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The mechanics of the heart: zooming in on hypertrophic cardiomyopathy and cMyBP-C

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ABSTRACT

Hypertrophic cardiomyopathy (HCM), a disease characterized by cardiac muscle hypertrophy and hypercontractility, is the most frequently inherited disorder of the heart. HCM is mainly caused by variants in genes encoding proteins of the sarcomere, the basic contractile unit of cardiomyocytes. The most frequently mutated among them is *MYBPC3*, which encodes cardiac myosin-binding protein C (cMyBP-C), a key regulator of sarcomere contraction. In this review, we summarize clinical and genetic aspects of HCM and provide updated information on the function of the healthy and HCM sarcomere, as well as on emerging therapeutic options targeting sarcomere mechanical activity. Building on what is known about cMyBP-C activity, we examine different pathogenicity drivers by which *MYBPC3* variants can cause disease, focusing on protein haploinsufficiency as a common pathomechanism also in nontruncating variants. Finally, we discuss recent evidence correlating altered cMyBP-C mechanical properties with HCM development.

Keywords: Hypertrophic cardiomyopathy, cardiac myosin-binding protein C, truncating *MYBPC3* variants, nontruncating *MYBPC3* variants, variants of uncertain significance, sarcomere contraction, RNA splicing, protein stability, protein nanomechanics, myosin.

Abbreviations:

ACMG/AMP: American College of Medical Genetics / Association for Molecular Pathology
ACTC1: Cardiac α-actin 1, human gene
ACTN2: α-actinin-2, human gene
ADP: Adenosine diphosphate
AF: Atrial fibrillation
AFS: Atomic force spectroscopy
AHA/ACC: American Heart Association / American College of Cardiology
ASA: Alcohol septal ablation
ATP: Adenosine triphosphate
CaM: Calmodulin
CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II
CD: Circular dichroism
cMyBP-C: Cardiac myosin-binding protein C
CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats / CRISPR-associated 9
DRX: Disordered-relaxed state
ELC: Essential light chain
ESC: European Society of Cardiology
FDA: Food and Drug Administration
FLNC: Filamin C, human gene
Fn3: Fibronectin-III

fsMyBP-C: Fast skeletal myosin-binding protein C
gDNA: Genomic DNA
GWAs: Genome-wide association studies
HCM: Hypertrophic cardiomyopathy
HF: Heart failure
hiPSC-derived CMs: Human-induced pluripotent stem cell-derived cardiomyocytes
HMM: Heavy meromyosin
ICDs: Implantable cardioverter-defibrillators
Ig: Immunoglobulin
IHM: Interacting-heads motif
Indel: Insertion/deletion
KI: *knock-in*
KO: *knock-out*
LMM: Light meromyosin
LV: Left ventricle / Left ventricular
LVH: Left ventricular hypertrophy
LVOT: Left ventricular outflow tract
LVOTO: Left ventricular outflow tract obstruction
MHC: Myosin heavy chain
MLCK: Myosin light chain kinase
M motif: MyBP-C motif
mRNA: messenger RNA
MST: Microscale thermophoresis
MVs: Modifier variants
MyBP-C: Myosin-binding protein C
MYBPC1: Slow skeletal myosin-binding protein C, human gene
MYBPC2: Fast skeletal myosin-binding protein C, human gene
MYBPC3: Cardiac myosin-binding protein C, human gene
Mybpc3: Cardiac myosin-binding protein C, mouse gene
MYH6: α-myosin heavy chain, human gene
MYH7: β-myosin heavy chain, human gene
MYL2: Regulatory myosin light chain 2, human gene
MYL3: Essential myosin light chain 3, human gene
NMD: Nonsense-mediated mRNA decay
P/A: Proline-alanine
P_i: Inorganic phosphate
PKA: Protein kinase A
PKC: Protein kinase C
PKD: Protein kinase D
PLN: Phospholamban, human gene
Pre-mRNA: Precursor Messenger RNA
PTC: Premature termination codon

PTMs: Posttranslational modifications
R6K: 90-KDa ribosomal S6 kinase
RCT: Randomized clinical trial
RLC: regulatory light chain
S1: Subfragment 1
S2: Subfragment 2
SCD: Sudden cardiac death
SERCA2: Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
SR: Sarcoplasmic reticulum
SRT: Septal reduction therapy
SRX: Super-relaxed state
sS1: Short subfragment 1
ssMyBP-C: Slow skeletal myosin-binding protein C
TnC: Troponin C
TnI: Troponin I
TNNI3: Cardiac troponin I 3, human gene
TNNT2: Cardiac troponin T 2, human gene
TnT: Troponin T
Tpm: α-tropomyosin
TPM1: α-tropomyosin 1, human gene
UPS: Ubiquitin-proteasome system
VT: Ventricular tachycardia
VUS: Variant of uncertain significance
WT: Wild-type

Introduction

This review summarizes the hallmark clinical aspects of hypertrophic cardiomyopathy (HCM), and introduces new therapies targeting underlying pathomechanisms (section *Hypertrophic cardiomyopathy*). HCM is mainly caused by variants affecting genes that encode components of the sarcomere, the basic contractile unit in cardiomyocytes, which we present in section *The cardiac sarcomere*. In this context, the genetic etiology of HCM is detailed in section *Genetic basis of HCM*, together with a structural and functional description of the main sarcomere components targeted by HCM-causing variants. Among them, a focus will be laid on the most frequently mutated gene in HCM, *MYBPC3*, which encodes the thick-filament associated protein cardiac myosin-binding protein C (cMyBP-C). The structure, interaction landscape and functional role of cMyBP-C are described in section *Cardiac myosin-binding protein C: structure and function*, focusing on the potential mechanisms by which this protein may play its regulatory role on sarcomere activity. Finally, disease mechanisms induced by both truncating and nontruncating *MYBPC3* variants are presented in section *HCM-causing MYBPC3 variants*. We would like to apologize for any contribution that we have not cited due to space constraints or unintended omission.

Hypertrophic cardiomyopathy

Definition of basic medical, anatomical or physiological concepts used throughout this section can be found in **Table 1**. Terms included in this table are marked with an asterisk in the text.

Definition, prevalence and clinical manifestations

HCM is the most frequent inherited heart muscle disease, with an estimated prevalence* of at least 0.5% in the general population [1-3]. The primary hallmark of HCM is the asymmetrical thickening of the left ventricular (LV) walls, which occurs in the absence of hypertension or other predisposing cardiac or systemic conditions. Other myocardial regions, such as the apex* or the interventricular septum*, can also be enlarged in HCM, which altogether reduces the size of the LV chamber [4] (**Figure 1**). Left ventricular hypertrophy (LVH)* has been related to heart muscle hypercontractility [5] (see section *Histological alterations and disease-associated functional hypercontractility*) and both have been proposed to contribute to the diastolic* dysfunction that characterizes this disease [4]. Left ventricular ejection fraction (LVEF)*, and thereby systolic* performance, is often conserved or enhanced, except in the event of obstructive HCM*, in which the blood flow from the LV into the aorta is hindered by LVH, causing left ventricular outflow tract obstruction (LVOTO)* (**Figure 1**). Obstructive HCM affects a significant proportion of patients and causes high intraventricular pressure, which can further contribute to LVH and impaired relaxation [4, 6, 7]. Regurgitation* due to anatomical alterations of the left atrioventricular mitral valve* is also typical in this condition (**Figure 1**) [8]. Mitral valve morphological defects and malfunctioning can also account for LVOTO [9] and may arise from the hypertrophy and abnormal

insertion of the papillary muscles* in the inner ventricular walls [10]. The presence of myocardial crypts*, *i.e*. small, narrow invaginations in the basal LV walls and in the interventricular septum, is also a phenotypic marker of HCM which usually occurs prior to the development of hypertrophy [11]. Myocardial bridging*, an abnormality that occurs when a segment of a major epicardial coronary artery goes intramurally through the myocardium, being compressed in every systole, is also a frequent HCM feature [12]. All the anatomical alterations listed in **Table 1** lead to cardiac dysfunction, which in turn underlies a broad range of HCM clinical manifestations, such as dyspnea*, fatigue, palpitations, lightheadedness, syncope and atypical chest pain [13]. Patients affected from LVOTO generally have a worse prognosis than those presenting nonobstructive HCM, in addition to more extenuating symptoms and exercise disability [6, 7, 14].

Cardiac arrhythmias, such as atrial fibrillation (AF)* or ventricular tachycardia (VT)* can also occur in a context of HCM [4]. Indeed, AF affects around 25% of patients with obstructive HCM, increasing the risk for thromboembolic stroke* [15]. HCM is the most common cause of sudden cardiac death (SCD) in the young, mostly affecting asymptomatic individuals and high-performance athletes [16]. VT and electrophysiological dysfunction of the heart due to cardiomyocyte* disarray and fibrosis (see section *Histological alterations and disease-associated functional hypercontractility*) seem to be the underlying cause of SCD in these cases [17].

One of the most incapacitating consequences and the current leading cause of death in HCM is heart failure (HF)*, which occurs in the absence of volume overload and pulmonary congestion. HF, which can also occur in nonobstructive HCM, can be pinpointed by an excessive shortness of breath during exercise [18, 19].

Histological alterations and disease-associated functional hypercontractility

At the tissue level, HCM cardiomyocytes* are disarrayed [20-27] and separated by areas of interstitial fibrosis [22-30], which in turn can result from hypertrophy-derived microvascular impairment and ischemia [31].

Mirroring what is observed at the macroscopic level, cardiomyocytes are also hypertrophied in the HCM myocardium, both in humans [25, 26, 28, 29, 32] and animal models [24, 27]. This phenotype is also observed in human-induced pluripotent stem-cell derived cardiomyocytes (hiPSC-derived CMs) [21, 33-37]. This cellular enlargement has been proposed to result from functional hypercontractility, *i.e.* enhanced contractile performance which may originate from disparate impairments in the contraction-relaxation cycle and which can be observed across different scales. For example, hypercontractility at the whole-organ level has been associated to an increased LVEF [38-40], meaning that a higher fraction of blood volume is ejected from the ventricle during each systolic contraction [41]. An increased fractional shortening* as an estimate of enhanced LV contractility [41], has also been observed in a context of hyperdynamic contraction [42]. On the other hand, an impaired myocardial relaxation has been linked to functional LV hypercontractility pinpointing an overt HCM phenotype [38]. At the cardiomyocyte level, the alteration of a variety of contractility-related parameters has been observed in the context of

hyperdynamic contraction. **Table 2** details abnormalities reported for some of these parameters that have been specifically associated with hypercontractility. As inferred from **Table 2**, hypercontractility has been consistently associated with the enhancement of contraction kinetics parameters and the depression and/or prolongation of muscle relaxation. However, no specific parameter alteration has been invariably observed in the context of disease, as supported by a comparative study summarizing experimental contractility data obtained for HCM human heart muscle samples [43]. Inconsistencies between parameters values reported in the literature may be explained by differences in the disease stage of HCM samples, experimental conditions, testing platforms and even size of the specimens. Future work unifying measurement criteria may enable the identification of universal HCM-linked contractility parameters as disease surrogates, which would also be instrumental for the better understanding of HCM pathomechanisms.

Parameters linked to hypercontractility at the molecular level, with a focus on HCM-related alterations affecting myosin as a major contractile sarcomere component, are presented in section *HCM-causing MYH7 variants*.

Diagnosis and clinical variability

As it will be presented below, HCM is a genetic disorder that is mainly caused by variants in proteins that build the sarcomere, such as myosin and cMyBP-C. However, more than 50% of HCM patients are genotype-negative, which means that no variant accounting for the disease can be identified [44, 45] (see **.** section *The eight "core" sarcomere genes in the development of HCM*). This situation forces diagnosis to be based on clinical manifestations rather than on genetic testing. As a result, the diagnosis of HCM is mostly triggered by the appearance of symptoms and consists on the detection of LVH by an imaging technique (echocardiography, cardiovascular magnetic resonance or computed tomography) [14]. However, the pattern and degree of hypertrophy is highly variable within HCM patients [46, 47]. Although hypertrophy is typically asymmetrical, mostly affecting the interventricular septum and the LV anterior wall, other forms can also occur, such as apical, septal, concentric or even right-ventricle related [48, 49]. Patients can suffer from HCM while presenting unobservable or LV thickness below the diagnosing threshold. Indeed, a subset of patients with advanced systolic dysfunction can develop end-stage HCM or burned-out cardiomyopathy*, defined by LV wall thinning [50, 51]. Moreover, HCM is characterized for its varying degrees of symptom severity and incomplete penetrance*, *i.e.* not all individuals with an HCM-linked genotype express the clinical condition. Both penetrance and symptom variability present disparities depending on the age at diagnosis, race and gender [52-54]. A significant proportion of HCM patients remain asymptomatic most of their life, whereas others suffer SCD forms or express additional clinical phenotypes such as angina* or arrhythmias [6, 14, 50]. HCM can appear at any age, including shortly after birth [46, 48, 55]. However, HCM is most frequently manifested in the post adolescence [56, 57].

The existence of HCM phenocopies*, *i.e.* systemic disorders with different genetic origin to HCM but also causing LV thickening, hinder the diagnosis of HCM and lead to clinical misclassification (see also section *HCM phenocopies*) [58]. In this same regard, LVH and even

LVOTO can also derive from long-lasting hypertension, aortic stenosis* and hemodynamic obstruction* [58]. High-level, young athletes can also develop physiological cardiac hypertrophy and remodeling in response to intensive training [59].

As presented, the heterogeneity in clinical manifestations and the existence of mimicking clinical conditions hamper a straightforward identification of HCM. This highlights the need for a multifactorial approach in the diagnosis of HCM encompassing careful clinical evaluation and genetic testing of patients and their relatives [14].

Treatment

Currently, HCM has no cure and affected individuals must follow long-term treatments towards symptom palliation or even resort to surgical procedures to improve their quality of life [6, 13, 60]. For the correct management of HCM, several organizations such as the American Heart Association together with the American College of Cardiology (AHA/ACC), or the European Society of Cardiology (ESC), regularly establish protocols to guide clinical practice [14, 50].

Most of the currently available pharmacological treatments are focused on relieving symptoms in obstructive HCM [14, 61, 62]. For that, drugs with negative inotropic* (reduction of the force of contraction) and chronotropic* (decrease in the heart rate) effects are used. As a general rule, nonvasodilating β-blockers* are the first option in the treatment of LVOTO. If these do not work or are not well tolerated, nondihydropyridine calcium channel blockers are used as an alternative. When neither of these treatments prove effectiveness, administration of the antiarrhythmic drug disopyramide is recommended. Albeit the combination of these drugs reduces disabling symptoms in 50-65% of the patients, only 25% benefit in the long term [61, 63].

If pharmacological treatment of obstructive HCM does not relieve symptoms, invasive surgical procedures can be considered [14, 61, 62]. Septal reduction therapy or SRT* (either by septal myectomy* or alcohol septal ablation, ASA*) is the most effective practice in the treatment of LVOTO. Septal myectomy consists on the thinning or removal of part of the hypertrophied septum and it has been reported to improve the quality of life, exercise capability and mid-long term survival of LVOTO patients through alleviation of myocardial regurgitation and reduction of LV remodeling [64, 65]. When surgical interventions are discouraged but drugs are no longer effective, ASA is the preferred invasive alternative [14, 61]. It consists on the localized delivery of absolute ethanol in the hypertrophied interventricular septum to provoke a controlled myocardial infarction for its long-term thinning [66]. However, the odds of reintervention and secondary implantation of a pacemaker are higher in ASA than in myectomy [67, 68].

The use of implantable cardioverter-defibrillators (ICDs)* is very effective in the prevention of SCD. Indeed, the better stratification of at-risk individuals who can benefit from ICDs has significantly lowered the mortality currently associated with HCM (≤1% per year, and mostly related to HF) [69, 70]. HF usually results from progressive LV dysfunction, increased replacement fibrosis and remodeling in nonobstructive HCM transitioning to burned-out stage. In these cases, heart transplantation is the only option for long-term survival [62, 71].

For next-generation treatments and personalized clinical management, enormous efforts are being focused on unravelling HCM molecular pathomechanisms. It is expected that the better understanding of the underlying causes of HCM will enable the design of therapies directed to its reversion, changing the natural history of the disease. Based on the cardiomyocyte hypercontractility observed in HCM (see section *Histological alterations and disease-associated functional hypercontractility*), recent randomized clinical trials (RCT)* have investigated the potential benefit of the deceleration of the cardiac contractile apparatus in the course of HCM. In this regard, the double-blind, placebo-controlled* phase III EXPLORER-HCM RCT assayed the administration of the drug mavacamten (previously known as MYK-461), a myosin inhibitor (see section *Regulation of contraction by myosin conformations* for a description of the molecular mechanisms of action), showing promising results in relieving obstruction and improving functional status, exercise capacity and life quality [72]. As mavacamten complied with all safety needs and successfully met all primary and secondary endpoints, it is already pending Food and Drug Administration (FDA) approval for the treatment of obstructive HCM [73]. In addition, the efficacy of mavacamten in both nonobstructive HCM and as an alternative to SRT in severe LVOTO cases, is being currently tested in the phase II MAVERICK-HCM [74] and phase III VALOR-HCM (NCT04349072) clinical trials, respectively. The safety, tolerability and clinical dose of CK-274, another small molecule with a mechanism of action comparable to that of mavacamten, is currently being assayed in a phase II RCT for the treatment of obstructive HCM (REDWOOD-HCM, NCT04219826) [61].

Unlike small molecules targeting the functional consequences of HCM-linked genetic defects, a new era of therapeutic strategies is currently under research to directly correct or repress disease-causing variants before clinical manifestation [75, 76]. Examples of these novel therapies are gene editing in early embryogenesis using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated 9) [77, 78], viral-mediated delivery of genes to compensate variant-induced deficits in contractile proteins [37, 79-81], and strategies directed towards correction of mutant transcripts [37, 82-84] or gene silencing [85, 86]. Although different technical concerns hamper their development and current application, these therapies hold promise to pave the way towards personalized HCM prevention.

The cardiac sarcomere

The cardiac tissue has a hierarchical, highly organized architecture based on the assembly of different functional units [87, 88]. At the top level of organization, muscle fascicles build up from fibers bundles, which are in turn composed of multiple cardiomyocytes connected through intercalated disks [88-90] (**Figure 2A**). These bridging complexes allow the electrical and mechanical coupling of cardiomyocytes, enabling their coordinated function in the fibers. The intracellular space of the cardiomyocytes, or sarcoplasm, is occupied by longitudinal arrays of spanning myofibrils (**Figure 2A**), which are the result of the longitudinal assembly of sarcomeres (**Figure 2A,B**) [87, 91-93]. The cardiac sarcomere, which is ~2 μm long, is composed of overlapping myosin-containing thick filaments and actin-based thin filaments (**Figure 2B**). Myosin heads extending

from thick filaments can hydrolyze adenosine triphosphate (ATP) and transduce the resulting chemical energy into the mechanical gliding of the thin filaments over the thick ones, resulting in net sarcomere shortening that is the basis of muscle contraction. A more detailed description of thick filaments function and composition will be provided in the section *The thick filament***.** On the other hand, globular cardiac α-actin monomers polymerize to form two-stranded thin filaments. The troponin complex (composed of troponins C, T and I, *i.e.* TnC, TnT and TnI, respectively), together with α-tropomyosin (Tm), associate to thin filaments, playing a key regulatory role in contraction [91, 94] (**Figure 2C**) (see section *The swinging lever arm model of muscle contraction*). While Tm dimers form coiled-coil structures wrapping thin filaments, troponins are small flexible proteins, consisting of helical domains separated by length-variable linkers. The structural organization of actomyosin filaments define different topographical regions within the sarcomere (**Figure 2B**) [87, 91, 93, 95]. The M-line divides the sarcomere in two symmetrical halves, providing structural support and equally distributing tension between them [91, 96]. The sarcomere is limited in its outermost edges by Z-lines, which do not only transmit force to neighboring units during contraction but also act as scaffold for signaling and mechanotransduction [97, 98]. The thick filaments are anchored to the M-line and span towards the Z-line, creating the A-band. The C-zone is defined as the region of the A-band that contains cMyBP-C [99-101]. Throughout most of the extension of the A-band, thin and thick filaments overlap, except for the so-called H-zone, which is also centered in the M-line and lacks superimposing thin filaments. Conversely, the thin filaments are attached to the Z-line and their stacking, with no overlay of thick filaments, creates the I-band distal region of the sarcomere. In the next section, an overview of the main components of the sarcomere and their function is provided.

Recently, a regulatory role of nonsarcomeric cytoskeleton in the function of the sarcomere in health and disease has been suggested [102, 103]. Indeed, an emerging hypothesis proposes that the intermediate filament protein desmin mediates the interaction between microtubules and sarcomeres, tuning cardiomyocytes mechanics and cytoskeletal-mediated mechanotransduction [104]. In this regard, an accumulation of detyrosinated microtubules and desmin has been observed in HCM myocardium, accompanied by increased cardiomyocytes stiffness and contraction deficits [105-107]. These data endorse the targeted reduction of microtubule detyrosination as a promising therapeutic approach for the improvement of cardiac function in HCM.

The thick filament

Myosin is one of the most abundant proteins in cardiac muscle, accounting for one third of the total sarcomere protein content [108]. Indeed, each thick filament, which is around 1.6 μm long and 10-20 nm wide [87, 109], is composed of ~300 myosin molecules [110]. Cardiac myosin belongs to the myosin II filament-assembly-competent subgroup [111, 112]. Each myosin unit is a hexameric complex composed of two myosin heavy chains (MHCs) and two pairs of accessory light chains [91, 110] (**Figure 2C**). In human adult ventricular myocytes, the pair of MHCs in myosin usually correspond to the β isoform (the product of the *MYH7* gene), although the presence of the highly

homologous α isoform (that of *MYH6* gene), more typical of the developing atria, has also been reported [91]. Near their C terminus, the two MHCs dimerize forming an α-helical coiled-coil tail called light meromyosin (LMM) or rod myosin region. These myosin tails bundle to form the basic scaffold of the thick filament [91, 110]. The LMM, together with the heavy meromyosin (HMM) fragment, can be obtained upon chymotrypsin-mediated digestion of the entire myosin molecule [109] (**Figure 2C**). The HMM fragment can be further digested by papain to produce the two myosin heads or myosin subfragment 1 (S1) [109] and myosin subfragment 2 (S2), an α-helical hinge region that mediates the connection of the globular heads to the myosin tail backbone [91, 109, 110] (**Figure 2C**). In sarcomeres, myosin heads extend from the thick filament backbone forming 14.3-nm-spaced myosin "crowns" arranged in a quasi-helical manner [113, 114]. These myosin heads are the motor domain of the myosin molecule, as they can bind and hydrolyze ATP to boost the sliding of the actomyosin filaments [91, 108, 110, 115]. The myosin heads are followed by an α-helical region called lever arm which also comprises the converter domain (**Figure 2C**). The lever arm acts as binding platform for both pairs of essential (ELC) and regulatory (RLC) myosin light chains in the hexameric complex, which regulate the orientation of the globular heads towards actin in the fine-tuning of actomyosin interactions and contraction dynamics [110, 115, 116] (**Figure 2C**). Both light chains are part of the EF-hand superfamily of calcium-binding proteins, although ELC has lost its ability to bind divalent cations [91].

The giant protein titin, which spans half the sarcomere length, from the Z-line to the M-line, is also part of the thick filament backbone (**Figure 2B,C**). Apart from its structural role in sarcomere assembly and stabilization, titin is an elastic protein which acts as a molecular spring, providing passive tension that limits sarcomere stretching during diastolic filling [117, 118]. More recently, a new role of titin in the powering and regulation of active sarcomere contraction has been proposed [119-122]. According to this hypothesis, force applied by myosin heads would relieve tension on titin, allowing the refolding of previously stretched domains. Domain refolding would then provide mechanical power, boosting sarcomere shortening. Furthermore, titin is a platform for mechanosensing and transduction in different signaling pathways [123]. The switching of titin isoforms, together with its targeting by different posttranslational modifications (PTMs), modulate titin mechanical properties [120, 124].

cMyBP-C is a thick-filament associated protein with complex regulatory roles in cardiac contraction (**Figure 2B,C**). It binds different components of the thin and thick filaments, regulating crossbridge kinetics, actomyosin gliding and sarcomere contraction dynamics [125]. Sections *Cardiac myosin-binding protein C: structure and function* and *HCM-causing MYBPC3 variants* below will summarize the role of cMyBP-C in contraction and its implication in HCM.

The swinging lever arm model of muscle contraction

Contrary to the old standard view that contraction is produced by coiling of continuous actomyosin filaments in the muscle [126], the sliding filament theory (proposed by HE Huxley and Hanson, and AF Huxley and Niedergerke in the 50s [127, 128]) stated that actin and myosin filaments are not continuous and that they overlap to different extents during the contraction cycle. The

sliding filament theory thereby defined muscle contraction as the propelling of thin filaments over thick filaments. In 1969, HE Huxley proposed the swinging crossbridge model, which contributed the fact that sliding was powered by the transduction of the chemical energy resulting from ATP hydrolysis into the mechanical motion of the myosin heads [129]. Nowadays this model is called the swinging lever arm model for muscle contraction [109, 130], due to further evidence implicating this myosin region in mechanical transduction.

The mechanical movement of the myofilaments is coupled to the electrical stimulation of cardiomyocytes [131, 132]. When the cardiomyocyte membrane depolarizes during action potential transmission, L-type voltage-dependent calcium channels in the T-tubules open and allow calcium ions to enter the sarcoplasm. This calcium influx stimulates the opening of the ryanodine receptors in the sarcoplasmic reticulum (SR). As a result, calcium also exits the SR, contributing to a rapid, high rise in intracellular calcium concentration, which initiates the contraction cycle. Free calcium cations bind to TnC, promoting a series of conformational changes in adjacent TnI and TnT which are finally transmitted to Tm. The resulting adjustment in the Tm wrapping around actin exposes myosin-binding sites [94], which enables contraction according to the mechanochemical cycle represented in **Figure 3** [91, 109, 133]. When the ATP in the myosin nucleotide-binding pocket is hydrolyzed to adenosine diphosphate (ADP) and inorganic phosphate (P_i), the converter domain changes its conformation, priming the myosin head in a pre-power stroke position [109] (**Figure 3**, *state 1*). In this configuration, myosin heads bend towards actin and bind to the cryptic sites revealed by Tm repositioning, forming the so-called actomyosin crossbridges [91, 133] (**Figure 3**, *state 2*). P_i release induces conformational changes leading to the flexion or rotation of the lever arm over the converter region [109], which induces the displacement of actin by ~10 nm, resulting in sarcomere shortening [134]. This myosin-head-mediated propelling of actin over the thick filaments is known as power stroke [135] (**Figure 3**, *state 3*). Finally, release of ADP and subsequent ATP binding to the nucleotide-binding site induce the detachment of the myosin head from actin (**Figure 3**, *state 4*).

Regulation of contraction by myosin conformations

In addition to the well-known calcium-based regulation of contraction described above, a myosin-mediated modulatory mechanism has been described in the last decade, adding an extra complexity layer to the fine-tuning of muscle contraction [136, 137]. In sarcomeres, three distinct myosin configurations can be found, each of them associated with a particular ATPase rate (**Figure 4**). As a result, the regulation of the proportion of these conformations allows the adjustment of energy consumption by cardiac muscle.

One of these configurations occurs mainly during activation of muscle contraction and consists on the strong myosin binding to actin *via* its two heads, resulting in maximum ATP hydrolysis rate (>1 s^{-1}) [138] (**Figure 4**). This myosin conformation can be mostly found in the C-zone [139]. On the other hand, during relaxation or diastole, two different myosin conformations, namely super-relaxed (SRX) and disordered-relaxed (DRX) states, coexist in a dynamic equilibrium [111, 140] (**Figure 4**). The SRX state is defined as a relaxed myosin configuration with an extremely slow

ATPase rate (< 0.01 s^{-1}) [137, 138, 141, 142]. In skeletal and cardiac fibers, 50-60% of myosins are estimated to be in the SRX state [138, 141]. The DRX state is characterized by intermediate ATP hydrolysis rate (> 0.03 s^{-1}) [137, 138, 141, 142]. Recent super-resolution data suggests that, despite being present across the entire thick filament, the myosin SRX state is enriched in the C-zone, whereas the DRX state is more abundant in flanking sarcomere regions [143].

Regarding the structural basis of these relaxed myosin configurations, the functional SRX state has been proposed to stem from the formation of the interacting-heads motif (IHM) or any similar configuration during relaxation [109, 138, 141, 142, 144-149]. In this hypothetical structural arrangement, one of the myosin heads, known as *blocked*, would fold back onto its myosin S2, lying onto the backbone of the thick filament. As a result, the ATPase activity of this *blocked* head would be sterically inhibited. At the same time, the actin-binding region of the *blocked* head would be also masked by its interaction with the converter domain of the partner *free* myosin head. Consequently, both myosin heads would be sequestered away from the thin filament, unavailable to form crossbridges nor hydrolyze ATP [144-146, 150, 151]. However, recent investigations with short myosin constructs have proven that SRX-typical hydrolyzing properties can result from structural arrangements other than a folded-back motif [152-154]. Indeed, homology models now suggest that the formation of a proper IHM-like structure is not essential to generate the SRX state, which could actually result from a particular configuration of the myosin lever arm in which the lever is primed in a pre-stroke conformation leading to a minimal ATPase activity [152]. For the sake of clarity, this review will exclusively refer to the SRX state when pertinent biochemical data is available.

Regarding the structural configuration of the DRX state, it has been hypothesized that it comprises a heterogeneous population of myosin conformations swaying in a variety of proximities to the thin filament, with troponin/tropomyosin-driven limited access to actin [111, 147, 155]. This disordered configuration of the myosin heads away from the thick filament shaft would be in line with earlier studies observing a random and broad rotational motion of myosin heads in the absence of actin interaction [156-158]. The regulation of the SRX:DRX equilibrium is key for the fine-tuning of contraction dynamics and energetics. The disordering of myosin heads towards a more active configuration is cooperative and it has been hypothesized to depend on a variety of factors [111, 140]. Among them, the phosphorylation of RLC would depopulate the SRX state [138, 142, 154, 159, 160] (**Figure 4**). A calcium binding-mediated [159] and ADP-related [138, 141, 143, 154, 161] destabilization of the SRX state have also been suggested to shift the SRX:DRX equilibrium to the more active disordered state. Temperature is another determinant factor and it has been proposed that SRX would serve as an energy saving mechanism, allowing animals to efficiently control muscle contraction in adverse conditions, such as during hibernation [162]. On the other hand, cMyBP-C interaction has been suggested to stabilize the myosin SRX configuration, and phosphorylation-mediated loss of this binding would shift the balance favoring the DRX state [163-167] (**Figure 4**) (see section *The complex regulatory role of cMyBP-C in sarcomere contraction*). Furthermore, a mechanosensing-based regulation of the thick filament in which load regulates the activation status of myosin has also been proposed [168].

As presented in the section *Treatment*, mavacamten is a recently developed drug for the treatment of HCM based on myosin inhibition. Regarding its molecular action, mavacamten primarily decreases the basal P_i release rate from the nucleotide-binding pocket of myosin (**Figure 3**), delaying the myosin mechanochemical cycle and reducing its ATPase activity [169]. This retention of the P_i may be explained by a mavacamten-mediated stabilization of the slow-nucleotide-hydrolyzing myosin SRX state [152, 153, 162] (**Figure 4**).

Genetic basis of HCM

HCM is a monogenic Mendelian condition that is mostly inherited in an autosomal dominant fashion. Up to date, more than 1,500 gene variants have been annotated as causing disease [13]. HCM is considered a *disease of the sarcomere* since the majority of these variants target genes encoding sarcomere-related components [108, 170-172].

The eight "core" sarcomere genes in the development of HCM

In the 1960s HCM was considered an idiopathic hypertrophic subaortic stenosis [173]. It was not until 1989 when the group of Christine and Jonathan Seidman first described a genetic cause for the disease. Using linkage analysis, they found a single base substitution in *MYH7* that correlated with the development of the condition in a large HCM pedigree [174-176]. After this first definition of the genetic etiology of HCM, over a dozen causal genes have been identified [108].

In this scenario of genetic causality, eight sarcomere genes have been unambiguously associated with HCM *via* cosegregation, frequency population and functional criteria [177] (**Figure 5**). Due to their unequivocal linkage to disease and the fact that variants in these genes explain a significant fraction of HCM cases, these genes are considered "core" or "definitive" in the etiology of HCM. These genes are *MYBPC3* [178, 179], *MYH7* [176], *MYL2* (regulatory myosin light chain 2) [180], *MYL3* (essential myosin light chain 3) [181], *TNNT2* (cardiac troponin T 2) [182], *TNNI3* (cardiac troponin I 3) [183], *TPM1* (α-tropomyosin 1) [182], and *ACTC1* (cardiac α-actin 1) [184]. Nearly 70% of HCM cases with an identified genetic cause (genotype-positive HCM) can be explained by variants in *MYH7* or *MYBPC3*. The remaining six genes collectively account for around 5% of HCM cases [14, 45, 171, 172] (**Figure 5**). Variants in *MYL2* and *MYL3* have been proposed to alter the SRX:DRX balance either by directly affecting the ordering of myosin heads in the thick filament or by altering SRX modulatory factors, such as RLC phosphorylation [111, 140, 149, 185]. Variants in thin filament-associated proteins have been described to increase calcium sensitivity and impair calcium transients, leading to sustained sarcomere shortening and prolonged action potentials during relaxation [35, 186-188]. The nature and disease mechanisms of *MYH7* and *MYBPC3* variants will be discussed in next subsection and section *HCM-causing MYBPC3 variants*, respectively.

HCM-causing MYH7 variants

MYH7 variants are responsible for >25% of HCM cases with an identified genetic cause [45] (**Figure 5**). Most HCM-causing *MYH7* variants are missense, *i.e.* single nucleotide substitutions resulting in nonsynonymous amino acid changes in the final mutant protein [92, 108, 189]. Missense myosin mutants are incorporated into the sarcomere, where they exert a dominant-negative effect by disrupting the normal function of the contractile apparatus. As a result, mutant myosin proteins are usually referred to as "poison peptides" [91, 92].

An early perspective on HCM pathomechanisms considered that sarcomere gene variants impaired the contractile capacity of the sarcomere, leading to compensatory hypertrophy [190]. However, this vision did not agree with the existence of genotype positive-phenotype negative HCM patients who showed increased systolic function and abnormal relaxation in the absence of hypertrophy [38]. In other words, clinical hypercontractility appears to be a direct effect of sarcomere gene variants and not a consequence of myocardial remodeling.

In this context, it has been hypothesized that HCM-causing *MYH7* variants lead to hypercontractility through a variant-induced increase in the global power output (P) of the myosin motor [5]. P is the product of the ensemble force that the actomyosin filaments produce, $F_{ensemble}$, and the contraction velocity, v (**Equation 1**) [5]:

$$P = F_{ensemble} \cdot v \qquad \textbf{(Equation 1)}$$

$F_{ensemble}$, in turn, can be defined according to **Equation 2** [5]:

$$F_{ensemble} = F_{intrinsic} \cdot N_a \cdot {}^{t_s}/_{t_c} \qquad \textbf{(Equation 2)}$$

where $F_{intrinsic}$ is the intrinsic force developed by the myosin motor working as an independent force generator, N_a is the number of myosin heads that are functionally available for their interaction with actin, and ${}^{t_s}/_{t_c}$ is the duty ratio (fraction of the total time of myosin mechanochemical cycle, t_c, when myosin is tightly bound to actin, t_s). At the same time, t_s is related to v through the standard definition of velocity ($v = d/t_s$, being d the actin displacement induced by the myosin power stroke) and t_c is conditioned by the ATPase rate, k, of the myosin mechanochemical cycle in accordance with the expression $t_c = 1/k$ [5]. In light of this model, it has been suggested that HCM-causing *MYH7* variants boost the sarcomere power output through the gain of function of the ATPase activity, the contraction velocity and/or the myosin intrinsic force [5, 191]. However, several reports on *MYH7* variants and how they affect myosin motor function have detected conflicting alterations in these parameters [167, 192-195]. Indeed, simultaneous changes in several of them may occur, affecting contractility in opposite directions. As a result, net changes in global power output have been difficult to assess.

More recently, variant-induced changes in N_a have been studied as a factor leading to hypercontractility. According to **Equation 2**, a higher number of myosin heads that are functionally available for their interaction with actin could effectively lead to an increased $F_{ensemble}$, and therefore, to a boosted sarcomere power output [5]. In this view, a pathological effect

of *MYH7* variants could be the weakening or disruption of key interactions involved in the putative sequestration of myosin heads in a folded-back, less active configuration (see section *Regulation of contraction by myosin conformations*) [5]. In this regard, the mesa hypothesis was put forward by the Spudich lab as a possible unifying explanation accounting for hypercontractility linked to both *MYH7* and *MYBPC3* pathogenic variants [109, 196]. The mesa is a relatively flat surface on the myosin motor domain which, according to this hypothesis, would be involved in electrostatic interactions with cMyBP-C or the proximal S2 for the stabilization of a folded-back, restrained myosin head configuration. HCM variants could destabilize this configuration, releasing myosin heads for their interaction with actin and subsequently resulting in hypercontractility [109, 152, 167, 191, 196-199]. In support of this hypothesis, many HCM-causing *MYH7* variants have been mapped to regions involved in the formation of this putative folded-back structure [109, 196, 198, 200]. Whether the folded-back configuration proposed in light of the mesa hypothesis correlates to the IHM and/or the functional myosin SRX state is still under debate [5].

Other sarcomere and sarcomere-related genes associated to HCM

The curation effort to identify HCM-causing variants has led to the recent incorporation of *PLN* (encoding phospholamban) and *FLNC* (filamin c) as two additional genes definitively associated to the development of disease [177]. Phospholamban acts as a negative regulator of cardiac contraction through SERCA2 (sarcoplasmic/endoplasmic reticulum calcium ATPase 2) inhibition [201, 202], while filamin c crosslinks actin filaments in the organization of cytoskeletal proteins [203, 204].

Less commonly, HCM-linked variants have also been proposed to target genes encoding other sarcomere proteins, such as components of the M-line or the Z-line. Among them, *ACTN2* encodes the major Z-line component α-actinin-2, which organizes the anchoring of thin filaments to this outlining sarcomere platform [205]. Clinical and genetic evaluation of HCM probands first established a moderate association of *ACTN2* variants with the development of HCM [206]. More recently, the implication of *ACTN2* variants in HCM pathophysiology has been further supported by experiments using hiPSC-derived CMs [36].

Additionally, alterations in other polypeptides with accessory functions in cardiac architecture and regulation, like elements of the calcium cycling homeostasis and the excitation-contraction coupling, have also been suggested to participate in HCM pathogenesis [91, 108].

HCM phenocopies

Only 30-40% of diagnosed HCM cases present with an underlying causative gene variant [45] (**Figure 5**). In this scenario, up to 10% of patients who are clinically diagnosed with the disease without harboring an identifiable genetic cause may suffer from a HCM phenocopy [171, 177, 207]. These phenocopies include disorders and syndromes that mimic the clinical expression of HCM but have a different genetic origin. As a result, 20 genes have been associated with HCM with moderate evidence because of their implication in phenocopies. It is estimated that variants in

these syndromic genes may account for up to 2% of HCM cases [208] (**Figure 5**). Importantly, these conditions have a family history and inheritance fashion different from that of HCM, and they also require a distinct clinical management. Hence, early diagnosis *via* genetic testing is key to differentiate HCM from its phenocopies for a correct clinical care [171, 177, 207].

These phenocopies include various types of metabolic and storage diseases, such as Wolff-Parkinson-White syndrome [209], Pompe disease [210], Danon disease [211], Anderson-Fabry disease [212], and familial amyloidosis [213, 214]. Other malformation syndromes (such as multigenetic Noonan disease [215, 216]), neuromuscular disorders (Friedrich's ataxia [217]) and rare mitochondrial diseases [218] have also been described as HCM phenocopies.

Compound and complex HCM genotypes

A minority of HCM patients present complex genotypes, *i.e*. more than one HCM-causing variant. The prevalence of patients presenting two variants associated with disease, either within the same gene, in one or two different alleles (compound heterozygosis), or within different genes (double heterozygosis) is estimated to be very low, underlying only 3-5% of HCM cases [219, 220]. Individuals with a homozygous genotype have also been reported [221-225]. Furthermore, < 1% of HCM patients present three different disease-causing variants [226].

The association of these complex genotypes with a worse prognosis or disease severity is controversial and highly dependent on the specific pair of variants [220]. Compound and double genotypes have been linked to a higher disease penetrance, a greater degree of hypertrophy and LVOTO, and a higher SCD incidence than patients harboring a single HCM-causing variant [219, 227-230]. Homozygous or compound heterozygous truncating *MYBPC3* variants have also been linked to extreme HCM severity, leading to severe neonatal cardiomyopathy and HF within the first year of life [220, 221, 225]. Furthermore, the presence of triple variants have been associated to an increased risk of end-stage HCM and ventricular arrhythmias [226]. All these observations support a gene-dosage hypothesis, which postulates that simultaneous disruption of different sarcomere proteins or profound impairment of a single component by compound variants can contribute to a more severe disease phenotype [207, 229]. The combination of several low-penetrant variants would explain some of the clinical variability characterizing HCM [108].

However, this gene-dosage effect has been mainly observed in small families and founder populations and some other studies have detected no changes in severity, but only an increased risk for symptomatic HCM in patients with double variants [231]. Furthermore, the pathogenicity status of the variants considered in traditional studies may not comply with current variant classification guidelines [220]. As a result, curation of databases and reassessment of these observations in the light of contemporary protocols is needed before considering the identification of multiple variants as a prognostic marker.

Genotype-phenotype associations in HCM

Considerable efforts have been put in trying to correlate specific genotypes to particular disease outcomes and phenotypes. Traditional studies have supported the vision that the underlying gene variants determine the resulting clinical phenotype, as different molecular defects could lead to disease *via* alternative pathways. The identification of these genotype-phenotype relationships may be a determinant prognostic value, guiding clinical management and opening the door to personalized treatments targeting specific underlying genetic causes.

In this regard, a special focus has been laid on identifying possible phenotype differences between *MYH7* and *MYBPC3* variants, since they account for the majority of HCM cases. *MYH7* variants have been traditionally associated with an earlier onset, more severe LVH and higher incidence of SCD and arrhythmias, while *MYBPC3* variants have been linked to later onset, slower disease progression and better prognosis [57, 232-234]. Indeed, this notion has been supported by recent studies and meta-analyses retrieving data from literature [235-237]. Different age onset has also been reported depending on the underlying sarcomere gene variant [236]. Furthermore, differential clinical expression has been associated to variants occurring on components of the thick or the thin filament, being the latter related to a milder and more atypical distribution of LVH, but also to an increased risk of HF [238]. Further increasing complexity, a distinct clinical expression has been suggested depending on the genetic nature of the variant or even on the affected protein region and its specific functionality [207, 239]. However, several recent studies disagree with those observations, pointing at no significant variant-specific clinical effects. For example, several reports indicate that *MYH7* and *MYBPC3* variants are both phenotypically and clinically indistinguishable and that their effects do not depend on the variant type or the affected protein domain [231, 240, 241]. As a result, no clear consensus exists nowadays on whether specific genotypes lead to a distinct disease expression or prognosis.

Disease modifiers

The existence of disease modifiers that can alter the phenotypic expression of HCM even in the context of the same underlying causing variant also limits the utility of genetic testing for genotype-guided prognosis. It is becoming increasingly clear that a particular disease manifestation results from the combined action of HCM variants in a certain genetic background, lifestyle and environment. The synergistic effect of these patient-specific factors could contribute to explain the high phenotypic diversity of HCM, which cannot be solely interpreted on the basis of a specific gene defect [108, 172, 207].

A significant proportion of patients carry not only a classical HCM-linked variant, but also multiple polymorphisms on other sarcomere and nonsarcomere genes that can influence disease expression [172, 207]. These polymorphisms are usually referred to as modifier variants (MVs) and, even though they are usually not sufficient nor necessary to cause disease, they can still influence the penetrance of other pathogenic variants [108]. Rare variants in genes encoding components of the desmosomes, ion channels, or the renin-angiotensin-aldosterone system have been described as MVs in HCM [242-246]. However, not only low-frequency variants, but also

common single nucleotide polymorphisms have been found to have an important impact in the disease phenotype and the risk of developing HCM [247, 248]. In sarcomere-positive patients, who have a basal high penetrance risk stemming from the mutation of at least one HCM-linked sarcomere gene, modest effects from these common variants can have a determinant impact on the final disease outcome. In genotype-negative patients, the additive effect of these common variants may be of special relevance. In this regard, recent genome-wide association studies (GWAs) have revealed that a polygenic risk score based on common HCM susceptibility variants may account for the phenotypic variability observed among carriers of disease-causing variants [247]. The same study led to the identification of new loci associated with HCM, dilated cardiomyopathy (DCM) and LV traits. Indeed, a genetic correlation has been described between myocardial traits in the general population and the susceptibility to HCM and DCM, with opposite direction of effects. Among these traits, an increased LV contractility has been proposed to be causally associated with HCM development [247]. Moreover, a causal relationship between HCM and other conditions, such as hypertension or obesity, has also been suggested in additional studies [248, 249]. Particularly, a high diastolic blood pressure was identified as a substantial risk factor for HCM in genotype-negative patients, which may indicate that these subjects develop HCM from an exaggerated response to hypertension in the context of a certain susceptible genetic background [248]. Consequently, not only HCM-linked variants would be important for genetic counselling and clinical management, but also a strong influence of polygenic risk factors, especially in certain basal conditions such as hypertension, should be considered. There are also other factors that can influence HCM expression. For example, sex has been proven to influence the clinical manifestation of HCM, presumably through the expression of certain hormones [250, 251]. Likewise, lifestyle habits, such as exercise and diet, can also play a role in disease manifestation and progression [252].

Challenges in variant annotation and interpretation

Genetic testing is routinely performed on HCM patients since the identification of causative variants is key for the correct clinical management of patients and the identification of at-risk relatives [253-255]. However, the high proportion of genotype-negative individuals limits the benefits of current genetic testing [44, 45]. Besides, most HCM-causing variants are considered “private”, as they are only described in a proband and their relatives or in a small group of families [256]. In many cases, cosegregation analysis is difficult to perform, which leads to classification of variants as of uncertain significance (VUS). This situation contributes a fraction of HCM cases for which a genetic cause cannot be ascertained [253-255]. Furthermore, the incomplete disease penetrance and the modest impact of some pathogenic variants also contribute to HCM underdiagnosis [108, 255, 256].

The advent of next-generation sequencing techniques has revolutionized the annotation of cardiomyopathy-related genes [253-255]. However, distinguishing disease-causing variants from nonpathogenic polymorphisms have remained challenging. Traditionally, gene-disease associations have been based on lax criteria, relying mostly on the absence of the tested variant in limited control populations, or in poor genotype-phenotype associations in small groups of

families [177]. As a result, many of the identified variants are now categorized as VUS, since they lack enough experimental and/or co-segregation data to define whether they are causative of HCM or disease-unrelated polymorphisms [253-255]. To overcome this problem, several genome and exome databases, such as the Exome Aggregation Consortium [257], or the 1000Genomes Project database [258], among others [259], have been created to compare the frequency of the variants in the general population with the expected HCM prevalence. Their application has led to reclassification of >10% of previously considered HCM-linked variants [177, 260, 261].

Nowadays, genetic variants must comply with several lines of evidence of pathogenicity, according to the American College of Medical Genetics and the Association of Molecular Pathologists (ACMG/AMP), before they are considered as disease-linked (see reference [259] for a complete list of classification criteria). For instance, variant enrichment in affected individuals compared with control populations strongly supports disease association (PS4 ACMG/AMP criterion). In this same direction, very low variant frequency in the general population, indicating that it is unlikely to be a polymorphism unrelated to disease, is considered moderate evidence of pathogenicity (PM2 criterion). Co-segregation analysis confirming that only affected individuals are carriers of the tested variants is also a supporting sign of causality (PP1 criterion). Moreover, nonsynonymous gene variants resulting in changes in the amino acid sequence, especially when affecting functional protein regions conserved throughout evolution, are more likely to be pathogenic (PM1). In this regard, *in silico* predictions have been proven very useful since they anticipate the functional outcome of variants in the target gene sequence or the resulting protein [262]. Indeed, multiple lines of computational evidence supporting a deleterious outcome are also useful in the assessment of variant pathogenicity (PP3 criterion) [259]. These predictors allow the selection of positive hits, *i.e.* variants with significant alterations in the parameter of interest, for further experimental validation, which entails considerable time and cost savings. In this regard, functional tests informing damaging effects of tested variants also provide strong evidence of pathogenicity (PS3 criterion) [259]. Current approaches for functional evaluation of variants include *in vitro* interrogation of disease-associated molecular features [263, 264] and more physiological examination of variant-induced effects by means of hiPSC-derived CMs [35-37, 265-267] and EHTs [268-272].

Cardiac myosin-binding protein C: structure and function

MYBPC3 genomic organization and cMyBP-C protein structure

Myosin-binding protein C (MyBP-C) was first identified as a co-purifying contaminant in myosin preparations from rabbit skeletal muscle extracts [273, 274]. The protein is approximately 40-nm long and 3-nm wide, and its molecular weight is around 140 kDa [125, 275]. There are three highly homologous MyBP-C isoforms [276] encoded by different genes: slow skeletal (ss)MyBP-C is encoded by *MYBPC1* in chromosome 12, fast skeletal (fs)MyBP-C is encoded by *MYBPC2* in chromosome 19, and cardiac (c)MyBP-C is encoded by *MYBPC3* in chromosome 11 [76, 125]. The

cardiac isoform was the last one to be discovered [277] and *MYBPC3* genomic localization was traced to chromosomal position 11p11.2 in humans [278]. The structure and sequence of the human *MYBPC3* gene were published in 1997, revealing that it consisted of more than 21,000 bp and 35 exons (**Figure 6**) [279]. Of these 35 exons, 34 are coding and two are unusually small, each consisting of only 3 bp. Interestingly, there is a striking correlation between the limits of the exons and those of the structural protein domains, with each domain being encoded by two or three exons in most cases [279].

cMyBP-C is exclusively present in the heart, where no other MyBP-C isoform is normally expressed [125, 280]. This restrictiveness avoids complementation of cMyBP-C expression by their skeletal counterparts. Conversely, the two skeletal isoforms concur in the skeletal muscle, where ssMyBP-C is first expressed in development and fsMyBP-C is detected at later stages. As a result, transcomplementation of the skeletal isoforms is possible in the event of alterations in MyBP-C stoichiometry [280].

The different MyBP-C isoforms belong to the intracellular immunoglobulin superfamily [125, 279], as they share a common modular architecture consisting of repetitions of globular domains of the immuglobulin (Ig) or fibronectin-III (Fn3) families, named C1-C10 (**Figure 6**). At the N-terminal region, a proline-alanine-rich region (P/A) can be observed, together with a 105-residue-long stretch called MyBP-C (M) motif connecting domains C1 and C2. Furthermore, the cardiac isoform cMyBP-C has distinctive structural features, *i.e.* an additional Ig domain C0 at the N terminus, connected to C1 through the P/A-rich region; a nine-residue-long loop in the M motif (LAGGGRRIS in the human sequence) containing an extra phosphorylatable serine residue, and a 28-residue-long loop in the C5 domain [278, 279, 281] (**Figure 6**).

cMyBP-C interactome

Several models have been proposed for the arrangement of cMyBP-C within the sarcomere [282]. In this regard, Winegrad *et al*. first proposed a scenario in which three cMyBP-C molecules would form a collar around the thick filament, with the three C-terminal domains of one molecule binding to the three N-terminal modules of the next [282, 283]. This region overlap would maintain tight packing of the thick filament backbone and restrict the movement of myosin heads. Upon adrenergic activation these interactions would be shifted to only involve domains C0 and C10 of each cMyBP-C molecule, hence loosening the myosin rods and freeing myosin heads for interaction [283]. Apart from this first proposal, an additional collar model was suggested in the light of *in vitro* binding assays revealing intramolecular interactions between cMyBP-C domains. In this case, cMyBP-C would trimerize to form a collar around the thick filament, with domains C5-C10 of each molecule wrapping around the backbone and domains C0-C4 extending into the interfilament space [282, 284].

The accuracy of these collar models has been questioned upon description of cMyBP-C interactions with components from both the thin and thick filaments, which indicates that cMyBP-C forms bridges with both types of filaments in the sarcomere. Indeed, direct visualization of cMyBP-C transverse tethers suggests that most of the protein extends away from the thick filament backbone, contacting actin [100, 125, 285-288]. Regarding specific interactions enabling

tethering, cMyBP-C interacts axially with thick filament components through its C-terminal domains, whereas its N terminus reaches the vicinity of the thin filament, either contacting actin and/or myosin S1 region (see below) (**Figure 6**). It has been suggested that the N terminus of cMyBP-C may switch between myosin and actin to fine tune muscle contraction [289] (see section *The complex regulatory role of cMyBP-C in sarcomere contraction*).

"C-protein" was first identified as a myosin-binding protein and subsequently named MyBP-C after this observation [274]. Since then, several studies have unraveled that cMyBP-C can interact with different myosin regions through its two termini (**Figure 6**). In this regard, early studies identified the strong affinity of the last Ig repeat in the C terminus of fsMyBP-C for the LMM region [290, 291]. An equivalent interaction has also been reported for the cMyBP-C isoform [292-294]. The M motif has been described to bind myosin S2 [295, 296], and this interaction would be sufficient for the incorporation of cMyBP-C into the cardiac sarcomere [295]. Furthermore, phosphorylation of key residues within the M motif by protein kinase A (PKA) (see section *cMyBP-C posttranslational modifications*) abolishes this interaction [296, 297], which has direct implications in the regulation of cardiac dynamics. The interaction with myosin S2, more specifically at the vicinity of the S1-S2 hinge, has also been detected for the N-terminal cMyBP-C C1 and C2 domains [298, 299] (**Figure 6**). In addition, the interaction between the cardiac-specific C0 Ig domain and RLC has been reported (**Figure 6**). Similarly to what happens with the interaction between the M motif and myosin S2, this contact could be phosphorylation-dependent through modification of RLC [300]. Moreover, human dephosphorylated full-length cMyBP-C or C0C2 have been shown to bind directly to human β-cardiac short S1 (sS1, a myosin head fragment lacking RLC) in a phosphorylation-dependent manner [199]. In this line, C1 has been identified as the major myosin head-binding domain within cMyBP-C N terminus by *in vitro* binding experiments using microscale thermophoresis (MST) [296]. Using MST, the interaction between C3 and myosin S1 has been recently found in two independent reports [264, 296] (**Figure 6**). Indeed, the interaction sites with the highest affinity for myosin S1 are not localized to cMyBP-C N terminus, but to central segments C2C4 and C5C7 [296]. These two segments also interact with the so-called miniHMM fragment, consisting of a short fragment of the myosin tail, ΔS2, with two bound RLCs. In addition, C5C7 also binds the bare ΔS2 myosin tail (**Figure 6**) [296].

Titin was also early identified as a major MyBP-C binding partner in the thick filament backbone [301]. Since then, different *in vitro* studies have demonstrated that the titin-binding region within cMyBP-C is located at its C-terminal end. Indeed, a 3D reconstruction of cardiac filaments based on electron microscopy analysis revealed that titin lies in an elongated strand along the thick filament axis, where it interacts with the three C-terminal domains of cMyBP-C and the myosin backbone [113] (**Figure 6**). Regarding interaction sites within titin, 11-residue-long super-repeats in this protein provide regularly spaced binding sites for cMyBP-C and myosin [100, 113]. Indeed, a series of super-resolution imaging studies have recently determined that the C-terminal end of cMyBP-C anchors at the interface between two titin super-repeats [302]. These observations suggest that the spatial distribution of these super-repeats is the major determinant controlling the regularly spaced positioning of cMyBP-C in discrete stripes in the C-zone of the sarcomere.

The first evidence of the interaction of MyBP-C with F-actin came from experiments with skeletal myofibrils. These *in vitro* assays also pointed out that the length of MyBP-C was sufficient to contact actin while remaining attached to the thick filaments [303]. Later on, electron tomography experiments on frog sartorius muscle confirmed that a major extension of MyBP-C reaches out beyond myosin crowns, contacting actin *in vivo* in physiological conditions [286]. In this same direction, recent super-resolution fluorescence microscopy experiments have demonstrated that the cMyBP-C N terminus is disordered and biased towards the thin filament both in activating and relaxed conditions in mice samples [288].

Different studies have identified specific actin-binding regions in cMyBP-C. In this regard, both N-terminal C0C1 and C0C2 fragments have been shown to bind F-actin, inducing its polymerization into filaments or helically ordered complexes [289, 304, 305]. The construct C0-C1-M-C2 binds actin through multiple sites, crosslinking F-actin filaments [306] (**Figure 6**). Furthermore, it has been described that actin interactions involving the cMyBP-C N terminus are reduced upon M motif phosphorylation [306, 307]. Additional experiments have shown that actin binding, particularly the interaction between C1 and Tm, could shift Tm conformation to the active state for thin filament sensitization, regulating myosin head accessibility to actin [305, 308]. In this interaction, C0 would be key to stabilize C1 contacts with actin [309]. Another study suggests that both C0 and C1 can bind actin in the same position, competing with myosin heads [310]. Albeit impeding myosin head recruitment, this interaction would also sensitize the thin filament through the displacement of Tm towards its open state. As a result, the interaction between cMyBP-C and actin would promote the activation of the thin filament, even in the absence of calcium. At high calcium levels, however, when Tm is predominantly in its open state, cMyBP-C binding to actin could conversely block the attachment of myosin heads, inhibiting thin filament sliding.

cMyBP-C posttranslational modifications

Phosphorylation of cMyBP-C has attracted a lot of attention in the last years, since it has a profound effect on cardiac contractility mainly through modulation of cMyBP-C interactions with other sarcomere partners [311, 312]. Indeed, together with phosphorylation of cTnI and phospholamban, cMyBP-C phosphorylation contributes to the positive inotropy and enhanced systolic function resulting from cardiac adrenergic stimulation [313, 314].

cMyBP-C has a number of key phosphorylatable residues in the M motif, among which Ser273, 282 and 302 in the mouse sequence have been more extensively characterized [312]. Gautel *et al.* first identified the PKA-mediated phosphorylation of these residues [278]. Indeed, the authors showed that these sites are targeted by PKA in a hierarchical manner in the sense that phosphorylation of Ser282 within the cardiac-specific LAGGGRRIS loop induces conformational changes that make the neighboring sites more accessible [278]. Furthermore, Ser282 was also described as a potential target for Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-mediated phosphorylation [278]. cMyBP-C is also phosphorylated by protein kinase C (PKC) [312]. Indeed, this kinase shares common phosphorylation sites with PKA, which are absent in MyBP-C skeletal isoforms [315]. Protein kinase D (PKD)-mediated phosphorylation of cMyBP-C Ser302

has also been proposed to accelerate crossbridge cycling kinetics [316]. This same effect has been observed upon Ser282 phosphorylation by 90-KDa ribosomal S6 kinase (R6K) [317]. The action of some of these kinases is no independent in the sense that phosphorylation by one of them can influence the activity of partner protein kinases affecting the same or neighboring targeting sites [318].

As deduced from the existence of this variety of phosphorylation-sensitive residues and targeting kinases, cMyBP-C is extensively phosphorylated *in vivo* in basal conditions and its dephosphorylation can be observed in the context of HF or pathological hypertrophy [319]. Indeed, replacement of endogenous cMyBP-C by a nonphosphorylatable form in mice causes depressed cardiac contractility, altered sarcomere structure and upregulation of hypertrophy-associated transcripts [319]. Furthermore, the expression of this nonphosphorylatable form in the context of *Mybpc3* null mice does not rescue its phenotype of cardiac hypertrophy, myofibril disarray and fibrosis [319]. All these data suggest that cMyBP-C phosphorylation is essential for normal cardiac function and architecture.

Moreover, different studies have revealed that the N-terminal region of cMyBP-C, especially through the M motif, interacts with calmodulin (CaM) in a calcium-dependent manner [320, 321]. CaM is an intracellular calcium receptor which binds to and modulates the activity of several target proteins upon calcium binding, such as calcium-dependent kinases. This way, the physical interaction between the M motif and CaM has been proposed to act as a structural conduit linking cMyBP-C with calcium signaling pathways to coordinate and integrate downstream phosphorylation by different CaM-dependent kinases, such as CaMKII or myosin light chain kinase (MLCK). The activity of these kinases regulates the interaction between cMyBP-C and myosin S2 or RLC, respectively, which has an impact on sarcomere performance [125, 321].

Apart from phosphorylation, cMyBP-C is target of other PTMs, such as redox modifications (carbonylation, S-nitrosylation and S-glutathionylation), acetylation and citrullination [311]. Although different effects have been proposed for these PTMs, such as protein degradation, modulation of protein-protein interactions and regulation of sarcomere contractility and calcium sensitivity, the role of these modifications in cardiac pathophysiology remains poorly understood.

The complex regulatory role of cMyBP-C in sarcomere contraction

Several studies performed on transgenic mouse models, which grew to adulthood even in the event of full transcriptional *Mybpc3 knock-out* (KO) [22, 23, 322, 323], evidence that cMyBP-C is not essential for viability, but its normal expression is crucial for sarcomere activity.

cMyBP-C exerts a dual effect on sarcomere contraction as both positive and negative effects have been described. This dual modulatory role depends on calcium concentration and is thought to be possible thanks to the interaction of the cMyBP-C N terminus with both actin and myosin, which allows cMyBP-C to shuttle between these two major players of sarcomere contraction [125]. We describe the current view on how cMyBP-C achieves modulation of sarcomere activity below.

At low calcium concentration, the N-terminal domains of cMyBP-C bind to actin, shifting Tm to its active structural state and therefore favoring myosin head accessibility to the thin filament [308-310, 324-327]. Furthermore, recent *in vitro* single-molecule imaging studies suggest that at this low calcium level cMyBP-C diffusively scans the thin filament, potentially searching for a binding access towards actin sensitization [326].

At high calcium levels, however, cMyBP-C inhibits contraction, resulting in slower crossbridge cycling rates and maximal sliding velocity, limited force production, and reduced myosin power output [328, 329]. Different mechanisms have been proposed as significant contributors to this effect.

On one hand, competition of cMyBP-C and myosin heads for the same actin-binding sites could prevent the formation of crossbridges and subsequent contraction cycling [325]. This competition hypothesis is supported by the fact that actin sites for myosin head recruitment are also targeted for binding by C0 and C1 domains [310]. Furthermore, the previously described diffuse cMyBP-C scanning on the thin filament turns to a more extensive and tight binding at high calcium concentration, potentially blocking myosin-binding sites [326].

On the other hand, orientation of the myosin heads with respect to the thick filament shaft can also influence their accessibility to the thin filament. Indeed, studies identifying the C0 and C1-M interaction sites on RLC and the vicinity of myosin S1-S2 hinge, respectively, have proposed that this N-terminal region can control myosin heads orientation and influence crossbridge formation rates [295, 297, 298, 300, 309, 330]. In this same regard, Witt *et al.* proposed that cMyBP-C interacts with the myosin head and neck simultaneously, imposing steric hindrance on the head and regulating crossbridge formation [331]. Moreover, the M motif-S2 binding could sequester myosin heads to the backbone of the thick filament, affecting their flexibility and preventing their swinging for actin interaction. In this sense, a more disordered structure of the myosin heads in isolated thick filaments from *Mybpc3* KO mouse models have been observed [100, 113, 332, 333]. Recently, a hypothetical model resulting from *in vitro* binding data has proposed that a constitutive interaction with cMyBP-C central domains would guide myosin heads to and away from the thin filament, tuning crossbridge formation rate [296].

As an additional potential scenario explaining contraction inhibition, cMyBP-C interaction with different sarcomere partners could create a viscous load dragging the sliding of actomyosin filaments [325, 326, 334]. Regarding this hypothesis, Previs *et al*. developed *in vitro* motility assays in which fluorescently labelled single actin filaments could be visualized moving over native cardiac thick filaments [335]. A reduction in the actomyosin motion generation in the C-zone could be detected, which suggested that cMyBP-C induced an internal load to lower power output

and energy utilization [335]. Several hypotheses have been presented with regards to the nature and protein regions implicated in the formation of the tethers. For example, Hofmann *et al.* early proposed that MyBP-C could directly tether myosin S2 to the thick filament, which could result in straining of long-lived crossbridges in a direction opposing filament sliding [327, 336].

Another possibility is that simultaneous interaction of cMyBP-C with both actin and myosin occurs [287, 288, 327, 336, 337]. These cMyBP-C protein tethers would create a mechanical load opposing shortening, and domains would be subsequently subject to mechanical force during contraction [134] (**Figure 7A**). Indeed, several reports have demonstrated the mechanical behavior of cMyBP-C domains under force using atomic force spectroscopy (AFS) [264, 337-340]. In the event of actomyosin bridging, it has also been proposed that cMyBP-C could also act as a force transducer, communicating titin-based strain in the thick filament to the regulatory apparatus of the thin filament and thereby contributing to the orchestration of sarcomere shortening [341].

A combination of various effects in the inhibition of sarcomere dynamics is also possible. Indeed, a mathematical model developed by Walcott *et al*. suggests that a combined drag-competition mechanism best explains the inhibitory effects of cMyBP-C on actin motility [334]. According to this model, cMyBP-C would primarily block myosin head recruitment but it would additionally create transient links with sarcomere partners, further restricting contraction. As presented, the dual role of cMyBP-C on the regulation of sarcomere contraction relies on its interaction with different sarcomere partners. Subsequently, the modulation of these interactions by phosphorylation or load can have an impact on contraction dynamics [125]. Experiments conducted on skinned fibers first demonstrated that the interaction between the M motif and myosin S2 regulated contractility [330]. Additional experiments have confirmed that this interaction limits actin sliding velocities in *in vitro* motility assays [328]. Upon adrenergic stimulation, phosphorylation of the M motif in key conserved residues leads to disruption of its interaction with myosin S2 [297], relieving a potential sequestration of myosin heads and making them more available for actin binding [342]. Importantly, the effect of phosphorylation on the inhibitory capacity of cMyBP-C would only be patent at low calcium levels. Maximum calcium levels at the peak of contraction would revert cMyBP-C structure to the one capable of binding to and sensitizing the thin filament, even in the event of phosphorylation [343].

Unexpectedly, results using a novel cut-and-paste method to study the effects caused by *in situ* replacement of endogenous C0C7 cMyBP-C fragments has also uncovered a role of the protein in spontaneous oscillatory contraction [344]. Specifically, the loss of C0C7 induces auto-oscillatory contractions at submaximal calcium concentration, which are damped upon addition of the dephosphorylated recombinant exogenous fragment but not on application of the phosphorylated analogue. These data suggest that cMyBP-C N-terminal domains play a role in halting spontaneous oscillatory contractions in the sarcomere, which may contribute to boost contractility upon adrenergic stimulation.

In line with cMyBP-C allowing for full relaxation in diastole [345], several studies have suggested a role of cMyBP-C in promoting the energy-conserving myosin SRX state [163-167] (Figure 7B). Indeed, it was first reported that skinned cardiac fibers from homozygous but not heterozygous *Mybpc3* KO mice have a significant decrease in the myosin SRX population as compared with wild-type (WT), supporting the idea that the loss of cMyBP-C results in the activation of myosin heads [163]. In this same line, Toepfer *et al*. used three different mouse models to show that a graded loss of cMyBP-C resulted in a concomitant reduction of the SRX state [166]. Moreover, a depopulation of this configuration has also been observed in HCM patients harboring *MYBPC3* variants [164]. As further supporting evidence, the addition of the C0C7 cMyBP-C subfragment to purified human β-cardiac myosin increases the population of myosin in the SRX state [167]. These data suggest that the decline of cMyBP-C content in HCM-causing truncating *MYBPC3* variants may contribute to disease development through depopulation of the myosin SRX state, which would increase the dynamics of crossbridge formation and induce hypercontractility. Remarkably, cMyBP-C phosphorylation has been proposed to regulate the SRX:DRX equilibrium [163, 165]. In this regard, a triple phosphomimetic version of cMyBP-C (substituting Ser273, 282 and 302 in the M motif to aspartic acid) in mice significantly depopulated the SRX state compared to WT and to mice expressing a phosphoablated version of the protein (same serine residues mutated to alanine) [165].

Regarding the structural basis of the cMyBP-C-mediated stabilization of the SRX population, examination of skinned myocardial preparations from WT and *Mybpc3* null mice have revealed that the loss of cMyBP-C induces a radial displacement of the crossbridges away from the thick filament [346]. Considering the hypothetical correlation between the SRX state and the structural sequestration of myosin heads (see section *Regulation of contraction by myosin conformations*), this observation would support a role of cMyBP-C in the formation of the SRX state through the tethering of myosin heads to the thick filament shaft. However, the specific protein regions involved in this hypothetical cMyBP-C-myosin-interaction-dependent regulation of the myosin ATPase activity have not been identified yet. In this sense, fluorescence microscopy experiments support the notion that the bulk of the cMyBP-C molecule extends radially towards the actin filament both in relaxing and activating conditions, positioning its N-terminal region far away from the myosin heads [287, 288]. Remarkably, very recent *in vitro* data suggest that the central region of cMyBP-C may bind myosin heads, potentially regulating their ATPase activity [296].

Apart from governing sarcomere contraction dynamics, an emerging hypothesis suggests that cMyBP-C is also implicated in excitation-contraction coupling [347]. In every contraction cycle, calcium is released from T-tubules and diffuses towards the center to fully activate contraction. This diffusion induces a nonuniform distribution of calcium and a consequent inefficient activation of contraction. In this scenario, cMyBP-C would sensitize the thin filament in a manner that counterbalances this calcium gradient, ensuring rapid and uniform force development during early systole.

HCM-causing *MYBPC3* variants

About 40% of HCM cases with an identifiable genetic defect are caused by variants in *MYBPC3*, the most commonly mutated gene in the development of this disease [45, 76, 348, 349] (**Figure 5**). The association of *MYBPC3* with HCM came from independent studies evidencing physical linkage between disease-causing variants and *MYBPC3* chromosomal location 11p11.2 [178, 179].

The spectrum of *MYBPC3* variants differs from the one reported for other sarcomere genes implicated in the disease. In this regard, whereas HCM-causing *MYH7* variants are almost exclusively missense (see section *HCM-causing MYH7 variants*), the majority of *MYBPC3* variants lead to prematurely truncated polypeptides which in many cases lack the titin- and myosin-binding C-terminal domains [76, 348, 349]. The rest of HCM-linked *MYBPC3* variants are nontruncating, *i.e*. single nucleotide substitutions resulting or not in an amino acid change in the mutant protein.

Truncating MYBPC3 variants causing HCM

Most known *MYBPC3* variants associated with HCM are nonsense, insertion/deletions (indels) or variants leading to splicing defects, which result in the appearance of a premature termination codon (PTC) and the truncation of the resulting mutant polypeptide. Whereas nonsense variants directly generate a PTC in the variant location, the appearance of a PTC in indels and splicing defects results from variant-induced changes in the reading frame (frameshift variants) [76, 348, 349]. While interpretation of nonsense and indel variants is straightforward, frameshift effects induced by splicing variants may be challenging to anticipate.

During transcription, the genetic information contained in the DNA is first transferred to precursor mRNA (pre-mRNA) molecules, which must then be processed into the final mature mRNA. The pre-mRNA molecule is made up of noncoding regions or introns and coding sequences or exons. An important step in the processing of pre-mRNA is called splicing and consists on eliminating the introns and juxtaposing the coding exons to generate the mature mRNA [350, 351]. The splicing process involves recognition of certain conserved sequences at the exon-intron boundaries, both at the beginning (5' splicing donor sites with an invariant initial GT dinucleotide) and at the end (3' splicing acceptor sites with an invariant final AG dinucleotide) of introns. Changes in these invariant dinucleotides alter the consensus splicing pattern of the pre-mRNA and consequently they are considered pathogenic in autosomal dominant diseases caused by protein loss of function [261, 352, 353]. Alternatively, variants can activate cryptic splicing acceptor or donor sites, which can compete with the native ones in the processing of the pre-mRNA. In either case, the alteration of the canonical splicing usually leads to the skipping or removal of coding sequences or to intron retentions [354], which normally results in the appearance of a PTC and the consequent truncation of the final mutant protein. Several of the first reports associating the chromosome 11p-linked form of HCM with *MYBPC3* variants are based on the identification of disease-causing splicing variants [178, 179, 279].

Truncating MYBPC3 variants: haploinsufficiency vs "poison peptide" as the disease mechanism

Several molecular effects have been proposed as the pathomechanism leading to HCM by truncating *MYBPC3* variants. If the mutant truncated protein is expressed and prevails, it can act as a "poison peptide" through a dominant-negative effect, altering the assembly and function of the sarcomere. Alternatively, if the mutant is not produced or it is rapidly degraded, disease can be a consequence of reduced cMyBP-C levels (protein haploinsufficiency) [76, 355]. A "Third Way" pathomechanism in which cell-to-cell imbalance in the expression of mutant proteins can lead to disease has been proposed for *MYH7* variants [356]. In principle, such a mechanism could also operate in HCM caused by *MYBPC3* variants.

During the late 90s-early 2000s, initial reports on truncating *MYBPC3* variants in engineered transgenic murine models proposed the "poison peptide" hypothesis as the most probable disease mechanism of these variants in the development of HCM [355]. In this regard, Yang *et al*. generated a transgenic mouse model expressing a truncated form of cMyBP-C, which constituted 40-60% of the total cMyBP-C pool but was not efficiently incorporated into the sarcomere. Although the expression of the truncated cMyBP-C also led to a reduction in the endogenous levels of WT cMyBP-C, the authors partly attributed the observed sarcomere dysgenesis to the "poison peptide" action of the truncated cMyBP-C [323]. Further evidence for this negative-dominant function came from transgenic mouse models expressing different C-terminally truncated cMyBP-C whose expression in the myocardium frequently accompanied an aberrant sarcomere architecture and functional defects [322, 357, 358]. Furthermore, the expression and myofibril incorporation of mutant proteins have not only been detected for C-terminally truncated proteins, but also for cMyBP-C lacking the N-terminal C1 domain and the P/A –rich region in both heterozygous and homozygous transgenic mice [331].

However, further observations in engineered murine models have evidenced that the truncated proteins were not always detectable and that either its expression or absence was accompanied by a reduction in the total levels of endogenous WT cMyBP-C [22, 23, 323]. These data directly confront the hypothesis that the "poison peptide" effect is the solely mechanism by which truncating *MYBPC3* variants generate disease. Furthermore, a definitive challenge to this dominant-negative notion came from key observations on human myocardial samples from HCM patients carrying nonsense or frameshift *MYBPC3* variants. In these human sources, truncated cMyBP-C has never been detected [359-366] and the absence of these mutants is usually accompanied by a reduction of the full-length WT cMyBP-C [361-366]. The absence of these truncated proteins in human heart tissue argues against its malignant incorporation in the sarcomere. On the contrary, the reduction of endogenous protein levels points at haploinsufficiency as the main disease mechanism by which heterozygous truncating *MYBPC3* variants cause HCM [76, 355] (**Figure 8A**).

Haploinsufficiency occurs when the heterozygous truncating variant generates a null allele, the expression and function of which cannot be compensated by those of the healthy allele [355]. In other words, the expression of a single functional copy of *MYBPC3* results in insufficient cMyBP-C protein content, below the threshold level required to carry out its normal function within the

sarcomere. Given that cMyBP-C is co-expressed with other thick filament components in early stages of myofibril development [280, 367], insufficient cMyBP-C could lead to an imbalance in the stoichiometry of sarcomere proteins, causing dysgenesis and altered contractility [279, 355, 368]. Mechanistically, myofilament sliding velocity has been shown to be enhanced in the C-zone of human HCM myocardium that is cMyBP-C haploinsufficient as a consequence of a truncating *MYBPC3* variant [366]. In addition, studies on skinned heart fibers from HCM patients bearing heterozygous truncating *MYBPC3* variants have revealed a 50% reduction in the myosin SRX state resulting in hypercontractility, impaired relaxation and excessive energy consumption [166] (see also section *The complex regulatory role of cMyBP-C in sarcomere contraction*). Protein haploinsufficiency can originate at the DNA, mRNA or protein stages [355]. For instance, epigenetic DNA modifications can repress the expression of a certain allele. A variant-induced reduction in the interaction of the target allele with regulatory elements, such as transcription factors, can also lead to the same outcome. On the other hand, the degradation of mutant transcripts and truncated proteins are key mechanism driving protein haploinsufficiency, as elaborated below.

Nonsense-mediated mRNA decay (NMD) is an evolutionary conserved mRNA quality control system that reduces the effective translation of truncated mutant proteins [368]. Generally, mRNAs bearing a PTC are exclusively and specifically targeted by rapid degradation by this quality control pathway [368]. Indeed, the participation of the NMD has been suggested in a number of studies using human samples and cell cultures in which a reduced amount of nonsense transcripts with respect to WT mRNA was observed [362, 369, 370]. However, NMD is not 100% efficient, and if the transcripts bear a PTC very close to their 3' terminus, they may escape targeted degradation [371]. The prevailing detection of low levels of nonsense mutant transcripts in human HCM myectomy samples [359, 360, 362] can be explained upon avoidance of this and other transcript quality control systems like the No-Go mRNA decay pathway, which stops translation in ribosomes and promotes RNA degradation through endonucleolytic pathways [372]. Studies on cardiomyocytes or mouse models have observed very low levels of truncated proteins despite robust expression of nonsense mRNA [357, 373]. All these observations indicate that further quality control systems operate at the protein level.

The absence of truncated proteins in human myectomy samples from HCM patients bearing nonsense or frameshift *MYBPC3* variants can be explained either by an inefficient translation of the mutant mRNA or by degradation of the truncated protein [355]. If the PTC-bearing mRNA adopts an aberrant folding, its translation efficiency can be slowed down or even disrupted [374]. Alternatively, truncated proteins can be targeted for degradation *via* different protein quality control systems. In particular, the ubiquitin-proteasome system (UPS) prevents accumulation of cytosolic, nuclear and myofibrillar proteins when they are mutated, damaged or misfolded [368].

Proteins targeted for UPS-mediated elimination need to be polyubiquitinated prior to their ATP-dependent degradation in the proteasome. The mechanisms identifying candidate proteins for polyubiquitination and degradation are not clear, although it has been suggested that the addition of new amino acids upon frameshift causes protein misfolding, favoring UPS recognition and action [368]. The participation of the UPS in the clearance of aberrant cMyBP-C truncated proteins has been suggested in cell cultures and *in vivo* mouse transgenic models [370, 373].

The accumulation of misfolded mutant protein can lead to the impairment of the UPS function. In this regard, it has been proposed that protein aggregates can overload the proteasome, directly interfering with its proteolytic activity [368]. In fact, UPS impairment and accumulation of ubiquitin-positive aggregates have been detected in neonatal rat cardiomyocytes expressing truncated forms of cMyBP-C [373]. Furthermore, proteasome activity was found to be markedly reduced in myectomy samples from HCM patients compared with nonfailing donors [375]. UPS impairment in these cases may prevent the proper degradation of other UPS substrates, such as prohypertrophic and proapoptotic factors, as well as channel proteins, contributing to HCM pathogenesis [368, 375, 376]. Furthermore, the efficiency of the UPS machinery decreases with age, what may explain the generally late HCM onset of *MYBPC3* carriers [377]. Combining these two effects, an study comparing the activity of the UPS in two different mouse models, an homozygous *Mybpc3*-targeted *knock-in* (KI) model expressing only a 10% of truncated protein [370] and another homozygous *Mybpc3*-targeted KO mice, expressing no cMyBP-C [22], revealed that after one year of life only the mice expressing the truncated protein showed specific impairment of the UPS, suggesting that UPS may be saturated after chronic degradation of mutant cMyBP-C [378]. Furthermore, adrenergic stress on the homozygous version of the KI mice revealed proteasome dysfunction correlating with the degree of LVH, suggesting that UPS impairment contributes to HCM pathophysiology [379].

Diverse strategies targeted at counterbalancing the effects of variant-induced null alleles have been explored [368, 380]. In this regard, partial inhibition of the NMD or UPS may be an option to rescue phenotypes induced by haploinsufficiency [368, 381]. PTC read-through to force the synthesis of the full-length protein may also work as an effective therapeutic strategy in the event of nonsense *MYBPC3* variants [355, 368]. The development of RNA-based therapies that can block cryptic splicing sites or exclude/include exons to restore the reading frame, is an active field of research nowadays [82, 83]. Similarly, gene replacement therapies using viral vectors are promising for the correction of cMyBP-C haploinsufficiency [37, 79-81].

Nontruncating MYBPC3 variants: plausible disease mechanisms

Nontruncating *MYBPC3* variants are a frequent cause of HCM. These variants induce single nucleotide substitutions in the DNA which can result (missense variants) or not (synonymous variants) in single amino acid changes at the protein level. Unlike truncating variants, nontruncating variants often lead to stable full-length proteins [76, 377]. The mechanisms by which nontruncating *MYBPC3* variants lead to the development of HCM are still obscure but, in light of their expression and incorporation into the sarcomere, it has been generally assumed that these variants cause disease through a "poison peptide" mechanism [76, 377]. This dominant-negative hypothesis has been supported by studies reporting an equal abundance of WT and mutant transcripts and preserved total cMyBP-C content in human myectomy samples carrying a missense *MYBPC3* variant [369]. Upon incorporation in the myofibrils, missense cMyBP-C mutants can exert dominant-negative effects through different mechanisms, such as enhancement of

calcium sensitivity leading to increased force generation and impairment of normal mechanical function of sarcomeres [377].

However, an alternative possibility is that nontruncating variants cause disease through protein haploinsufficiency (**Figure 8B**). In this regard, and similarly to what has been already described for truncating variants, nontruncating variants could induce the destabilization and subsequent degradation of mutant mRNA and protein through quality control systems. In support of this hypothesis, the clinical expression of truncating and nontruncating disease-causing *MYBPC3* variants is similar, suggesting that nontruncating variants may also cause HCM through protein haploinsufficiency [382-384]. Indeed, a reduction in total cMyBP-C has been detected in myectomy samples from patients harboring missense *MYBPC3* variants with respect to nonfailing hearts [361, 364]. Furthermore, a recent report has described the specific induction of two major haploinsufficiency drivers, *i.e*. RNA splicing alterations and protein destabilization (**Figure 8C,D**), by HCM-linked nontruncating *MYBPC3* variants [263]. In this scenario, the examination of RNA splicing alterations and protein destabilization as disease-associated molecular traits provided supporting evidence of pathogenicity of 11% of nontruncating *MYBPC3* VUS in the ClinVar database [263]. The disease mechanisms of these two haploinsufficiency drivers in the context of nontruncating *MYBPC3* variants are described in the following subsections.

Rare exonic putative nontruncating variants that do not alter the invariant dinucleotides in splicing canonical sites but affect nearby sequence features in the exon-intron boundaries can impair splicing [263, 350, 352, 385, 386] (**Figure 8C**). Indeed, variants can decrease the performance of natural splicing sites, create alternative inappropriate splicing sites or alter sequence recognition by splicing regulatory elements. However, while canonical splice site variants have a straightforward loss-of-function effect, the functional outcome of sequence variation in other regions is not completely characterized and as a result most of these nontruncating variants are categorized as VUS [385, 386].

In recent years, there has been interest in recognizing splice-altering variants among nontruncating *MYBPC3* VUS, in an attempt to improve variant classification. In this regard, *in vitro* prioritization of potentially disrupting variants and experimental validation by minigene splicing assays has been applied [263, 385, 386]. Splicing alterations could be detected for nontruncating *MYBPC3* variants, especially in those from cardiomyopathy and clinical databases compared with general population registries. These results support the idea that rare *MYBPC3* variants classified as nontruncating can impair canonical RNA splicing, leading to HCM-causing frameshifts and truncated polypeptides.

Variant-induced protein destabilization can lead to more frequent unfolding and consequent degradation of the mutant protein, potentially resulting in cMyBP-C haploinsufficiency [387, 388] (**Figure 8D**). In addition, unstable mutant proteins can also overload the UPS, exerting a toxic effect on the cell and activating the unfolding protein response (**Figure 8D**). In this regard, mutant cMyBP-C E334K causes cellular proteasome impairment, increasing the relative proportion of proapoptotic proteins and potentially contributing to cardiac dysfunction in

HCM [389]. General UPS impairment has also been detected in myectomy samples from a group of HCM patients including missense *MYBPC3* variant-harboring subjects [375].

Different HCM-causing missense *MYBPC3* variants have been found to profoundly destabilize the structure of mutant domains [263, 298, 390, 391]. Indeed, the observation that a missense *MYBPC3* VUS in C4 domain severely compromised its stability has led to the reappraisal of its pathogenicity [392]. Further supporting variant-induced protein destabilization and degradation, heterologous expression of mutant cMyBP-C harboring the HCM-causing missense variant p.E334K in COS-7 cells and neonatal rat cardiomyocytes led to reduced levels of the mutant protein due to its higher polyubiquitination and elimination by the UPS [389]. In this same regard, a series of missense mutants in the C-terminal C10 domain of cMyBP-C failed to incorporate into the sarcomere in rat ventricular myocytes and their degradation rates were markedly accelerated [382].

As already described for RNA splicing examination, the characterization of variant-induced changes in protein stability can be first guided by *in silico* algorithms [263, 393, 394]. In this regard, mutational modeling of HCM-causing missense *MYBPC3* variants have shown that a group of pathogenic variants in C1 domain can disturb its native structural integrity by different means [395, 396]. In this same direction, Thompson *et al*. recently applied STRUM, a structure-based algorithm, to predict the effect of missense *MYBPC3* VUS in subdomain unfolding [394]. They found that those HCM patients carrying VUS with a positive prediction of protein destabilization presented a higher probability of suffering adverse clinical events. As a result, the authors proposed that this *in silico* tool could aid in the reappraisal of *MYBPC3* VUS. Furthermore, when using STRUM over a set of missense *MYBPC3* variants with a defined pathogenicity status, they could predict protein destabilization in 32% of the pathogenic variants but only 7% of the nonpathogenic variants, further promoting the idea that detection of variant-induced protein destabilization can provide supporting evidence of pathogenicity.

However, and given the limited specificity of bioinformatics predictions, further experimental validation is needed to certainly conclude whether missense variants lead to protein destabilization. Variant-induced alterations in protein structure and changes in thermodynamic stability can be characterized by different experimental approaches [397]. On one hand, the recombinant expression of mutant domains can inform about variant-induced effects on protein stability [263, 398, 399]. Indeed, the inability of *E.coli* to produce mutant engineered domains under the same expression conditions used to generate their WT counterparts can be interpreted as a sign of variant-induced protein destabilization. If expression of the engineered mutant domain is achieved, different methods can then be used to characterize protein structure and stability, including circular dichroism (CD), calorimetry or differential scanning fluorimetry [400-403]. In this context, bacterial recombinant expression and CD data on protein stability has been recently used to estimate a 89% specificity and 60% sensitivity for STRUM predictions of cMyBP-C-variant-induced domain destabilization [404]. However, the latter may be increased when predictions are applied on domains with high-resolution structural information. Moreover, the expression of mutant proteins on specific cell lines may be especially useful for the examination of variant-induced protein destabilization phenotypes in a cellular context [382].

While RNA splicing alterations and protein domain destabilization caused by nontruncating *MYBPC3* variants have been specifically linked to induction of HCM, there are several pathogenic variants including p.R502W, the most common cause of HCM [261], that preserve both properties. Prompted by recent experimental observations, we discuss plausible alternative pathomechanisms triggered by these variants.

A number of HCM-linked missense *MYH7* variants have been proposed to lead to hypercontractility by drastically reducing binding to cMyBP-C, which would presumably reduce the fraction of myosin molecules in the SRX state [295, 297]. Similarly, variants affecting residues on the surface of cMyBP-C domains could impair its interaction with myosin or other sarcomere partners [299, 388]. For example, some HCM-linked missense variants in C1, C5 and C6 domains have been reported to substantially reduce binding affinity for myosin S2 [296, 298], whereas an abrogation of the interaction between domain C0 and RLC has been proposed for several HCM-linked missense *MYBPC3* variants [300]. Structural biology, molecular modeling and computational tools can also inform about the predisposition of a certain variant to change surface charge distributions, potentially impacting binding with other protein partners [395, 396, 405]. Assuming the proposed role of a potential myosin-cMyBP-C interaction in the regulation of the SRX:DRX myosin equilibrium, the disruption of this binding could also depopulate the SRX state, leading to contractile defects (**Figure 9A**). In support of this hypothesis, human myectomy samples with either truncating or missense *MYBPC3* variants have shown an overall deprivation of the SRX state compared to control donors [164]. However, it remains uncertain whether this hypothetical regulatory model based on cMyBP-C-myosin interactions is compatible with available imaging and structural data [287, 288].

Considering that cMyBP-C may act as a negative regulator of crossbridge cycling dynamics by imposing an internal viscous load to actomyosin sliding (see section *The complex regulatory role of cMyBP-C in sarcomere contraction*), variant-induced impairment of cMyBP-C mechanical properties could result in a defective braking function, thus leading to hypercontractility and HCM (**Figure 9B**). In this regard, AFS experiments have recently detected slight mechanical destabilization of mutant C3-R495W at low forces and increased folding rate of mutant C3-R502Q. Neither of these nanomechanical phenotypes were found in two nonpathogenic variants targeting domain C3 [264]. The authors hypothesized that the mechanical destabilization induced by HCM-linked p.R495W variant could lead to more frequent unfolding of targeted C3 domains during myosin power strokes, inducing a deficient cMyBP-C braking function during sarcomere contraction. How an increased folding rate can alter modulation of sarcomere contraction remains elusive. Future work will need to elucidate the prevalence of nanomechanical phenotypes in *MYBPC3* variants. In this regard, AFS experiments showed that at least two HCM-linked missense C3 variants (p.R495Q and p.R502W) preserve WT mechanical stability and folding rate [264], suggesting that missense variants could alter the mechanical behavior of the protein by alternative mechanisms. For example, they can impair the normal folding trajectory of the protein, affect folding/unfolding intermediates [406, 407] or, enticingly, perturb force-sensitive interaction sites [408, 409]. Both aspects are challenging to observe using conventional AFS due to limited stability and force resolution. In this regard, the application of alternative, recently

developed force-spectroscopy techniques, such as magnetic tweezers, can help unraveling how protein nanomechanics may be impaired by HCM-linked missense *MYBPC3* variants [410, 411].

Conclusions and perspectives

Variants targeting the sarcomere component cMyBP-C underlie most HCM cases with an identified genetic defect. We have reviewed the intricate regulatory role of cMyBP-C in sarcomere contraction and presented potential pathomechanisms associated with both truncating and nontruncating *MYBPC3* variants. While truncating *MYBPC3* variants have been generally described to cause disease through protein haploinsufficiency, the pathomechanisms associated with nontruncating variants have remained obscure. In this regard, the possibility exists that nontruncating variants may cause disease by lowering total cMyBP-C content. Alterations in RNA processing or protein destabilization are presented as two major protein haploinsufficiency mechanisms triggered by nontruncating variants, with the potential to guide VUS reappraisal. To examine *in vivo* the effects of a given nontruncating *MYBPC3* variant, the measurement of RNA and protein levels but particularly animal models would be of great benefit, given the limited availability of human samples. Although variant-induced protein haploinsufficiency may be a predominant pathomechanism in *MYBPC3*-variant-driven HCM, a number of variants do not appear to cause RNA splicing alterations of protein destabilization, pointing to the existence of alternative HCM pathomechanisms. For example, nontruncating variants may disrupt key binding sites mediating the interaction of cMyBP-C with other sarcomere partners. Alternatively, as recently described, nontruncating variants that alter the protein's mechanical properties may lead to defective braking function and hypercontractility. Further work aiming at increasing the number of pathogenic and nonpathogenic variants tested will be necessary to dissect these alternative molecular pathomechanisms and to define specific pathogenic variants-pathomechanism associations.

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Figure legends

Figure 1. Comparison between a healthy heart and an HCM counterpart.

The main cardiac cavities, valves, arteries and veins are indicated. In the disease state, the LV walls, together with the interventricular septum and the apex, get abnormally thicker, reducing the volume of the LV chamber. Apart from contributing to diastolic dysfunction, this pathological hypertrophy can work together with mitral valve abnormalities to hamper blood flow from the left ventricular outflow tract (LVOT)* into the aorta, causing obstructive HCM. All terms included in this figure are defined in **Table 1**.

Figure 2. The sarcomere is the contractile unit of cardiomyocytes.

A) Hierarchical organization of the cardiac muscle. Cardiac fibers are composed of cardiomyocytes, which are interconnected through the intercalated disks. The intracellular space of cardiomyocytes is occupied by transversal arrays of myofibrils, which are composed of multiple sarcomeres in series. **B)** Schematics of the cardiac sarcomere. The gliding of actin-containing thin filaments over myosin-based thick filaments and towards the M-line powers sarcomere contraction. The C-zone within the A-band defines the topographical region where cMyBP-C is located. **C)** Myosin and cMyBP-C localization in the sarcomere. Myosin is a hexameric complex composed by two MHCs and two pairs of myosin light chains (ELC and RLC). Enzymatic digestion of HMM releases myosin S1 and the proximal region of myosin S2. S1 comprises the myosin heads and the lever arm region, which serves as binding platform for the light chains. The converter domain swings in the transition between pre- and post-stroke states and this movement is amplified by the lever arm in the boosting of thin filament sliding and contraction. The dimerization of the MHCs in their C terminus creates the myosin S2 or myosin tail. The packing of myosin tails builds the LMM region, which ensembles the thick filament backbone. The troponin/tropomyosin complex regulates contraction activation in response to calcium. cMyBP-C is a thick-filament associated protein which acts a modulator of sarcomere activity through interaction with components from both the thin and thick filaments.

Figure 3. Swinging lever arm model of muscle contraction.

The different steps of the myosin mechanochemical cycle during sarcomere shortening are presented. The yellow star on myosin heads depicts the converter domain. Myosin light chains are represented in purple tones. The troponin/tropomyosin complex is omitted for simplicity. **State 1)** ATP hydrolysis changes converter domain conformation, priming myosin in a pre-stroke configuration. **State 2)** In this configuration, myosin heads can interact with actin and form crossbridges between thin and thick filaments. **State 3)** Upon P_i release, the lever arm amplifies the rotation of the converter domain, inducing the bending of the myosin heads and the subsequent displacement of the thin filaments by ~10 nm. **State 4)** When ADP is released from the nucleotide-binding pocket and ATP reoccupies this position, myosin heads detach from actin.

Figure 4. The dynamic equilibrium of myosin configurations adjusts energy consumption during mechanochemical contraction cycles.

In the myosin SRX state (*left*), the ATPase rate is very slow, whereas an intermediate ATP hydrolysis activity is associated with the DRX state. Both DRX and SRX myosin states are mainly found during cardiac relaxation. While RLC or cMyBP-C phosphorylation promote the DRX state, the small molecule mavacamten and interaction with cMyBP-C stabilize the SRX configuration. During active cardiac contraction (*right*), when calcium and ATP are available, the ATPase activity is maximal.

Figure 5. Current HCM diagnostic yield by genetic testing.

Genotype-positive individuals present an identifiable disease-causing genetic variant upon genetic testing. Most HCM cases with an underlying gene defect are caused by variants in *MYH7* and *MYBPC3*. Variants in the remaining six "core" genes in HCM etiology account for around 5% cases [261, 412]. As a result, the yield of sarcomere-positive HCM (*i.e.* finding a causative variant in sarcomere components) is ~32%. Additionally, sarcomere gene variants with an uncertain pathogenicity status in the context of HCM pathology (see section *Challenges in variant annotation and interpretation*) are also found in 9% of HCM probands [412, 413]. HCM phenocopies are caused by variants targeting genes that cause syndromic diseases (see section *HCM phenocopies*) [208]. Genotype-negative HCM refers to diagnosed cases for which no causative variant can be identified. Within this group, nonfamilial HCM comprises diagnosed individuals with no affected relatives even after sustained clinical follow-up [412]. On the other hand, gene-elusive patients present a family history of disease but lack an underlying disease-causing gene variant. Percentage data obtained from [45].

Figure 6. *MYBPC3* genomic organization and cMyBP-C protein structure.

Top: *MYBPC3* genomic DNA (gDNA) is composed of 35 exons, 34 of which are coding (boxes filled in white). *Middle*: *MYBPC3* messenger RNA (mRNA). *Bottom*: there is a spatial correlation between exons and cMyBP-C protein structure, with 2-3 complete exons encoding each domain in most cases. cMyBP-C is composed of eight Ig-like domains (ovals) and three Fn3-like domains (rectangles). The linker P/A-rich region and the M motif are also indicated. Cardiac-specific protein features are highlighted in red. Protein binding partners and cMyBP-C domains involved in specific interactions are indicated at the bottom of the figure. Figure adapted from [76].

Figure 7. Models for cMyBP-C regulatory role on contraction.

A) cMyBP-C interacts with components from both the thin and thick filaments, potentially establishing protein tethers in the interfilament space if both types of interactions happen simultaneously. This bridging would impose an internal load during myosin-driven actin gliding, limiting force generation during contraction. The establishment of anchor points in gliding actomyosin filaments would impose strain in the central domains of cMyBP-C and thus the mechanical properties of this region could be important for the viscous load induced by cMyBP-C on contraction. **B)** Further contributing to the regulation of contraction dynamics, cMyBP-C favors the SRX state, characterized by a very slow ATPase activity (see section *Regulation of contraction by myosin conformations*). The promotion of this energy-conserving myosin state would presumably result from the interaction between both proteins in the sarcomere.

Figure 8. Haploinsufficiency disease mechanisms in HCM-linked *MYBPC3* variants.

A) HCM-causing truncating *MYBPC3* variants lead to the targeted degradation of the mutant transcripts or the truncated protein, reducing total cMyBP-C protein content (haploinsufficiency). **B)** The mechanisms by which nontruncating *MYBPC3* variants cause disease are less characterized. A fraction of them can also result in cMyBP-C haploinsufficiency through RNA splicing alterations or protein destabilization. **C)** RNA splicing alterations induced by putative nontruncating *MYBPC3* variants. Variants can induce either the appearance of an alternative aberrant site, or the loss of a canonical splicing site. In either case, splicing alteration can induce frameshifts and the appearance of stop codons. **(D)** Nontruncating variants can disrupt the native structure of cMyBP-C domains, leading to its unfolding and degradation *via* the proteasome. In the event of an excess of mutant proteins, the proteasome can be overloaded, leading to accumulation of UPS substrates that may further contribute to HCM pathogenesis.

Figure 9. Potential alternative disease mechanisms in HCM-linked nontruncating *MYBPC3* variants.

A) Nontruncating *MYBPC3* variants can impair the interaction of cMyBP-C with other sarcomere partners. In the example depicted here, the abrogation of a potential cMyBP-C binding to certain myosin regions may hamper cMyBP-C-driven stabilization of the myosin SRX state, contributing to enhanced crossbridges rates, faster ATPase activity and hypercontractility. **B)** Given the mechanical role of cMyBP-C within the sarcomere, variant-induced alterations in the mechanical properties of cMyBP-C could impair its dragging role in actomyosin sliding during contraction, also leading to hypercontractility.

1 **Tables**

2

3 **Table 1. Definition of medical, anatomical and physiological terms.**

Term	Definition
Alcohol septal ablation (ASA)	Type of septal reduction therapy consisting on the localized delivery of absolute ethanol in the hypertrophied interventricular septum to provoke a controlled myocardial infarction for its long-term thinning
Angina	Chest pain caused by reduced blood flow into the heart
Aorta	Blood vessel carrying blood from the left ventricle to the systemic circulation
Aortic stenosis	Abnormal narrowing of the aortic valve, which restricts blood flow from the left ventricle to the aorta
Aortic valve	Cardiac valve regulating blood flow between the left ventricle and the aorta
Apex	Most inferior, anterior and lateral part of the heart. Tip of the left ventricle
Atrial fibrillation (AF)	Arrhythmogenic heart condition characterized by a chaotic and abnormally fast beating of the atria
Atrium	Upper cardiac chamber transferring blood received from the pulmonary veins (left atrium) or the superior vena cava (right atrium) to its corresponding ventricle
Burned-out hypertrophic cardiomyopathy	End-stage hypertrophic cardiomyopathy characterized by wall thinning, myocardial fibrosis and systolic dysfunction
Calcium sensitivity of force development	Term used to express the fact that force varies depending on the concentration of free available calcium during contraction. An increased calcium sensitivity implies that a lower concentration of calcium is needed to reach a given force value
Cardiomyocytes	Cardiac cells building up the cardiac muscle
Chronotropic	Relative to the rate of contraction
Contraction duration	Time between peak velocity of contraction and peak velocity of relaxation
Contraction/relaxation velocities	Speed at which muscle length changes along time during the contraction or relaxation phases, respectively, of the contraction cycle
Diastole	Phase of the cardiac cycle during which the heart fills with blood
Double-blind placebo-controlled randomized clinical trial (RCT)	Medical study in which volunteers are randomly assigned to different groups and neither the participants nor the researchers know which group is taking the tested drug or the placebo
Duration of the slow phase relaxation	Rapid, complete calcium removal induces a biphasic relaxation that includes a first slow tension decay phase followed by an exponential tension decrease until baseline levels. The duration of the slow phase relaxation refers to the time taken by this step to occur
Dyspnea	Shortness of breath
Fractional shortening	Percentage change in left ventricle diameter during systole. Estimate of left ventricular contractility
Heart failure (HF)	Progressive cardiac condition in which the cardiac muscle is no longer able to maintain the blood flow through the body
Hemodynamic obstruction	Impediment of the normal dynamics of the blood flow
Hypertrophic cardiomyopathy phenocopies	Syndromes that mimic the clinical expression of hypertrophic cardiomyopathy but have a different family history and genetic origin
Implantable cardioverter-defibrillator (ICD)	Electronic device connected to the heart that allows rate monitoring and suppression of abnormal cardiac electrical activity
Inotropic	Relative to the force of contraction
Interventricular septum	Wall separating the ventricles

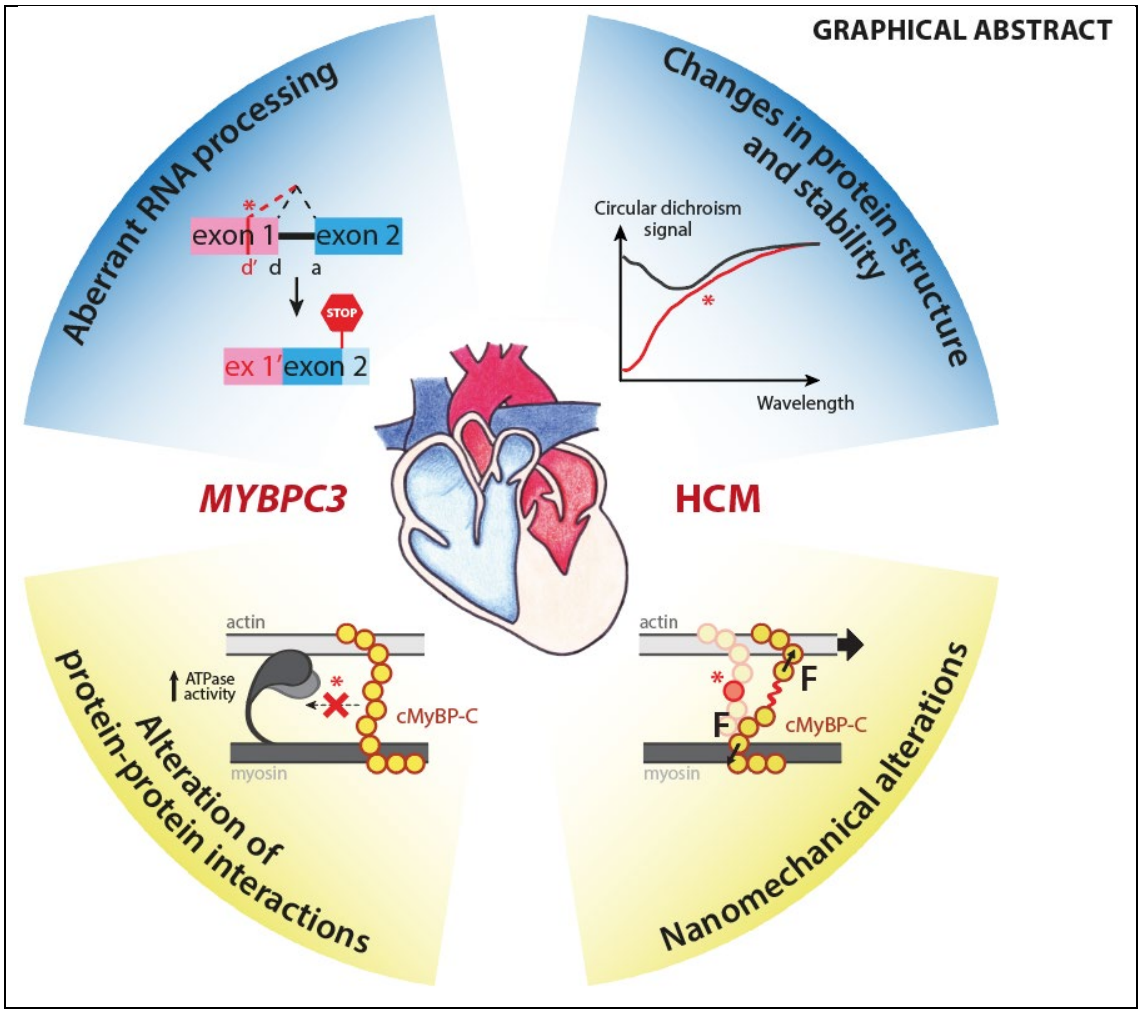
Left ventricular ejection fraction (LVEF)	Measurement (in %) of how much blood is ejected from the left ventricle to the systemic circulation in every contraction
Left ventricular hypertrophy (LVH)	Abnormal thickening of the left ventricular walls
Left ventricular outflow tract (LVOT)	Structure of the left ventricle through which the blood is pumped from the ventricle towards the aorta
Left ventricular outflow tract obstruction (LVOTO)	Condition in which the blood flow from the left ventricle into the aorta is impeded or reduced by the thickening of the left ventricular walls and the abnormal movement of the mitral valve
Maximum contraction velocity	Maximum speed at which muscular length changes during contraction
Maximum force	Also called peak force. Maximum force developed during a contraction cycle
Mitral valve	Cardiac valve regulating blood flow between the left atrium and ventricle
Myocardial bridging	Cardiac abnormality occurring when a segment of a major epicardial coronary artery (*i.e.* arteries running along the outermost layer of the heart) goes intramurally through the myocardium, being compressed in every systole
Myocardial crypts	Small, narrow invaginations in the left ventricular walls and in the interventricular septum
Nonvasodilating β-blockers	Drugs that inhibit the sympathetic activation of β-adrenergic receptors without widening the lumen of blood vessels
Obstructive hypertrophic cardiomyopathy	Hypertrophy cardiomyopathy condition in which the abnormal thickening of the interventricular septum hampers the ejection of blood from the ventricles, causing left ventricular outflow tract obstruction
Papillary muscles	Small muscles at the inner face of the ventricles which attach to atrioventricular valves to ensure their proper movement during contraction cycles
Penetrance	Proportion of individuals harboring a genotype who express the related condition
Prevalence	Proportion of individuals in the population harboring a particular genotype
Pulmonary artery	Blood vessel carrying blood from the right ventricle to the lungs
Pulmonary valve	Cardiac valve regulating blood flow between the right ventricle and the pulmonary artery
Regurgitation	Backflow of blood into the heart or between chambers
Relaxation duration	Time that relaxation takes during a contraction cycle
Relaxation half-time	Time required for the force to decrease from 95% to 50% of the maximum developed force after the last contraction. Estimate of the relaxation performance
Resting tension	Also called passive tension. Pulling force resulting from the stretching and subsequent recoiling of certain elastic foldable proteins trying to recover their original configuration
Sarcomere shortening	As a measurement of sarcomere contractility, it indicates how much the sarcomere shortens (%) during a contraction cycle
Septal myectomy	Type of septal reduction therapy consisting on the surgical removal of part of the hypertrophied interventricular septum
Septal reduction therapy (SRT)	Therapy for the treatment of hypertrophic cardiomyopathy which aims at alleviating symptoms caused by the abnormal hypertrophy of the interventricular septum
Superior vena cava	Blood vessel supplying venous blood from the upper half of the body to the right atrium
Systole	Phase of the cardiac cycle during which blood is pumped out from the ventricles
Thromboembolic stroke	Stroke caused by a blood clot that is formed in or travels to a blood vessel supplying blood to the brain
Tricuspid valve	Cardiac valve regulating blood flow between the right atrium and ventricle
Twitch force	Force developed upon a twitch contraction, *i.e.*, contraction-relaxation period of a muscle after a single, brief stimulation. Given the contemporary

	nature of twitch contraction, twitch force is lower than the maximal contraction force
Ventricle	Lower chamber of the heart. The right ventricle receives blood from the right atrium and pumps it to the lungs *via* the pulmonary artery. The left ventricle distributes blood received from the left atrium to the circulatory system through the aorta
Ventricular tachycardia (VT)	Arrhythmogenic heart condition characterized by a fast heart rate of the ventricles

Table 2. Contractility-related parameters whose enhancement or prolongation has been linked to functional hypercontractility of the muscle.

The definition of all listed parameters is provided in **Table 1**. EHTs: engineered heart tissues.

Enhanced/Prolonged contractile parameter informing hypercontractility	Sample	Reference
Sarcomere shortening* Relaxation duration*	Cardiomyocytes from mouse models and human heart tissue + hiPSC-derived CMs	[162]
Calcium sensitivity of force development*	Human muscle fibers (vastus lateralis muscle)	[414]
Twitch force* Resting tension* Maximum contraction velocity* Relaxation half-time*	hiPSC-derived CMs	[415]
Maximum force* Contraction duration*	hiPSC-derived CMs	[416]
Maximum force Calcium sensitivity of force development Contraction and relaxation velocities*	EHTs composed of mouse cardiomyocytes	[268]
Calcium sensitivity of force development	LV mouse muscle fibers	[331]
Calcium sensitivity of force development Duration of the slow phase relaxation*	LV rat muscle fibers	[417]


GRAPHICAL ABSTRACT
Aberrant RNA processing
exon 1
exon 2
d' d a
STOP
ex 1' exon 2
Changes in protein structure and stability
Circular dichroism signal
Wavelength
MYBPC3
HCM
actin
ATPase activity
cMyBP-C
myosin
Alteration of protein-protein interactions
actin
F
F
cMyBP-C
myosin
Nanomechanical alterations

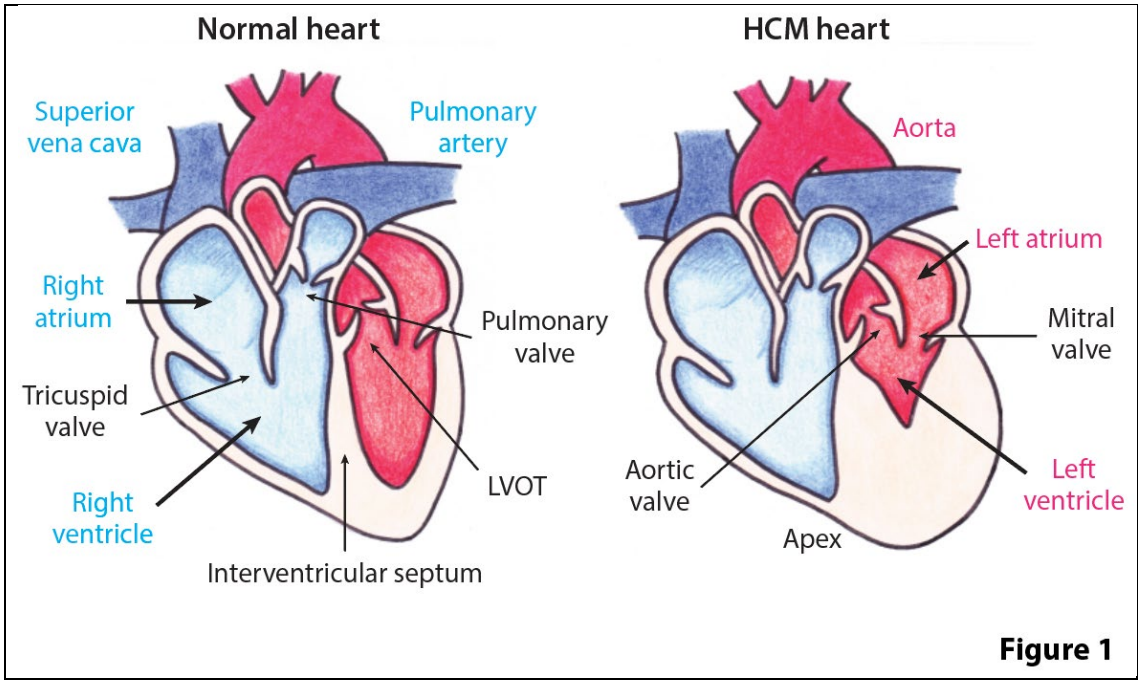
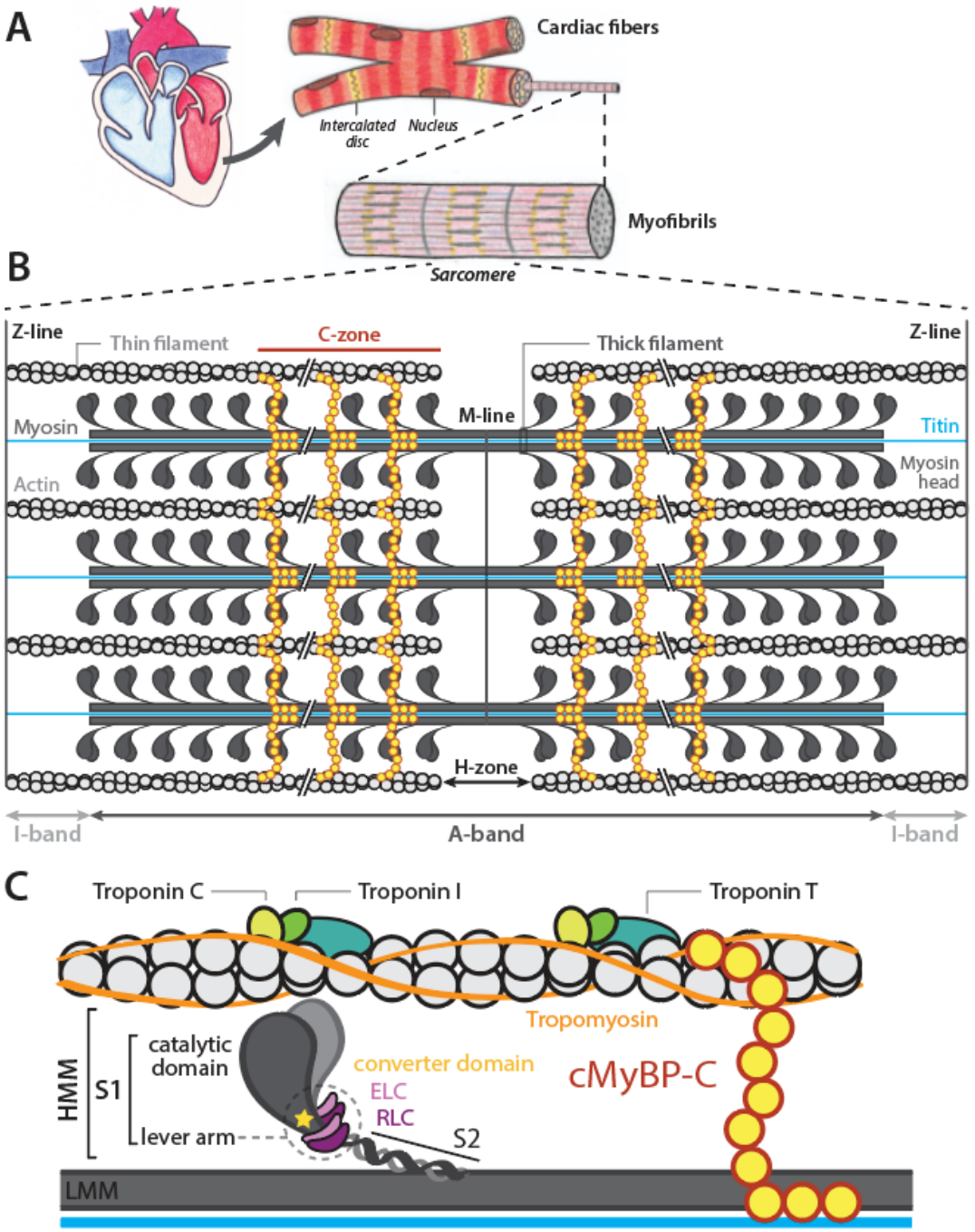

Normal heart
HCM heart
Superior vena cava
Pulmonary artery
Right atrium
Pulmonary valve
Tricuspid valve
LVOT
Right ventricle
Interventricular septum
Aorta
Left atrium
Mitral valve
Aortic valve
Left ventricle
Apex

Figure 1

Figure 2


A
Cardiac fibers
Intercalated disc
Nucleus
Myofibrils
Sarcomere
B
Z-line
Thin filament
C-zone
Thick filament
Z-line
Myosin
M-line
Titin
Actin
Myosin head
H-zone
I-band
A-band
I-band
C
Troponin C
Troponin I
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converter domain
ELC
RLC
cMyBP-C
lever arm
S2
LMM

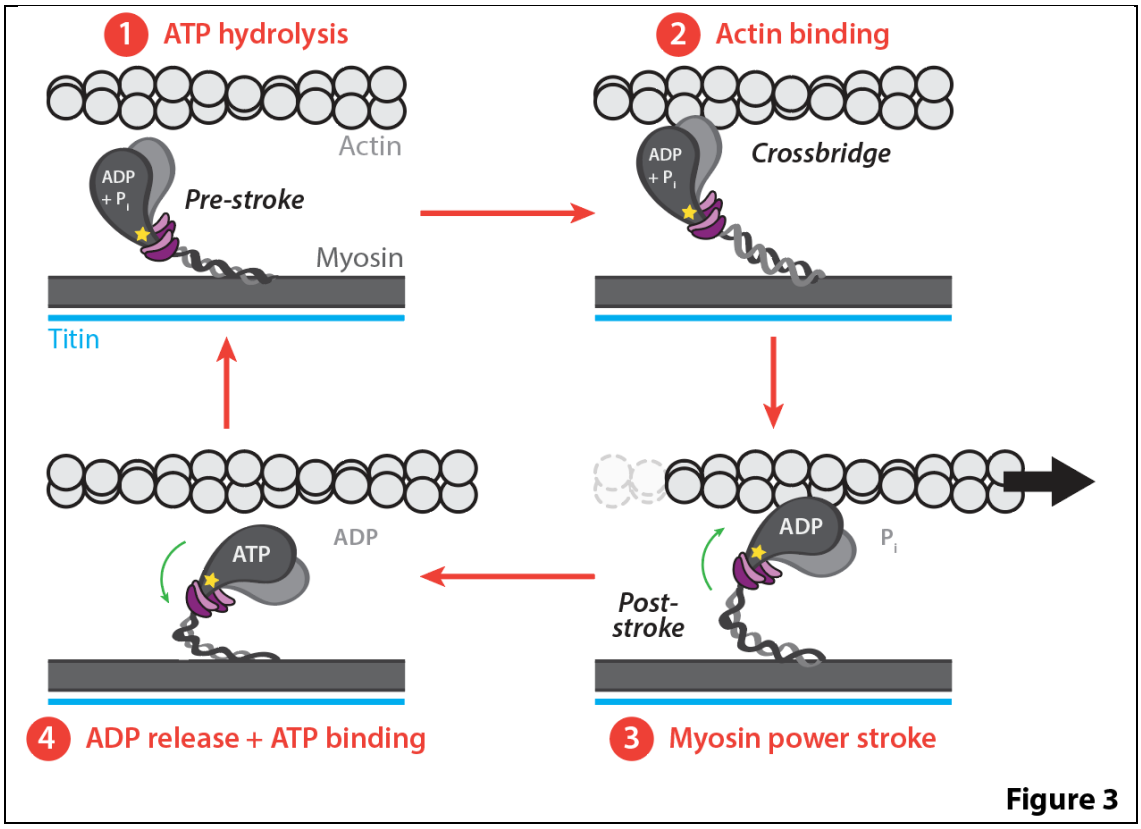

Figure 3

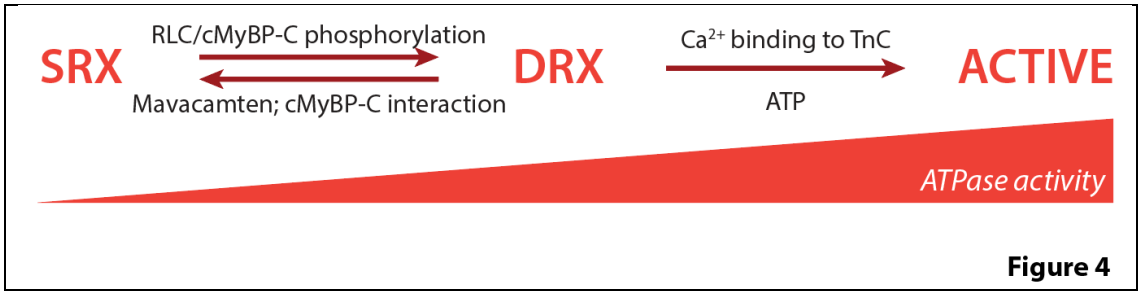

Figure 4

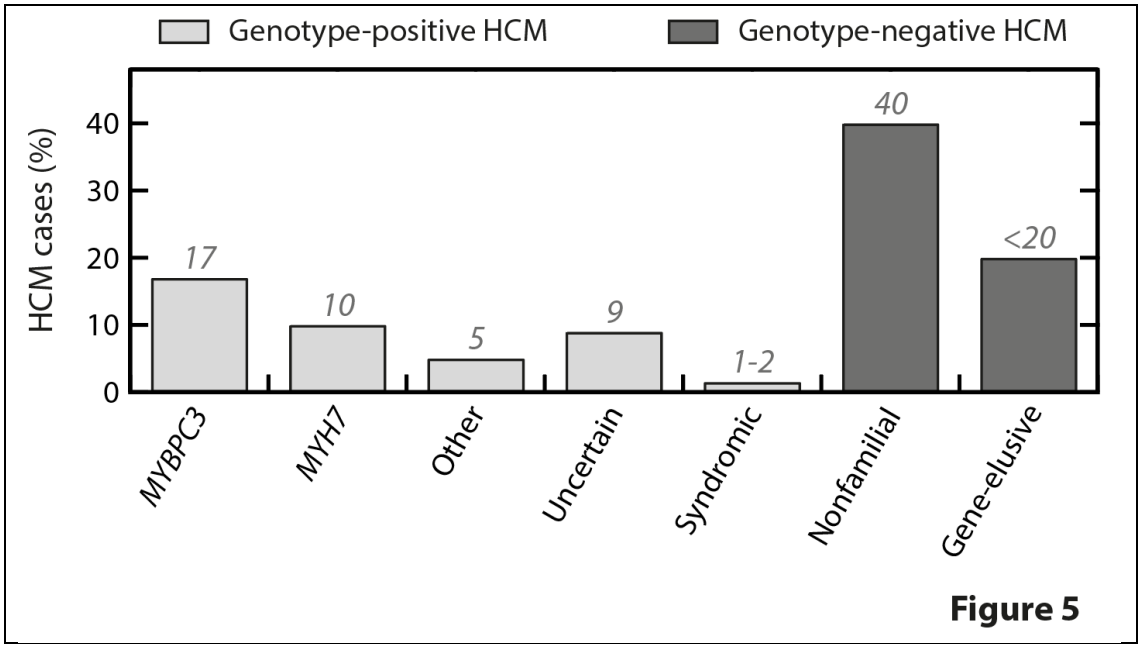

Figure 5

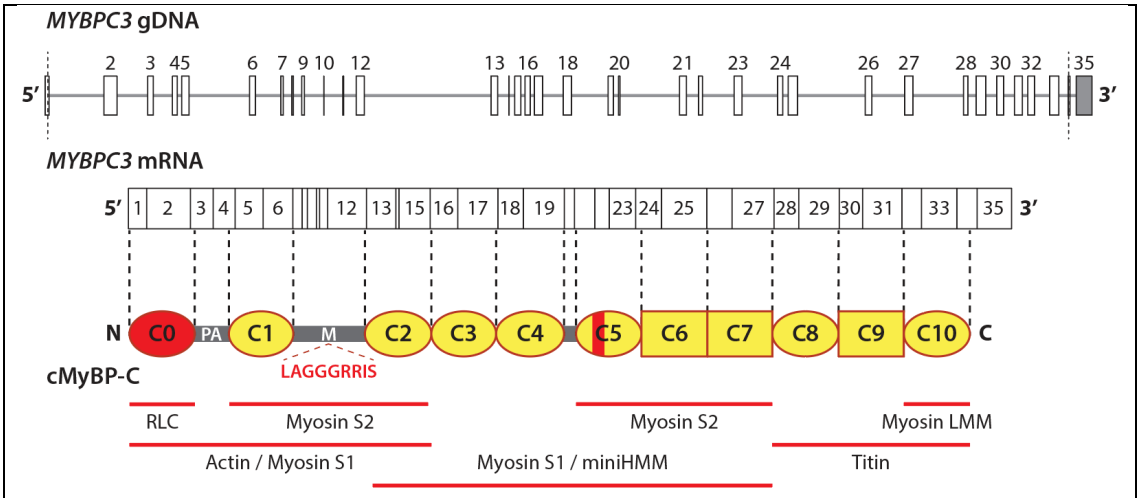

Figure 6

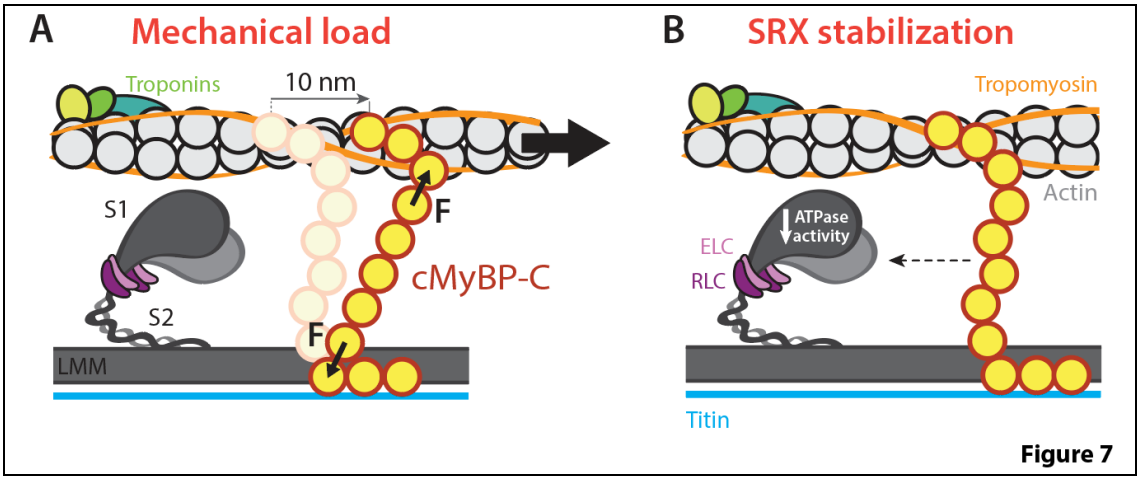

Figure 7

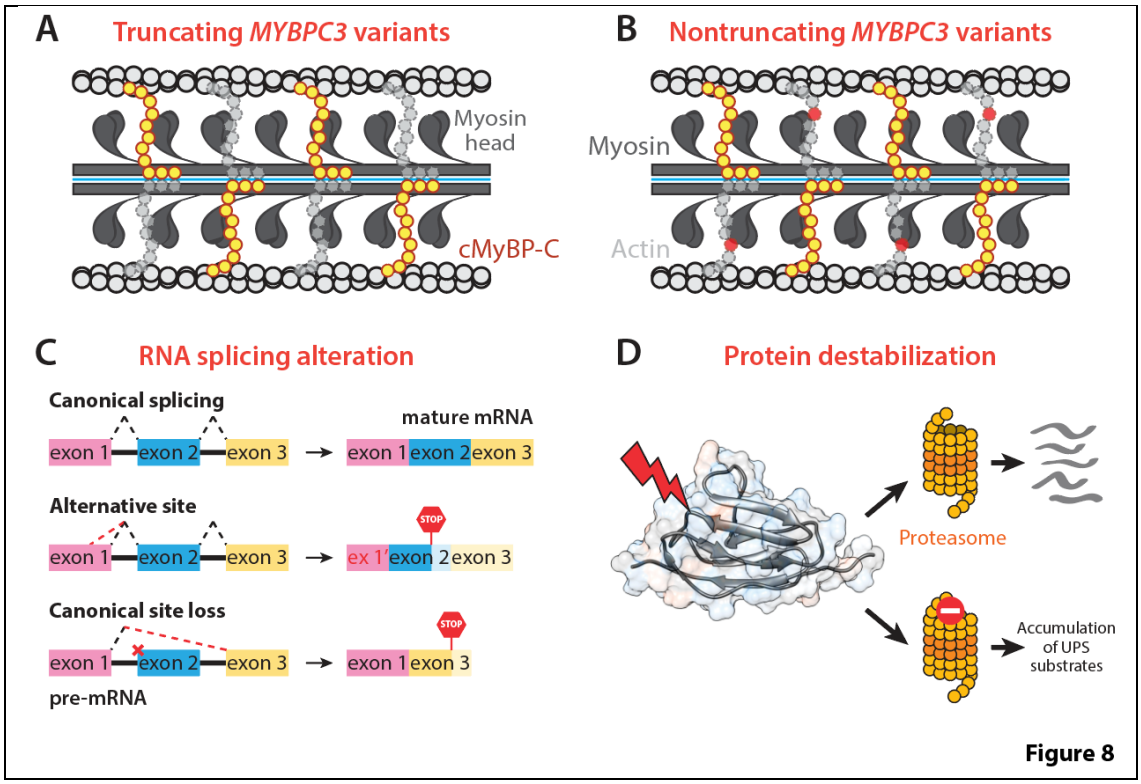

Figure 8

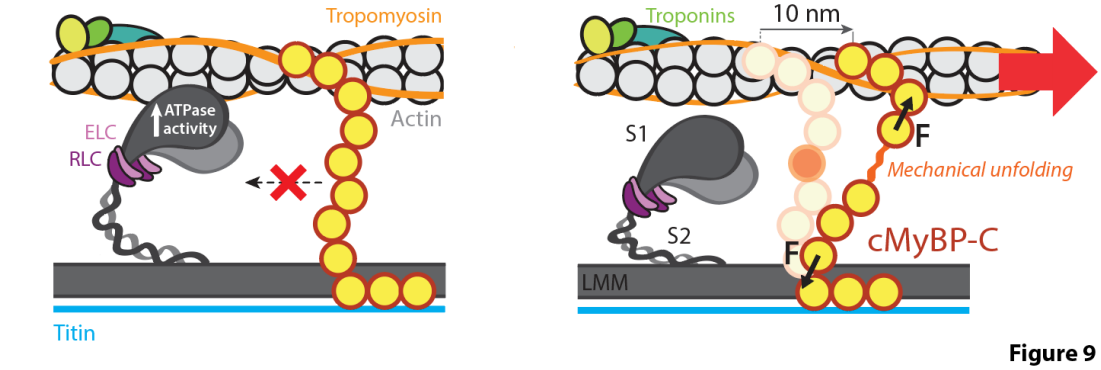

Figure 9