Thin Filament Mutations
Developing an Integrative Approach to a Complex Disorder

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Abstract: Sixteen years ago, mutations in cardiac troponin (Tn)T and α-tropomyosin were linked to familial hypertrophic cardiomyopathy, thus transforming the disorder from a disease of the β-myosin heavy chain to a disease of the cardiac sarcomere. From the outset, studies suggested that mutations in the regulatory thin filament caused a complex, heterogeneous pattern of ventricular remodeling with wide variations in clinical expression. To date, the clinical heterogeneity is well matched by an extensive array of nearly 100 independent mutations in all components of the cardiac thin filament. Significant advances in our understanding of the biophysics of myofilament activation, coupled to the emerging evidence that thin filament linked cardiomyopathies are progressive, suggests that a renewed focus on the most proximal events in both the molecular and clinical pathogenesis of the disease will be necessary to achieve the central goal of using genotype information to manage affected patients. In this review, we examine the existing biophysical and clinical evidence in support of a more proximal definition of thin filament cardiomyopathies. In addition, new high-resolution, integrated approaches are presented to help define the way forward as the field works toward developing a more robust link between genotype and phenotype in this complex disorder. (Circ Res. 2011;108:765-782.)

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The publication of the original study in 1990 establishing the genetic linkage of the β-myosin heavy chain gene (βMyHC) to FHC, the familial form of hypertrophic cardiomyopathy (HCM), was, like all seminal scientific findings, both a beginning and an end.1 It represented an “end point” in that it provided a definitive answer to a question that had been discussed and debated for more than 30 years. The initial description of a patient with HCM by Teare in 1958 had noted that multiple family members were also affected by this “unexplained” ventricular hypertrophy.2 The linkage results also prompted a spirited round of “of course!” from muscle biologists, because it seemed to make perfect sense that a mutation in the enzymatic domain of the primary cardiac motor protein could lead to a massive hypertrophic...
response in the heart. Thus began the molecular era of HCM.

From the outset, it was clear that the oft-noted clinical heterogeneity of HCM was likely to be matched by considerable genetic heterogeneity, as suggested by the initial observation that the inheritance of HCM and the initial DNA marker loci were discordant.1 Subsequent genome-wide linkage analysis initially identified 3 additional disease loci. As considerable phenotypic overlap between the affected families was noted, additional components of the cardiac sarcomere were selected as strong disease gene candidates. The subsequent identification of the 15q2 locus was facilitated by the finding that the murine α-tropomyosin (α-TM) gene mapped to a region of chromosome 9 immediately adjacent to a syntenic portion of human chromosome 15, representing both the first use of the mouse in the study of HCM, and the extension of the disorder to mutations in the thin filament.4 It is interesting to note that the chromosomal locations of several of the human thin filament proteins including the cardiac isoform of troponin T (cTnT) were not known before the linkage studies and thus the studies identifying α-TM and cTnT as important disease loci also provided basic information regarding genomic location, a significant advance. This interrelationship between elucidating the pathogenesis of a complex primary cardiac genetic disorder and understanding the basic biology of the cardiac thin filament has continued to this day.

### Linking Genotype to Phenotype in HCM: A Moving Target

The extension of the causative mutations to the regulatory thin filament changed the definition of HCM from a disease of the cardiac motor to a disease of the cardiac sarcomere and greatly expanded the breadth of the potential molecular pathogenic mechanisms. In the following decade causative mutations were linked to the genes encoding the remaining cardiac thin filament proteins.5–7 One of the earliest hypotheses regarding disease mechanism posited that the varied clinical prognoses in the familial form of HCM (FHC) could be directly linked to mutations in independent protein components of the sarcomere. Although the original goal to link genotype to phenotype remains, it is now clear that the overall complexity of both the potential molecular mechanisms at the level of the cardiac sarcomere and our evolving understanding of the dynamic myocardial and ventricular remodeling that occurs in patients with thin filament mutations will require a far more subtle understanding on both levels.

Before addressing the potential mechanisms whereby thin filament protein mutations can cause cardiomyopathies, it is important to consider the complexity of the end cardiac phenotype. The basic definition of HCM is deceptively simple and dates back to the original findings by Teare of unexplained “asymmetrical ventricular hypertrophy.”2 Thus, HCM represents a classic diagnosis of exclusion where left ventricular (LV) remodeling occurs in the absence of known external causes. This straightforward clinical definition obscures the vast complexity of both the pathological and physiological findings in HCM, many of which were documented in a comprehensive 1968 Circulation article by Frank and Braunwald documenting a striking variation in LV wall thickness between affected patients.8 Although it is now well established that “extreme” degrees of LV hypertrophy (maximal wall thickness of >30 mm) represent a significant risk factor for sudden cardiac death (SCD), one of the more intriguing clinical observations was that the degree of functional impairment in patients with HCM was not necessarily directly related to the degree of LV hypertrophy.9 In particular, abnormalities in relaxation parameters that were out of proportion to wall thickness were noted in several studies and led to the rather prescient prediction that there was likely to be an unknown impairment in function at the myocellular level.10 These observations have been extended to FHC where significant differences in LV mass were found in patients with independent gene mutations, different mutations within the same gene and, surprisingly, within affected families carrying the same gene mutation.11 Subsequently, mutations in sarcomeric genes (including the thin filament proteins cTnT and cTnI) have been linked to both dilated (DCM) and restrictive (RCM) cardiomyopathies.12–14 This seemingly endless degree of phenotypic variability has limited meaningful genotype–phenotype correlations for most mutations, calling into question the feasibility of using genotype information in patient management.15

The most likely mechanism(s) whereby mutations in the same functional domain or even identical mutations can lead to different patterns of ventricular remodeling would be the effects of modifier genes and/or environment that influences the downstream, secondary cellular responses to thin filament mutations. It is also possible that sarcomeric gene variants or polymorphisms may act as secondary phenotypic modifiers. More recently, it has become apparent that 5% to 6% of patients with FHC have either double (2 mutations in the same gene) or compound (2 or more mutations in multiple genes) genotypes.16 Although most of the involved multiple mutations are in β-MyHC or MyBP-C, the resultant effects include earlier presentation and often rapid disease progression. These findings have established the importance of including a complete panel of sarcomeric genes in screening strategies. There is, however, an additional and perhaps...
central issue that confounds our ability to match genotype to phenotype in FHC, the progressive nature of the cardiac pathogenic process. In the past several years, a series of longitudinal studies of patients with thin filament protein mutations have documented the dynamic nature of the ventricular remodeling, including late-onset HCM, progression from HCM to DCM, stable HCM with worsening restrictive physiology and primary DCM.\textsuperscript{13,17} Thus, it is apparent that focusing only on the end phenotype as the supposed “link” to the molecular mechanism is not only limiting but also likely to be misleading. Surmounting this significant limitation will require additional longitudinal studies of early stage or “genotype-positive, phenotype-negative” cohorts on the clinical side. The potential power of this approach was recently demonstrated in a study by Ho et al, in which early profibrotic signaling was detected in patients with MYH7 and MYBPC3 mutations before the onset of hypertrophic growth or detectable fibrosis.\textsuperscript{18} Of note, differential activation patterns were observed between patients carrying independent gene mutations, thus providing a potential mechanism to explain gene mutation-specific patterns of ventricular remodeling. Overall, the predicted result of focusing on the earliest stages of cardiac remodeling (before the activation of potentially complex signaling cascades) would be that the observed molecular and biophysical effects of thin filament mutations would provide a more robust mechanistic link between genotype and phenotype. Moreover, from the standpoint of developing more specific therapeutic options for this complex disorder, it is likely to be far easier to alter the natural history of an early pathogenic process as opposed to achieving the regression of an end-stage cardiomyopathy. Just as a more “proximal” assessment of the clinical disease process is likely to reveal a less variant phenotype, to directly determine the potentially unique role of thin filament mutations in FHC, it is important to begin with the assumption that the primary biophysical insult at the level of the cardiac sarcomere remains the single pathogenic constant among all patients with a given mutation. Thus, by definition, the mechanism of FHC begins with the cardiac cross-bridge cycle.

The Cardiac Thin Filament As a Dynamic Multisubunit Regulatory “Machine”

The fundamental source of cardiac force is the cross-bridge and represents a complex mechanochemochemical interaction between the S1 head of the MyHC and the thin filament backbone, comprised of cardiac actin (reviewed elsewhere\textsuperscript{19}). Myosin is an enzymatic energy-transducing molecule. It converts energy in the pyrophosphate bond of ATP to mechanical energy. Although questions remain as to whether this energy transduction is direct or the result of a “stochastic ratchet,” it is clear that each step of the myosin motion over the actin backbone drives the hydrolysis of ATP.\textsuperscript{20} The actomyosin interaction can occur in the absence of the troponin (cTn) complex and TM. The essential regulation of contraction, however, wherein the hemodynamic demands of the body are directly transduced to the cardiac sarcomere, requires the presence of the complete regulatory thin filament.\textsuperscript{21} As shown in Figure 1, the basic functional unit of the cardiac thin filament is a highly evolved multiprotein machine comprised of 7 actin monomers, 1 troponin complex (cTnI:cTnT:cTnC), and 1 TM coiled-coil dimer. As originally noted by Ebashi in his elegant 1968 study, the role of Ca\textsuperscript{2+} as the activator of striated muscle contraction is fully dependent on Ca\textsuperscript{2+} binding to the Tn complex.\textsuperscript{22} Forty years later, there remains considerable debate as to the molecular mechanisms that underlie the dynamic changes in structure and function that govern myofilament activation following Ca\textsuperscript{2+} binding to cTnC. The initial hypothesis explaining the complex activation process was proposed by Huxley, who suggested 2 specific positions (states) of TM that were dependent on Ca\textsuperscript{2+} binding to cTnC.\textsuperscript{23} In the Ca\textsuperscript{2+}-off state (“blocked” or B state), TM was predicted to sterically block the interaction of actin and myosin. Myofilament activation would subsequently occur via Ca\textsuperscript{2+}-binding induced changes in the protein–protein interactions within the Tn complex, causing a significant azimuthal shift in the position of TM (to the “closed” or C state) that would allow for actin–myosin binding and force generation. Although a broad array of subsequent biophysical and kinetic studies supported the 2-state steric-blocking model, it became clear that myosin binding was required for full activation, eg, Ca\textsuperscript{2+} binding alone was insufficient. This limitation of the 2-state model was directly addressed by McKillop and Geeves, who showed that the kinetic and equilibrium data could be reconciled by the addition of a third TM position (the “open” state or M state) that was induced by an isomerization promoting the transition of the initial weak actin–myosin interaction to a strong, force-generating crossbridge.\textsuperscript{24} Subsequent structural studies have since confirmed and extended the 3-state model of myofilament activation.\textsuperscript{25} As shown in Figure 2, the average position of TM in each of the 3 “states” determines the interaction between actin and myosin that in turn leads to force generation. Given that thin filament regulation is fully dependent on the precise coordinated movements of multiple proteins, it is perhaps not surprising that a wide range of thin filament protein mutations can disrupt the function of this highly tuned molecular machine and cause complex cardiomyopathies.

Mutations in Thin Filament Proteins Alter Structure and Dynamics to Disrupt Myofilament Function and Cause FHC

The original findings that mutations in thin filament proteins caused FHC led to an initial series of important in vitro
investigations that identified many of the basic effects of disease mutations on myofilament activation. Impairments in ATPase activity, the Ca\textsuperscript{2+} sensitivity of force generation and the importance of mutant protein dosage on function were all detailed in early work from several independent groups.\textsuperscript{26–28} Interestingly, the disease linkage discovery was also concurrent with the basic studies establishing the biophysics of the 3-state model of myofilament activation (reviewed elsewhere\textsuperscript{29}) and contributed to a renewed interest in understanding the unique biology and structure of the cardiac sarcomere. This effort extended the original NMR and FRET-based approaches and led to the first high-resolution crystal structure of the human Ca\textsuperscript{2+}-activated cardiac thin filament core domain in a landmark study by Takeda et al.\textsuperscript{30} Subsequent studies using reconstituted thin filaments followed by 3D reconstruction confirmed and extended the Takeda model and the current extant structure of the thin filament represents the basis of any approach to link genotype to phenotype.\textsuperscript{31} As noted above, whereas crystal structures are, by definition, static representations of atomic position at a given time point, proteins are inherently dynamic. The inherent flexibility of the thin filament (especially cTnT and TM) has been previously noted; in fact, the flexibility of cTnT N-terminal region contributed to the difficulty in obtaining crystals of the Ca\textsuperscript{2+}-activated cTn core domain.\textsuperscript{30} The concept of single protein flexibility can be addressed at multiple levels of resolution and is an important component of the basic concept of allostery, whereby local changes in structure are translocated to distant regions of the protein and affect function. More recently, studies in enzymes have revealed that mutations at a distance from the active site can significantly alter the rate of catalysis via “time-dependent motions” (protein dynamics).\textsuperscript{32,33} Work from our laboratory originally applied these computational approaches (molecular dynamics [MD]) to evaluate local changes in protein flexibility caused by cTnT FHC mutations.\textsuperscript{34} Since that time, the use of MD to extend the resolution of our molecular understanding of dynamic changes in thin filament function to the atomic level has become a component of an approach to evaluate the precise effects of independent thin filament mutations on structure and dynamics.\textsuperscript{35,36} In the next sections, we detail how these high-resolution approaches to understanding the molecular pathogenesis of thin filament related FHC have begun to bridge the divide between genotype and phenotype.

**cTnI and cTnC: The Ca\textsuperscript{2+} Sensor and Trigger for Myofilament Activation**

As noted above, cTnI and cTnC together comprise a functional complex that directly transmits the Ca\textsuperscript{2+} binding status of the single functional regulatory site (site II) on cTnC to the thin filament via cTnT (Figure 3). It is this basic binding step that triggers the initial blocked (B) – closed (C) transition and

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**Figure 2. The 3-state model of myofilament activation.** The 3 average positions of TM are depicted. TM resides at the outer actin domain in the blocked state (red). Ca\textsuperscript{2+} binding to cTnC results in an azimuthal shift to the weakly bound closed state (yellow) in the actin inner domain, and myosin binding drives the final shift to the force-producing open state (green).

**Figure 3. cTnI exons, structure, and functional domains.** Listed mutations are discussed in the text. **Ser 23/24** PKA-dependent phosphorylation sites. Mutations in gray have been linked to primary DCM and mutations in italic have been linked to RCM. Inset, predicted cTnI secondary structure.
is absolutely required for myofilament activation. Because these interactions are fully interdependent, it is illustrative to consider the effects of disease-causing mutations on the functional cTnI:cTnC complex.

Since the first reported linkage study in 1997, mutations in cTnI represent the most extensively characterized clinical subset in FHC. Although the original linkage study did not provide clinical profiles, a subsequent report focusing on an in-frame deletion of a residue directly adjacent to the second actin–TM binding site of cTnI (ΔLys183) provided a comprehensive characterization of 25 individuals across 7 families carrying the mutation. This study was notable for high disease penetrance, age-independent SCD, extreme variability of the observed ventricular remodeling both with respect to degree of left ventricular hypertrophy (LVH), and regional distribution and evidence of progressive LV dilatation after age 40 years. The broad range of clinical phenotypes caused by the same gene mutation, including individuals within the same family, was surprising. The clinical characterization was made more complex by the observation that mutations in cTnI contributed to the development of restrictive physiology both within HCM families and as an apparent rare primary manifestation. Important considerations regarding the interpretation of many of the initial family studies are the persistent effects of small family size and referral bias. Subsequent studies directly addressed this concern by assessing the frequency of sarcomeric gene mutations in large populations of unrelated patients with HCM. Although the overall frequency of thin-filament mutations ranged from 3% to 8%, the landmark kin-cohort study by Mogensen et al provided a uniquely detailed, longitudinal characterization of the striking phenotypic variation in cTnI-related FHC, derived from 748 HCM families, encompassing 100 mutation carriers with 13 different cTnI mutations. The basic findings regarding the distribution of disease-causing mutations (largely exons 7 and 8), the presence of HCM and RCM within single families and the wide range of ages for initial disease presentation (second to eighth decade of life) largely confirmed the results of previous reports. Of particular note, the 10-year follow-up period again established the plasticity of the ventricular remodeling with clear disease progression. More recently, 2 cTnI mutations were definitively linked to autosomal dominant DCM that was notable for rapid clinical progression after presentation (Lys36Gln, Asn185Lys). Finally, although ≈8 independent cTnI missense substitutions have been associated with primary cardiomyopathies, with the exception of the Gly159Asp DCM mutation, the clinical data are restricted to individual patients or very small families, thus greatly limiting the phenotypic characterization. In the main, it is apparent that the end cardiac phenotypes caused by mutations in cTnI:cTnC are complex, progressive, and often strongly dependent on when the disease is first diagnosed, an important consideration for establishing mechanistic links between genotype and clinical phenotype.

To begin to elucidate the structural mechanism(s) of cTnI:cTnC cardiomyopathies, it is useful to first examine the distribution of cTnI mutations within the known functional domains, as shown in Figure 3. The basic structure of cTnI represents a common motif among the 3 proteins of the cTn complex. Highly α-helical domains are separated by linker sequences of varying lengths and convey a high degree of flexibility to the overall structure. Approximately 35 independent mutations in cTnI have been linked to FHC. A striking degree of structural “clustering” is apparent with >75% of the known mutations falling within either the highly charged inhibitory region (cTnI\textsubscript{\text{I}} In: residues 137 to 148) or the proposed mobile region that contains the second actin–TM binding domain (cTnI\textsubscript{\text{TM}}: residues 164 to 210). Both of these regions were poorly defined within the extant crystal structure of the Ca\textsuperscript{2+}–activated cTn core domain, likely because of their high inherent motility. This mutational clustering within the highly flexible domains of the troponin core is a recurrent observation and may reflect a “tolerance” of the thin filament to functional alterations in dynamic components of the regulatory thin filament. These are regions that change in orientation and position as part of normal activation, and thus mutations within these domains likely alter these dynamic movements without fully abolishing the regulation of thin filament activation (reviewed elsewhere). Conversely, the regions that form rigid structural components of the cTn core and/or extended protein–protein interactions are virtually devoid of known mutations. A case in point is the highly conserved cTnI components of the rigid IT arm comprised of the H1 (cTnC–C-lobe bound) and H2 (cTnT-bound) helices, where the Pro82Ser cTnI mutation is located within the flexible linker that bridges the 2 helices and thus is unlikely to significantly disrupt the overall structure of the extended IT arm.

In contrast, the HCM/DCM-linked mutations in cTnC are evenly dispersed throughout the complex bilobed structure comprised of multiple α-helices connected by flexible linkers.

A broad range of in vitro studies have been performed to assess the primary functional effects of cTnI:cTnC mutations on the regulation of myofilament activation. It is informative to consider these studies in the context of 2 of the basic physiological roles of the complex, to directly translate the binding of Ca\textsuperscript{2+} to site II of cTnC and trigger the onset of the B-to-C-to-M state transitions and to transduce an increase in hemodynamic demand via protein kinase (PK)A-induced phosphorylation of Ser23/24 in the N-terminal domain of cTnI to the cardiac sarcomere.

cTnC has 3 discrete Ca\textsuperscript{2+} binding sites, and initial myofilament activation is triggered solely via oscillatory Ca\textsuperscript{2+} binding to site II in the regulatory N-lobe. The resultant exposure of the hydrophobic “patch” in the N-lobe that binds to the cTnI switch domain leads to the release of the adjacent inhibitory and second actin–TM binding domains, facilitating the movement of TM and subsequent formation of weak actomyosin cross-bridges (Figure 3). As noted above, these 2 domains contain >75% of all cTnI mutations and several groups have investigated the functional effects of disrupting these regions of the protein. An initial report by Elliott et al showed that both the cTnI Arg145Gly and Arg162Trp mutations led to a significant increase in the Ca\textsuperscript{2+} sensitivity of ATPase regulation and a decrease in the inhibition of the actin–TM activated ATPase activity. These findings were confirmed and extended in an extensive study using cTn exchange into skinned rabbit muscle fibers to directly mea-
sure the Ca\(^{2+}\)-sensitivity of force development. \(^{44}\) Virtually all of the cTnI mutations studied (Arg145Gly, Arg145Gln, Arg162Trp, ALys183, and Lys206Gln), spanning all 3 of the C-terminal cTnI functional domains, caused a fundamental increase in Ca\(^{2+}\) sensitivity, a result consistent with an increase in myofilament activation at submaximal [Ca\(^{2+}\)]. The predicted physiological result would be a significant impairment in cardiac relaxation, a finding that was fully confirmed at the whole-heart level in the subsequent Arg145Gly transgenic mouse model. \(^{45}\) These studies established the basic observation that mutations in cTnI linked to a “hypertrophic” (or more accurately, “nondilated”) phenotype adjacent to the Asp190His and Asp192His mutations) demonstrate that the extreme C-terminal domain of cTnI (immediately adjacent to the Asp190His and Asp192His mutations) acts to stabilize TM in the Ca\(^{2+}\)-activated state. \(^{47}\) The basic mechanism posits an electrostatic-mediated search function for the mobile domain of cTnI via a “fly-casting” mechanism once it is released from its B-state position (bound to actin) after Ca\(^{2+}\) binds to TnC. \(^{48}\) As a corollary to this role in the Ca\(^{2+}\) activated state, it is likely that a successful “search” would facilitate a rapid return to the B state (relaxation) when Ca\(^{2+}\) is released. Therefore, as predicted by Kobayashi and Solaro and in a subsequent study by Mathur et al, mutations in the C-terminal domain of cTnI lead to an apparent increase in the Ca\(^{2+}\)-sensitivity of ATPase activity by altering the expected equilibrium position of TM, and this mechanism, in part, represents the most proximal cause of cTnI-related cardiomyopathies at the myofilament level. \(^{46,49}\)

The maintenance of cardiac output via the beat to beat regulation of contractility and relaxation is a central component of cardiovascular function. \(^{21}\) The PKA-mediated phosphorylation of cTnI at Ser23/24 in response to \(\beta\)-adrenergic activation represents a basic regulatory mechanism to directly transduce this signal to the myofilament level. The combined physiological effects of cTnI Ser23/24 phosphorylation include a decrease in the Ca\(^{2+}\) sensitivity of force generation, an increase in the off rate of Ca\(^{2+}\) from TnC site II, an increase in the cross-bridge cycling rate, and an increase in the rate of relaxation (reviewed by Metzger and Westfall \(^{40}\)). It had long been noted that patients with HCM exhibited blunted or abnormal cardiac responses to \(\beta\)-adrenergic stimulation. The discovery that mutations in cTnI caused HCM (and more recently DCM) raised the important question of whether this integral function of cTnI as a modulator of the \(\beta\)-adrenergic response was altered by disease mutations. There are at least 2 possible mechanisms whereby cTnI:cTnC mutations might interfere with this basic function. First, mutations could alter the PKA-mediated phosphorylation “potential” at Ser23/24, leading to a decrease in the expected level of phosphorylation for a given stimulus. Second, the expected physiological response to the phosphorylation event may be altered by mutation. Initial studies by Deng et al addressed the latter question using reconstituted thin filaments in actin-S1 ATPase assays to assess the effects of the cTnI Arg145Gly, Gly203Ser, and Gly206Gln mutations. \(^{51,52}\) Although the Arg145Gly mutants exhibited the expected increase in the Ca\(^{2+}\) sensitivity of ATPase activity, the expected desensitization after bisphosphorylation was not observed. Neither of the C-terminal mutations had an effect on Ca\(^{2+}\) sensitivity in the dephosphorylated state. It is interesting to speculate that the predicted effects of the Arg145Gly mutation would be particularly severe because the increase in Ca\(^{2+}\) sensitivity coupled to the loss of the PKA-mediated desensitization would affect both baseline diastolic function and the ability to recruit additional cardiac reserve in the context of increased hemodynamic demand. Of note, a subsequent analysis of the Arg21Cys cTnI mutation by Gomes et al revealed a significant increase in the Ca\(^{2+}\) sensitivity of force development in skinned fibers at baseline and a minimal desensitization in response to PKA phosphorylation, similar to the findings for Arg145Gly. \(^{53}\) In contrast, however, the impaired response was caused by a decrease in the level of Ser23/24 phosphorylation, consistent with a potential local change in the phosphorylation potential, perhaps in part because of the loss of the Arg residue at 21 that is part of the PKA recognition sequence. Thus, cTnI mutations can exhibit direct effects on the posttranslational regulation of myofilament activation.
More recently, significant advances have been made in elucidating both the structure of the N-terminal domain of cTnI and the nature of the inter- and intramolecular interactions within the cTnI:cTnC complex that are modulated by phosphorylation at Ser23/24. As noted previously, several regions of the Ca\(^{2+}\)-activated cTn core domain including the cardiac-specific N-terminal domain (residues 1 to 30) were not included in the crystal complex.\(^{30}\) The lack of high-resolution structure for this crucial domain has limited our understanding of the regulatory mechanism. A broad range of biophysical studies using an array of methodologies including FRET, ESR, and solution NMR had established that the N-terminal domain is highly flexible and maintains a relatively weak affinity for the N-lobe of cTnC in the dephosphorylated state and that phosphorylation at Ser23/24 induces a significant conformational change of the N terminus (reviewed elsewhere).\(^{54}\) To develop an atomic level model of troponin, Howarth et al recently used solution NMR of fragments of the N terminus of cTnI in multiple states, including bisphosphorylated, unphosphorylated, and DD-fragments of the N terminus of cTnI in multiple states, troponin, Howarth et al recently used solution NMR of fragmenting the interaction with the highly basic inhibitory domain via the Ca\(^{2+}\)-dependent affinity of the N terminus for the N-lobe of cTnC and, in C-terminal helix (residues 21 to 30) that further decreases the N-terminal extension of cTnI and the N-lobe of cTnC (and decrease Ca\(^{2+}\) affinity) as described above. These results were confirmed by a later study that revealed a significant uncoupling between Ser23/24 phosphorylation of cTnI and the Ca\(^{2+}\) sensitivity of force generation in skinned cardiac myocytes isolated from a patient carrying the Gly159Asp cTnC mutation.\(^{60}\) Thus, although the mechanism whereby this primary effect on the Ca\(^{2+}\) sensor for tuning the physiological response to increased hemodynamic demand would lead to a primary dilated cardiomyopathy remains unclear, the development of more integrated and specific approaches to address precise changes in thin filament dynamics caused by independent mutations has begun to elucidate the complex links between biophysics and physiology in FHC.

**cTnT: Altering the Flexibility and Orientation of the Central Thin Filament Adapter Has Far-Ranging Effects on Myofilament Activation**

cTnT was one of the first thin filament proteins to be linked to FHC, and although more than 30 mutations have been identified to date, our understanding regarding the molecular mechanism(s) that underlie this subset remain limited, in part because of the lack of a high-resolution structure of the N- and C-terminal domains. As noted by Tobacman in his extensive 1996 review, cTnT represents the “glue” of the thin filament because it links the cTnI:cTnC complex to TM–actin.\(^{64}\) In our laboratory, it is the “homerun protein” because it “touches them all”; the intricate and highly dynamic array of protein–protein interactions that determine myofilament activation are fully dependent on the highly flexible cTnT molecule.

Although cTnT was one of the first thin filament proteins to be linked to FHC, relatively few large family studies are available, and thus the clinical characterization remains somewhat in flux. In particular, the question of whether patients carrying a subset of cTnT mutations exhibit a high frequency of SCD in the context of relatively mild LVH has been of particular interest because it suggests that the disease mechanism is inherently myocellular as opposed to being dependent on ventricular morphology, a central issue regarding the ability to noninvasively diagnose the disorder. This
potential “dissociation” between the degree of ventricular morphology and the risk of sudden or disease-related death in cTnT-linked FHC was first noted by Watkins et al in the original clinical characterization of 8 independent mutations across 26 kindreds.62 Although several subsequent studies did not fully support this observation, given the continuing limitations regarding the relatively low frequency of cTnT mutations, small family size and referral bias, the definitive conclusion remains elusive.63 Again, as with cTnI:cTnC mutations, it is important to note that if mild LVH (eg, subclinical and/or asymptomatic) is a distinguishing characteristic, patients with this clinical profile would be underrepresented in any HCM clinic, especially those with large surgical referral cohorts in which many of the patients have obstructive physiology (rarely observed in patients with thin filament mutations). Two subsequent reports identified a second independent mutation at residue 92 of cTnT (Arg92Trp).64,65 In the study by Moolman et al,64 the patient profile was similar to the previously described cTnT mutations, with minimal LVH, low disease penetrance via echocardiogram, and a high frequency of SCD, especially in males. More recently, a long-term follow-up study based on the same patient cohort revealed late-onset (>35 years of age) hypertrophy of the interventricular septum in 46% of carriers, with no evidence of progression to a dilated phenotype.17 Whereas patients with the cTnT Arg92Gln/Trp mutations exhibit a relatively consistent early-onset clinical phenotype, this is not the case with several of the more C-terminal mutations, for example Arg278Cys, where late-onset, symptomatic septal hypertrophy has been observed.66 Finally, a recent report by Menon et al described a multigenerational family carrying the Ile79Asn cTnT mutation exhibiting a fully mixed HCM/RCM phenotype.67 Thus, similar to the complexity evidenced by patients carrying mutant cTn proteins, patients with cTnT-related FHC defy easy morphological characterizations and require extended follow-up, and “mild hypertrophy” does not necessarily correlate with a “low” risk for SCD.

In 2000, Kamisago et al published the first linkage of primary DCM to an in-frame single amino acid deletion of cTnT Lys210 (ΔLys210).12 The clinical findings were notable for early-onset DCM without any evidence of precedent hypertrophy, thus suggesting that the pathogenic pathways for diluted and nondilated forms of cTnT-linked cardiomyopathies were likely to be distinct, a hypothesis that continues to be actively investigated to this day. Subsequent family studies identified several additional DCM-linked cTnT mutations (Arg131Trp, Arg141Trp, Arg205Leu, and Asp270Asn).56,68 Unlike the mixed morphologies noted in the HCM cohorts, many of the DCM-linked families exhibited high penetrance and severity, with early cardiac transplantation a not-infrequent outcome. Of note, mutations that cause primary DCM are relatively common in cTnT, comprising ≈20% of all known mutations. Moreover, as shown in Figure 4, the distribution of the HCM and DCM mutations are tightly interspersed within the C-terminal domain of TNT1 and the immediate N-terminal domain of TNT2, an intriguing finding for such disparate clinical entities that likely reflects the highly tuned function of this integral adapter protein within the cardiac sarcomere.

As shown in Figure 5, similar to the findings for cTnI: cTnC, disease-causing mutations in cTnT are highly clustered within 2 primary locations, all located in regions of the protein that are predicted to be highly mobile. The “classic” organization of cTnT into 2 major functional domains (TNT1, TNT2) is largely based on chymotryptic digest studies and likely demarcates regions of the protein that are accessible to proteolysis.69 The TNT2 domain is directly bound to cTnC: cTnC comprises a significant portion of the rigid IT arm (residues 203 to 271). This region was included in the Ca<sup>2+</sup>-activated core Tn crystal structure and consists of 2 α-helices (H1: 204 to 220; H2: 226 to 271) connected via a short 5-aa linker sequence.30 Several of the known TNT2 mutations occur at the extreme N-terminal portion of the H1 helix and have been exclusively associated with DCM.12,39,70 The remaining regions of the protein include the extended N-terminal (comprised of the N-terminal “hypervariable” region and the largely α-helical TNT1) and the extreme C-terminal (C-TnT) domains that were either not included in the cTn complexes that were crystallized or could not be resolved in the extant structures, likely because of the high degree of inherent protein flexibility required to modulate the physical position of TM in response to Ca<sup>2+</sup> binding. In the main, these unresolved domains include more than 90% of the known disease-causing mutations in cTnT, and this lack of structural information remains an important limitation to elucidating the molecular mechanisms of this complex and often severe disease subset. The basic functional role of cTnT is to directly modulate the average position of TM between the 3 known states of myofilament activation. The response of the thin filament to both basal and increased hemodynamic demand is highly regulated by Ca<sup>2+</sup> binding, posttranslational modifications, and the direct effects of myosin and stretch. These inputs are modulated locally by direct interactions between cTnI and cTnC within the globular core
domain of cTn. The critical mechanism(s) whereby changes in these local protein–protein interactions are transduced to the elongated N-terminal domain of cTnT, the region directly responsible for both modulating the position of TM along the actin groove and stabilizing the head-to-tail array of contiguous TM molecules is unclear. Conversely, cTnT mutations in the N-terminal domain, in particular, occur at a great physical distance from the regulatory cTnI:cTnC complex. Thus, the disease mechanism underlying this large subset of cTnT-linked FHC mutations is likely to be strongly influenced by the allosteric propagation of the local changes in cTnT function to the rest of the thin filament “machine.”

The central role of cTnT in modulating myofilament activation coupled to the large number of known mutations (with distinct clinical phenotypes) has led to a broad array of studies by our laboratory and others, using biochemical, biophysical, computational, and animal model methodologies. Despite the lack of a high-resolution structure for much of cTnT, this in silico to in vivo approach has begun to provide important mechanistic insight. Missense substitutions, single in-frame amino acid deletions, and premature termination mutations have all been linked to cTnT-related FHC. As shown in Figure 5, there is significant mutational clustering between residues 69 to 110, with 17 independent mutations and 3 “hotspots” at residues 92, 94, and 110. Although no primary DCM mutations have been mapped to this region, the observed clinical phenotypes are highly variable both within single families (Ile79Asn) and between independent substitutions at the same amino acid (Arg92Gln/Trp/Leu).62,71 This clustering suggests that the N-terminal domain plays a complex modulatory role, whereby mutations alter but do not destroy normal function. Initial biophysical studies had described the elongated troponin tail as a Ca\(^{2+}\)-independent anchor between cTn and the TM–actin complex.72 In 1999, Hinkle et al used a series of full-length and truncated bovine cTnT molecules in binding studies to show that the residues 1 to 153 of the cTnT tail domain promoted TM binding to actin in the absence of cTnI:cTnC.73 This observation along with previous reports raised the important question of whether cTnT independently contributed to the stabilization of TM in the low Ca\(^{2+}\) B state. In a subsequent study, myosin-activated ATPase, regulated in vitro motility and myosin binding assays were used to demonstrate that residues 1 to 153 of cTnT alone exerted a strong inhibitory effect on the interaction of myosin with the rest of the thin filament.74 Moreover, electron microscopy and 3D reconstruction on reconstituted thin filaments plus cTnT 1 to 153 performed in the absence of Ca\(^{2+}\) revealed that cardiac TM was localized to the outer domain of actin, consistent with a strengthening of the TM–actin interaction and suggested that the N-terminal tail played an important role in the inhibition of myofilament activation. Disruption of the native inhibitory function of the N-terminal tail by FHC-linked mutations would thus likely alter the distribution of TM between the B and C states under conditions of low Ca\(^{2+}\) and be manifest as an increase in the Ca\(^{2+}\) sensitivity of myofilament activation, a prediction that was fully confirmed by an extensive series of studies by multiple groups using reconstituted systems, both solution-based and in skinned fibers.27,28,75,76 It is important to note that although these findings are consistent, as previously shown for mutations in cTnI:cTnC, measurements of Ca\(^{2+}\) sensitivity, per se, are relatively low resolution and represent the complex outcome of multiple inputs and are thus somewhat limited in the context of determining precise mechanisms for nondilated mutations.

Two independent studies had directly examined the biophysical effects of N-terminal mutations on TM-dependent properties of cTnT in both fragments (residues 70 to 170) and
full-length protein complexes. Several mutations that immediately preceded the crucial TM binding domain at residues 112 to 136 (Arg92Gln/Trp/Leu, Ala104Val, and Phe1101lle) all decreased cTnT TM affinity and were less effective at promoting TM–actin binding. These effects were likely attributable to changes in a-helical stability that would be predicted to significantly alter the flexibility of the N-terminal tail. Interestingly, mutations outside this range (Ile79Asn, Δ160Glu, and Glu163Lys) did not disrupt TM-dependent properties. Thus, similar to the findings for the C-terminal cTnI mutations, the degree of flexibility within this crucial cTnT domain acts to regulate the dynamic interactions with TM that, in part, determines the average position of TM as it transitions between the 3 states. Although the original studies could predict the qualitative effects on N-terminal flexibility, to further delineate the potential impact of individual mutations on flexibility and structural dynamics at the atomic level, our laboratory has applied MD approaches. Mutation-specific alterations in peptide flexibility for each of the 3 known hotspot substitutions at residue 92, Arg92Gln/Trp/Leu were observed. Of note, patients with each of these substitutions have been identified and exhibit mutation-specific patterns of ventricular remodeling. Average snapshots of the predicted motion reveal a striking “hinge” at residue 104 (Arg92Leu⇒Arg92Trp⇒Arg92Gln), 12 amino acids distant from the mutation site, a region that is known to be important for protein solubility and the site of a mutation linked to complex cardiac remodeling (Ala104Val). Based on this finding, we recently performed additional analyses to determine how the mutations at residue 92 lead to helical unwinding at residue 104. Examination of carbon–carbon distances (n=4) for the wild type and Arg92Trp, Arg92Leu revealed that the Trp and Leu substitutions induce a local helical electrostatic compaction and a subsequent helical expansion in the residue 104 region. Thus, the “bunching” of residues in the helix turns at the mutation site eventually results in a relative expansion of the helix turns in the first turn composed of hydrophobic residues resulting in mutation-specific unwinding and an increase in flexibility. These findings are fully consistent with both the previous biophysical studies and our original hypothesis that the predicted dynamic changes induced by the residue 92 mutations would alter the structural character in the hinge region while maintaining the overall helical character of the peptide, thus “modulating” as opposed to destroying thin filament function. In addition, the observation that the molecular effects of single amino acid substitutions can be propagated throughout the helix to effect structural and dynamic changes at a significant physical distance suggests that mutations at independent residues can lead to similar effects on function. In this manner, the mutations clustered immediately proximal to the cTnT-TM binding domain at 115 to 139 may have similar effects on function by acting at a distance to alter the residue 104 hinge region, a conclusion that could not easily be obtained via experiment.

Although the vast complexity of the cardiac remodeling and clinical phenotypes observed in the hypertrophic or nondilated patient cohorts has been mirrored by the biophysical findings, the DCM-linked cTnT mutations are, in general, more consistent on both levels. The first cTnT mutation to be linked to DCM was the in-frame deletion of a single Lys residue at 210. As noted above, many of the DCM-linked thin filament mutations cause a relatively severe, early-onset dilated cardiomyopathy that, in most cases, appears to be a primary event, as opposed to transitioning through a hypertrophic phase. Initial biophysical studies showed that, unlike virtually all of the HCM-linked thin filament mutations, the DCM-linked ΔLys210 and Arg141Trp mutations led to a primary decrease in the Ca^{2+} sensitivity of tension when exchanged into skinned rabbit fibers, a result that would likely affect the ability of the heart to generate force. This surprising observation was subsequently expanded to nearly all of the known cTnT DCM mutations in a study by Mirza et al. Using reconstituted human thin filament proteins in actin–TM activated ATPase and in vitro motility assays performed at both 100% and the expected physiological 1:1 ratios of WT and mutant cTnT, they demonstrated that the DCM-linked mutations all caused varying degrees of Ca^{2+} desensitization and decreases in thin filament activation. Thus, the prevailing hypothesis for the clear discordance in cardiac remodeling between HCM and DCM was that the primary pathogenic mechanism is determined by the changes in Ca^{2+} sensitivity at the myofilament level. This was directly tested by Du et al by generating a knock-in murine model of the cTnT ΔLys210. Both the heterozygous (+/ΔLys210) and homozygous (ΔLys210/ΔLys210) mice were studied from the myocellular to whole heart levels, with the ΔLys210/ΔLys210 animals exhibiting a particularly severe early-onset and rapidly progressive course. Several important observations stand out. First, isolated fibers from both lines exhibited a decrease in Ca^{2+} sensitivity without a significant decrease in maximal isometric tension in the context of normal cTnI phosphorylation. Second, the amplitude of the Ca^{2+} transient and SR Ca^{2+} load were elevated in both mutant mouse lines, a potential compensatory change to a perceived lack of Ca^{2+} “responsiveness” at the myofilament level. Lastly, in a direct test of the potential mechanism, pimobendan, a potent phosphodiesterase III inhibitor and direct myofilament cTnC Ca^{2+} sensitizer was orally administered to both mutant lines starting at 1 month of age. Treated ΔLys210/ΔLys210 mice exhibited a significant abrogation of virtually all of the observed pathogenic parameters including cardiac remodeling, increased Ca^{2+} transients, and improved survival despite persistence of the initial decrease in Ca^{2+} sensitivity at the myofilament level. Although this reversal is supportive of the basic hypothesis, both the severity of the ΔLys210/ΔLys210 phenotype (with rapid activation of apoptotic activity and precedent episodes of torsades de pointes and ventricular fibrillation) and the mild response to pimobendan in the +/ΔLys210 mice suggest that multiple downstream pathogenic signaling pathways are highly activated in the homozygous animals. Regardless, the ability to disrupt the pathogenic cascade with pimobendan is an encouraging and original observation from the clinical standpoint. Despite this high degree of consensus regarding the role of a decrease in the Ca^{2+} sensitivity of force development as a primary cause of DCM (in a field that is usually notable for the lack thereof), the structural mechanism(s) remain unclear. As is
shown in Figure 4, virtually all of the known DCM-linked cTnT mutations fall within the C-terminal portion of TNT1 or the extreme N-terminal portion of TNT2 and are closely interspersed with known nondilated mutations including a hotspot at 160 to 163. Residues distal to 203 were included in the core domain structure and comprise the initial component of the H1T2 helix (and the IT arm) and thus may directly affect Ca\(^{2+}\) sensitivity via interactions with the C-lobe of cTnC, although direct evidence is lacking. In contrast, although the TM-binding domain of TNT1 is thought to be \(\sim 70\%\) \(\alpha\)-helical, the C-terminal end of the region includes a highly charged sequence (157- RREEEEENRR -166) that is likely to be accessible to solvent and may unwind to form a long flexible linker to TNT2. This domain is very highly conserved, and a better understanding of the structure and function of this unresolved region would provide a framework for determining the molecular mechanism that underlies the development of HCM versus DCM in cTnT-linked cardiomyopathies.

**TM: The “Gatekeeper” of Myofilament Activation**

TM and cardiac actin form a unique functional unit that plays both structural and dynamic roles in sarcomeric function. Moreover, both of these integral thin filament proteins impact the thin filament beyond the single basic 7:1:1 functional unit. The head-to-tail array of contiguous TM coiled-coil dimers is a central component of the cooperative activation of the myofilaments, and actin interacts with the myocellular cytoskeleton via attachments at the Z-band; thus, mutations in either of these thin filament proteins have the potential for far-reaching and complex effects on cardiac function.

Although TM was one of the first thin filament proteins to be linked to FHC, it remains an infrequent cause of the disease, accounting for \(\sim 3\%\) of sarcomeric gene mutations. Many of the available studies rely on relatively small clinical cohorts and suggest a strong influence of genetic modifiers and environment on the end phenotype. A case in point is the Asp175Asn mutation, identified in the original linkage study and subsequently found in both Northern European and Japanese kindreds. Initial clinical profiles of the initial patient population described a highly variable pattern of mild-to-moderate LVH and an overall favorable prognosis with a loss of cardiac reserve that is thought to arise in the context of myocarditis. This is perhaps a structural analog to the precipitous loss of systolic function that occurs in the context of myocarditis. This pattern of early-onset, severe phenotypes. Interestingly, the Glu99Lys mutation in cardiac actin has been linked to the development of apical hypertrophy in 2 independent studies, and this atypical pattern of cardiac remodeling is thought to represent the influence of specific, unknown genetic modifiers. Thus, mutations in these 2 integral components of the thin filament contribute to complex clinical patterns that are strongly modulated by genetic and environmental factors, an observation that was validated experimentally by 2 independent transgenic mouse models of the Glu180Gly TM mutation where the resultant animals exhibited highly divergent phenotypes that were attributed to the effects of different genetic backgrounds.

Mutations in both TM and cardiac actin have been linked to familial DCM. Olson et al used a candidate gene approach to screen for TPM1 mutations in 350 unrelated patients with idiopathic DCM and identified 2 novel mutations, Glu40Lys and Glu54Lys. Although the family sizes were small and the age of presentation variable, both mutations led to a primary dilated LV phenotype without evidence of an intermediary hypertrophic stage. A similar approach by the same group had previously identified 2 mutations in cardiac actin associated with primary DCM, Arg312His and Glu361Gly. Definitive DCM linkage via segregation analysis was recently established for a novel TM mutation (Asp230Asn) in a extensive study by Lakdawala et al where 2 large independent multigenerational families carrying the Asp230Asn mutations were identified and studied longitudinally. Many of the initial clinical observations were similar to those noted for the A210Lys DCM mutation in cTnT, including early-onset primary ventricular dilatation in children and young adults and a high frequency of SCD. The extended clinical follow-up of these unique kindreds also revealed a bimodal pattern of disease expression with distinct “peaks” of presentation in early childhood (severe LV dilatation) and middle age (mild-to-moderate LV dilatation). Of particular note, as previously described in other thin filament-related cases of DCM, many of the affected children responded to aggressive supportive medical therapy with stabilization of the disease process and significant recovery of LV systolic function by early adulthood. The authors posited that the presence of the Asp230Asn TM conferred a susceptibility to secondary cardiac injury, for example, amplifying the precipitous loss of systolic function that occurs in the context of myocarditis. This is perhaps a structural analog to a loss of cardiac reserve that is thought to arise in the context of sarcomeric gene mutations that decrease the energetic efficiency of the heart. It is possible that this potential “second-hit” mechanism for the Asp230Asn TM mutation represents another example of the unique sensitivity of TM and cardiac actin mutations to extrasarcomeric modifiers including environmental, genetic, and external pathogenic stimuli. As noted above, these 2 proteins are very highly conserved, both play essential structural and regulatory roles, and disease-causing mutations are relatively rare. Although speculative, some TPM1 and ACTC mutations may only be tolerated when their baseline effects on sarcomeric function are relatively mild, and thus more severe manifestations occur.
only in the context of additional factors. The findings from the recently published mutant cardiac actin Glu316Gly transgenic mouse model generally support this hypothesis in that the 50% replacement mouse exhibited a minimal baseline phenotype at all levels studied and required β-agonist infusion to uncover a pathogenic response.95

Taken to its logical conclusion and given both the ordered structure and the dynamics of the movements that govern the Ca$^{2+}$ regulation of myofilament activation, it is somewhat surprising that mutations in either TM or cardiac actin are tolerated in nature. Indeed, the relatively low incidence of FHC-related mutations in TM and cardiac actin likely reflect a relative intolerance to structural and/or functional disruption of this essential interaction. TM represents a classic coiled-coil semirigid structure comprised of 2 α-helices arranged in parallel (reviewed elsewhere96). Each TM α-helix is defined by a continuous series of 40 short-range heptad repeats (7 residues, a-b-c-d-e-f-g) that extend over 2 helical turns. Residues at positions αd/εd are generally apolar and act to internally stabilize the dimer, whereas acidic residues at αe/εf of the heptad repeats interact with the basic residues on the flat surface of actin to form an electrostatic “sliding surface,” facilitating the TM movements that determine the 3 states of myofilament activation and contribute to stability of the overall structure. These basic amino acids also define a long-range periodicity ≈40 residues long that matches the pitch of consecutive actin monomers. Each TM supercoil is composed of 7 such “periods” that are not 100% regular in spacing and contain small clusters of core alanine residues at positions αd/εd that serve to locally destabilize/narrow the structure, leading to a “stagger” and resultant bend in the coiled-coil that is central to the actin–TM interaction.97,98

An elegant series of mutational and “period-swapping” studies by Singh and Hitchcock further established that both the acidic surface residues and the instability conferred by the alanine clusters are required for TM–actin binding. Moreover, this requirement defines a hierarchy whereby periods 1 and 5 represent primary and 2 to 3 and 6 to 7 are secondary actin binding sites.99 This hypothesis requires an inherent local flexibility because the primary sites serve as pseudoanchors, whereas the secondary sites “search” for optimal interactions with actin, a dynamic process that is likely central to myofilament activation and may be directly affected by TM-related disease mutations. Finally, whereas the interactions between a single TM coiled-coil and 7 actin monomers defines the basic contractile functional unit, TM forms a contiguous array of head-to-tail dimers that form complex overlapping structures and strongly stabilize the actin filament throughout its length. Although the overall structure of these crucial TM overlap regions remains unclear, they play a central role in the cooperativity that is essential to the coordination of contractile function at the molecular level.100,101

As shown in Figure 6, most of the known HCM-related mutations in TM are clustered within or flanking 2 major regions of the protein, the N terminus (Gly62Glu, Ala63Val, Lys70Tyr, Val95Ala) and period 5 (Ile172Thr, Asp175Asn, Glu180Gly, Glu180Val, Leu185Arg, Glu192Lys). Two of the original TM mutations identified (Asp175Asn, Glu180Gly) occupy positions in the heptad repeat predicted to stabilize the coiled-coil via electrostatic interactions and are within a domain of TM that weakly binds to the globular head region of Tn (via TNT2). They have been extensively studied both in vitro and via transgenic animal models. To date, the findings have been strikingly consistent across both species and experimental approaches. Both Asp175Asn and Glu180Gly increase the Ca$^{2+}$ sensitivity of myofilament activation.102,103 Similar results were obtained for all of the known HCM-linked mutations tested, thus fully recapitulating one of the central observations regarding the effects of HCM-linked thin filament protein mutations. Based in part on the predicted effects of εf substitutions on TM stability, several groups determined whether there were mutation-specific effects on thermal properties and/or actin binding. Again, there was strong consensus across methodological approaches because several groups showed that the Glu180Gly substitution had a greater effect (eg, a decrease in actin binding or an increase in thermal unfolding) than Asp175Asn.104,105 Because of the heterogeneity in the known patient populations, it remains unclear whether these measurable mutation-specific differences in TM local structure specifically influence clinical outcomes. Results from transgenic animal models of the Glu180Gly and Asp175Asn TM mutations (both maintained on the FVB/N background), however, strongly suggest that differential downstream pathogenic signaling pathways are activated by the independent mutations, because the phenotype of the Glu180Gly mice is markedly more severe and accelerated as compared to the Asp175Asn mice at similar...
levels of transgene expression. Although the basic mechanisms involved are unclear, recent advances in understanding the complex functional role of period 5 of TM (where both mutations reside) can guide future studies. One possible mechanism, the so-called “additive effect,” could be applied to many of the thin filament protein mutations, whereby independent impairments in specific aspects of thin filament activation lead to more severe phenotypes based on cumulative functional disruption that may overwhelm myocellular compensatory responses. In the present example, because Glu180Gly alters both of the known functional roles of period 5 (actin binding and Ca$^{2+}$ sensitivity), whereas Asp175Asn largely affects Ca$^{2+}$ sensitivity, the overall effects of Glu180Gly may cause more severe remodeling via significant disruption of local flexibility. Moreover, as previously noted, mutations that lead to a greater effect on component protein–protein interactions such that primary structure is significantly disrupted may have a disproportionate effect on end phenotype.

Based on the known structure, mutations in the N- and C-terminal overlapping domains of TM would be predicted to have distinct effects on myofilament structure and activation. Heller et al directly addressed the basic question of whether the HCM-linked Ala63Val and Lys70Thr mutations in the TM N-terminal domain would affect cooperative activation via changes in flexibility within the crucial head-to-tail overlap region. Similar to the previously described Asp175 and Glu180Gly mutations, both Ala63Val and Lys70Thr increased the Ca$^{2+}$ sensitivity of actin-activated ATPase and thin filament sliding speed. Surprisingly, despite significant decreases in folding stability that would likely alter the flexibility of the mutant N terminus and indirectly affect the C-terminal region of TM at a considerable distance, no measurable effects on cooperativity were observed. Although it is possible that mutations that significantly affect cooperativity are simply nonviable, the similarity of the observations among the HCM-linked mutations in different functional domains argues that existing approaches do not have sufficient molecular resolution given the complexity of the overall complex. Of note, all the known primary DCM-linked TM mutations are located at either end of the molecule. Although the available clinical information is limited, the Glu40Lys and Glu54Lys mutations in TM have extensively studied at both the in vitro and in vivo levels. These mutations do not appear to cause the bimodal distribution of clinical expression exhibited by other thin filament DCM mutations and may cause disease via unique mechanisms. The Glu40Lys and Glu54Lys substitutions cause a significant increase in the local positive potential at position $e$ of the heptad (2 turns apart) and would be predicted to alter protein stability. Mirza et al evaluated the potential structural effects of the N-terminal DCM mutations via CD and differential scanning calorimetry and found both local destabilization (40/54) and distant stabilization of the helix (54 alone), suggesting that Glu54Lys may affect TM structure at a considerable distance, which may account for the more complex functional disruption. The complexity of the biophysical findings for the N-terminal DCM mutants were matched by the Glu54Lys murine model, recapitulating some of the clinical phenotype, albeit with a profound dosage effect on survival. In the main, the potential mechanistic links between the biophysical effects at the molecular level and the observed changes in myofilament function remain obscure for both the HCM- and DCM-linked mutations in TM, especially regarding predicted structural effects and flexibility where there is considerable overlap between mutations. One potential explanation for the difficulties in linking predicted local changes in TM structure to the observed functional effects lies in the potential for the propagation of structural effects through the helix as originally observed via MD for cTnT N-terminal mutations. These effects “at a distance” were supported by the findings of Heller and Mirza. More recently, Nirody et al used electron microscopy and MD to evaluate the effects of amino acid substitutions on TM flexibility at the atomistic level. Not only did they find that the substitution of Leu for a highly conserved Asp at residue 137 caused a significant increase in both flexibility and the curvature of TM but also that the bulk of the increase in TM curvature occurred around residue 175 (within a major mutational clustering site) with only minor changes in the local structure at observed about residue 137. Thus, the predicted effects on myofilament activation would be expected to be more consistent with the functional role of the more distant site, a highly testable hypothesis that reinforces the utility of coupling computational approaches to experiment to obtain information regarding both local and propagated changes in protein function and dynamics caused by thin filament mutations. This recurring observation that independent mutations in disparate regions of the thin filament “machine” can cause similar functional effects from a distance suggests that a new classification of mutations based on their effects on myofilament activation as opposed to protein site may provide a more robust organization for future study.

Bridging the Gap Between Biophysics and Physiology: Animal Models

As noted here and in multiple prior reviews, the availability of transgenic and knock-in animal models of thin filament cardiomyopathies are essential to translating the primary molecular effects of mutations in sarcomeric proteins to the resultant impact on cardiac function. A case in point is the potential link between thin filament protein mutations and SCD. For many of the patient cohorts, the degree of LVH did not directly correlate with the risk of SCD, a central concern for clinical risk stratification and management. Animal model studies of thin filament mutations have identified 2 main primary mechanisms, myocellular energetics and Ca$^{2+}$ regulation. First, independent single amino acid substitutions at cTnT residue 92 in the N-terminal domain have been shown to directly increase the energetic cost of contraction at all workloads as measured via 31P-NMR spectroscopy in contracting hearts. Of particular note, despite near-identical increases in the Ca$^{2+}$ sensitivity of force generation for each of the 3 independent residue 92 mutations, the measured increases in the free energy of ATP hydrolysis (representing a baseline increase in energy utilization required to perform the identical workload) were mutation-specific with regard to magnitude and likely attributable to precise changes in local
protein–protein dynamics within the N-terminal tail domain. The net effect would be a significant decrease in contractile reserve that would be dramatically accentuated in the context of increased hemodynamic demand and potentially trigger SCD.115 Moreover, the long-term energetic compromise could contribute to cardiomyopathic remodeling. Both clinical NMR and animal studies suggest that abnormal cardiac energetics may represent a more general mechanism in HCM linked to sarcomeric gene mutations and thus represent a potentially important therapeutic target.116

A second central disease mechanism involves the complex and dynamic role of myocellular Ca\(^{2+}\) handling in thin filament cardiomyopathies. Alterations in the cellular distribution and myofibrillar response to Ca\(^{2+}\) have been documented at every level of investigation, from changes in the Ca\(^{2+}\) sensitivity of ATPase activity in reconstituted myofilaments to abnormal Ca\(^{2+}\) transients in murine transgenic myocytes to altered Ca\(^{2+}\) dependency of force generation in human patient samples. It is not entirely surprising that disrupting the function of the myofilament Ca\(^{2+}\)-sensing “machine” would directly alter the Ca\(^{2+}\) regulation of activation. In fact, the oft-noted (and measured) changes in the Ca\(^{2+}\) sensitivity of force generation have been touted as a general disease paradigm based on the observed end-stage cardiac remodeling, whereby an increase leads to a “hypertrophic” decrease to a dilated response.117 The latter observation has been a more consistent finding, with the prediction that the decrease in Ca\(^{2+}\) sensitivity (coupled to a decrease in the cross-bridge recycling rate) would lead to a baseline primary defect in force generation that causes a compensatory ventricular dilatation to maintain cardiac output. Likewise, the observed increase in Ca\(^{2+}\) sensitivity for many of the HCM-linked thin filament mutations has been posited to result in a compensatory hypertrophic response. Given the overall lack of correlation between the magnitude of the increase in Ca\(^{2+}\) sensitivity and the degree of LVH, not to mention the existence of families with a broad range of ventricular remodeling in the context of a single mutation (as recently shown for cTnT Ile79Asn), the “compensatory” hypertrophy hypothesis remains unproven.67,118 Moreover, as noted in the cTnC Gly159Asp discussion above, recent work by the Solaro and Marston laboratories has shown that the supposed link between DCM and a decrease in Ca\(^{2+}\) sensitivity may be an oversimplification because of the important influence of cTnI phosphorylation.59,60 This does not mean that the observed effects of thin filament mutations on Ca\(^{2+}\) sensitivity do not contribute to disease pathogenesis. Jagathseesan et al used a transgenic chimeric TM mouse model to genetically reverse the increased Ca\(^{2+}\) sensitivity of force generation in their TM Glu180Gly mouse model, demonstrating that this property could serve as a primary therapeutic target.119 Along similar lines, increasing myofilament Ca\(^{2+}\) sensitivity via the Ca\(^{2+}\) sensitizer EMD 57033 lowered the arrhythmia threshold of WT mice, whereas decreasing Ca\(^{2+}\) sensitivity in mutant cTnT mice with blebbistatin exhibited a protective effect by increasing the arrhythmia threshold.120 Thus, the observed primary alterations in Ca\(^{2+}\) sensitivity at the myofilament level are, at the very least, an indication that a central aspect of the Ca\(^{2+}\)-dependent sarcomeric activation is disrupted by thin filament mutations. The precise mechanistic role, however, remains unclear, although recent experimental advances to measure cTnC Ca\(^{2+}\) affinity in more complex systems will significantly advance our understanding of this central question.121

As noted by Alves et al in their recent review, the effects of sarcomeric mutations on Ca\(^{2+}\)-dependent functions are not limited to the myofilament level.122 Analysis of isolated adult ventricular myocytes from several mutant thin filament mouse models have revealed significant downstream alterations in myocellular Ca\(^{2+}\) kinetics. Two mutant cTnT models have been extensively studied, the Arg92Trp/Leu transgenic mice and the \(\Delta Lys210\) knock-in, linked to HCM and DCM, respectively.83,123,124 At baseline, skinned fibers isolated from the Arg92Trp mice had revealed an increase in the Ca\(^{2+}\) sensitivity of force generation, and fibers from the \(\Delta Lys210\) mice exhibited a decrease. Complex alterations in Ca\(^{2+}\) homeostasis were noted in both model systems and several observations are particularly noteworthy (for the following discussion, the comparisons are between the models with matched mutant protein levels, the Arg92Trp-50% and the \(\Delta Lys210^{+/+}\)). First, whereas the peak amplitudes of the Ca\(^{2+}\) transients were decreased in the Arg92Trp mice, they were increased in myocytes isolated from the \(\Delta Lys210\) animals. This is an intriguing finding because it suggests that the altered Ca\(^{2+}\) sensitivity at the myofilament level is directly coupled to the downstream response. For example, a decrease in myofilament Ca\(^{2+}\) sensitivity may be “sensed” by the cell as insufficient cytoplasmic Ca\(^{2+}\), and thus the transient is increased in response (and vice versa). The mechanism of the sensing is unclear, but the close coupling is supported by a second observation: that these changes in Ca\(^{2+}\) kinetics occur at early time points (<2 months of age), and in the case of the Arg92Trp mice, change significantly over time. Although the initial changes in myocellular Ca\(^{2+}\) are likely compensatory, chronic dysregulation of Ca\(^{2+}\) handling could cause multiple downstream pathogenic effects including secondary activation of Ca\(^{2+}\)-regulated signaling pathways, cardiac remodeling, decreased arrhythmia thresholds, and eventually apoptosis. Some of these secondary effects have been observed in mutant thin filament protein murine models, and partial disease regression has been achieved with Ca\(^{2+}\) blocker treatment.125 Therefore, it is clear that a better understanding of the underlying Ca\(^{2+}\)-mediated mechanisms at both the myofilament and cellular levels is clinically relevant and represents an excellent therapeutic target for this currently untreatable disorder.

Conclusion and Future Directions

In the main, our basic understanding of how thin filament mutations cause complex cardiomyopathies has significantly evolved on both the molecular and clinical levels. On the molecular side, mutation-induced local changes in protein dynamics and structure that disrupt the normal Ca\(^{2+}\)-activated distribution of TM have been shown to have widespread effects on virtually every aspect of myofilament activation. These effects disrupt basal function, the ability of the sarcomere to respond to hemodynamic demands, and downstream Ca\(^{2+}\) handling. The role of phosphorylation,
both PKA- and, more recently, PKC-mediated in the context of thin filament mutations is particularly important and again, must be evaluated in the context of a dynamic system as phosphorylation is often a transient effect, especially during the early compensatory response to contractile dysfunction. On the clinical side, there is renewed interest in both the earliest stages of the disease process and the overall time course of disease progression. For example, Ho et al recently showed that patients with preclinical HCM (sarcomeric gene mutation-positive, no overt LVH) exhibited abnormal relaxation with preserved systolic function with a later progression to both diastolic and systolic abnormalities. It is important to note that the latter observations required newer methodologies to measure longitudinal peak systolic strain and systolic strain rate because LV ejection fraction was not sufficiently sensitive, thus demonstrating how a higher "resolution" approach to an established clinical parameter can potentially provide novel insight into disease progression. Coupled to the aforementioned findings regarding preclinical activation of profibrotic signaling in patients with FHC linked to MYBPC3 and MYH7 mutations, it is clear that study of these cohorts will be very informative and that these approaches should be extended to both patients with thin filament mutations and to available animal models. Along similar lines, the potential role of abnormal protein processing via the ubiquitin–proteasome system has recently been identified as a contributor to abnormal remodeling in HCM, representing another potential point of intervention. Finally, as recently noted by Kaski et al (and in support of earlier studies), the growing literature regarding the surprisingly high frequency of sarcomeric gene mutations in infants and children with HCM strongly suggests that our present view regarding the pathogenesis and progression of FHC needs to be reconsidered, especially regarding age of presentation. This renewed focus on the earliest stages of the disorder will undoubtedly provide a more "proximal" phenotypic characterization. Armed with a robust molecular understanding and coupled to a less "end-stage" phenotype, the still elusive link(s) between genotype and phenotype will be uncovered. Of note, a recently published "white paper" discussing the proposed research priorities for the next stage of HCM investigations addresses many of the issues raised in the present review and expands on others; it outlines an exciting way forward for the field. In the end, this improved and integrated approach to studying thin filament cardiomyopathies will lead to novel and perhaps mutation- or gene-specific approaches to interrupt the progression of this complex and currently untreatable disorder.

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References


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**Generation of the human Ca\(^{2+}\)-activated thin filament model (Shown in Figure 1)**

The figure was obtained by docking cardiac troponin T to overlapping tropomyosin molecules, orienting the cardiac troponin and tropomyosins to the actin backbone and performing a 50ps simulation in equilibrium at 70 K using CHARMM33b1. Cardiac troponin was constructed using an atomistic model of the entire cTn complex from PDB entry 1J1E, an atomistic model of TM based on Holmes’ coordinates, an overlapping model of TM-TM based on PDB entry 2Z5I and cTn – TM interactions based on PDB entry 2Z5H. Missing regions of cardiac troponin were constructed using secondary structure prediction (PSIPRED) and homology with chicken fs Tn. The native stoichiometry of the thin filament 7:1:1, (actin:tropomyosin:troponin) may be more accurately described as 14:2:1 (actin:tropomyosin:troponin) where one troponin requires two overlapping tropomyosins to properly bind, thus requiring 14 actin monomers and 2 tropomyosins per each cTn complex. The actin backbone for the overlapping tropomyosins was created using Holmes’ coordinates and creating a 360 degree rotation about the long axis in the diagram, resulting in a 25.7 degree azimuthal rotation per monomer with a linear displacement of 57.25 Angstroms between monomer centers of mass. Recent evidence suggests a more likely rotation in the diagram would be an approximately 334 degree rotation about the long axis.

**References:**