in cardiac troponin T: identification of a critical tropomyosin-binding region. *Biophys. J.* 81:2827–2837. https://doi.org/10.1016/S0006-3495(01)75924-3

Pavadai, E., W. Lehman, and M.J. Rynkiewicz. 2020a. Protein-Protein Docking Reveals Dynamic Interactions of Tropomyosin on Actin Filaments. *Biophys. J.* 119:75–86. https://doi.org/10.1016/j.bpj.2020.05.017

Pavadai, E., M.J. Rynkiewicz, A. Ghosh, and W. Lehman. 2020b. Docking Troponin T onto the Tropomyosin Overlapping Domain of Thin Filaments. *Biophys. J.* 118:325–336. https://doi.org/10.1016/j.bpj.2019.11.3393

Richards, S., N. Aziz, S. Bale, D. Bick, S. Das, J. Gastier-Foster, W.W. Grody, M. Hegde, E. Lyon, E. Spector, et al. ACMG Laboratory Quality Assurance Committee. 2015. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet. Med.* 17:405–424. https://doi.org/10.1038/gim.2015.30

Robinson, P., P.J. Griffiths, H. Watkins, and C.S. Redwood. 2007. Dilated and hypertrophic cardiomyopathy mutations in troponin and alpha-tropomyosin have opposing effects on the calcium affinity of cardiac

thin filaments. *Circ. Res.* 101:1266–1273. https://doi.org/10.1161/CIRCRESAHA.107.156380

Schwartz, K., L. Carrier, P. Guicheney, and M. Komajda. 1995. Molecular basis of familial cardiomyopathies. *Circulation.* 91:532–540. https://doi.org/10.1161/01.CIR.91.2.532

Siddiqui, J.K., S.B. Tikunova, S.D. Walton, B. Liu, M. Meyer, P.P. de Tombe, N. Neilson, P.M. Kekenes-Huskey, H.E. Salhi, P.M. Janssen, et al. 2016. Myofilament Calcium Sensitivity: Consequences of the Effective Concentration of Troponin I. *Front. Physiol.* 7:632. https://doi.org/10.3389/fphys.2016.00632

Szczesna, D., R. Zhang, J. Zhao, M. Jones, G. Guzman, and J.D. Potter. 2000. Altered regulation of cardiac muscle contraction by troponin T mutations that cause familial hypertrophic cardiomyopathy. *J. Biol. Chem.* 275:624–630. https://doi.org/10.1074/jbc.275.1.624

Talbot, J.A., and R.S. Hodges. 1981. Synthetic studies on the inhibitory region of rabbit skeletal troponin I. Relationship of amino acid sequence to biological activity. *J. Biol. Chem.* 256:2798–2802.

Tardiff, J.C. 2011. Thin filament mutations: developing an integrative approach to a complex disorder. *Circ. Res.* 108:765–782. https://doi.org/10.1161/CIRCRESAHA.110.224170

Thierfelder, L., H. Watkins, C. MacRae, R. Lamas, W. McKenna, H.P. Vosberg, J.G. Seidman, and C.E. Seidman. 1994. Alpha-tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: a disease of the sarcomere. *Cell.* 77:701–712. https://doi.org/10.1016/0092-8674(94)90054-X

Tikunova, S., N. Belevych, K. Doan, and P.J. Reiser. 2018. Desensitizing mouse cardiac troponin C to calcium converts slow muscle towards a fast muscle phenotype. *J. Physiol.* 596:4651–4663. https://doi.org/10.1113/JP276296

Tobacman, L.S. 1996. Thin filament-mediated regulation of cardiac contraction. *Annu. Rev. Physiol.* 58:447–481. https://doi.org/10.1146/annurev.ph.58.030196.002311

Tobacman, L.S. 2021. Troponin revealed: Uncovering the structure of the thin filament on-off switch in striated muscle. *Biophys. J.* 120:1–9. https://doi.org/10.1016/j.bpj.2020.11.014

Tobacman, L.S., M. Nihli, C. Butters, M. Heller, V. Hatch, R. Craig, W. Lehman, and E. Homsher. 2002. The troponin tail domain promotes a conformational state of the thin filament that suppresses myosin activity. *J. Biol. Chem.* 277:27636–27642. https://doi.org/10.1074/jbc.M201768200

Vibert, P., R. Craig, and W. Lehman. 1997. Steric-model for activation of muscle thin filaments. *J. Mol. Biol.* 266:8–14. https://doi.org/10.1006/jmbi.1996.0800

Walsh, R., K.L. Thomson, J.S. Ware, B.H. Funke, J. Woodley, K.J. McGuire, F. Mazzarotto, E. Blair, A. Seller, J.C. Taylor, et al. Exome Aggregation Consortium. 2017. Reassessment of Mendelian gene pathogenicity using 7,855 cardiomyopathy cases and 60,706 reference samples. *Genet. Med.* 19:192–203. https://doi.org/10.1038/gim.2016.90

Wang, Z., M. Grange, T. Wagner, A.L. Kho, M. Gautel, and S. Raunser. 2020. Molecular plasticity of the native mouse skeletal sarcomere revealed by cryo-ET. *bioRxiv.* https://doi.org/https://doi.org/10.1101/2020.09.13.295386 (Preprint posted September 13, 2020)

Watkins, H., H. Ashrafian, and C. Redwood. 2011. Inherited cardiomyopathies. *N. Engl. J. Med.* 364:1643–1656. https://doi.org/10.1056/NEJMra0902923

Watkins, H., D. Conner, L. Thierfelder, J.A. Jarcho, C. MacRae, W.J. McKenna, B.J. Maron, J.G. Seidman, and C.E. Seidman. 1995a. Mutations in the cardiac myosin binding protein-C gene on chromosome 11 cause familial hypertrophic cardiomyopathy. *Nat. Genet.* 11:434–437. https://doi.org/10.1038/ng1295-434

Watkins, H., W.J. McKenna, L. Thierfelder, H.J. Suk, R. Anan, A. O'Donoghue, P. Spirito, A. Matsumori, C.S. Moravec, J.G. Seidman, et al. 1995b. Mutations in the genes for cardiac troponin T and alpha-tropomyosin in hypertrophic cardiomyopathy. *N. Engl. J. Med.* 332:1058–1064. https://doi.org/10.1056/NEJM199504203321603

White, S.P., C. Cohen, and G.N. Phillips Jr. 1987. Structure of co-crystals of tropomyosin and troponin. *Nature.* 325:826–828. https://doi.org/10.1038/325826a0

Yamada, Y., K. Namba, and T. Fujii. 2020. Cardiac muscle thin filament structures reveal calcium regulatory mechanism. *Nat. Commun.* 11:153. https://doi.org/10.1038/s41467-019-14008-1

Zheng, W., S.E. Hitchcock-DeGregori, and B. Barua. 2016. Investigating the effects of tropomyosin mutations on its flexibility and interactions with filamentous actin using molecular dynamics simulation. *J. Muscle Res. Cell Motil.* 37:131–147. https://doi.org/tlsb

Supplemental material

One table is provided online in an Excel file. Table S1 delineates each troponin residue's characterization as one of three types with respect to mutation or as unclassified with respect to mutation effect.