Review Article

The Role of Cardiac Myosin Binding Protein C3 in Hypertrophic Cardiomyopathy- Progress and Novel Therapeutic Opportunities†

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ABSTRACT

Hypertrophic cardiomyopathy (HCM) is a common autosomal dominant genetic cardiovascular disorder marked by genetic and phenotypic heterogeneity. Mutations in the gene encodes the cardiac myosin-binding protein C, cMYBPC3 is amongst the various sarcomeric genes that are associated with HCM. These mutations produce mutated mRNAs and truncated cMyBP-C proteins. In this review, we will discuss the implications and molecular mechanisms involved in MYBPC3 different mutations. Further, we will highlight the novel targets that can be developed into potential therapeutics for the treatment of HMC. This article is protected by copyright. All rights reserved

1 Introduction

Hypertrophic cardiomyopathy (HCM) is an inherited autosomal dominant condition that occurs as a result of mutations in the gene encoding for different types of sarcomeric proteins (Marian et al., 2001). The main characteristic of HCM is left ventricular hypertrophy (LVH) associated with myocardial and myofibrillar disarray in addition to a marked increase in interstitial fibrosis, which occurs in the absence of secondary cardiovascular triggers (Schlossarek et al., 2011). HCM is the second most common form of heart muscle disease, the latter is dilated, restrictive or arrhythmogenic right ventricular cardiomyopathy (Cecchi et al., 2012).

HCM can affect 1:500 from the general population (Tian et al., 2013). Approximately 700,000 Americans are thought to be affected by HCM (Marsiglia and Pereira, 2014), while in Europe, the estimate exceeds one million individuals (Olivotto et al., 2013). A recent study of the Arab/Middle Eastern population has suggested that approximately 160,000 individuals are believed to be affected by HCM in the Egyptian population alone (Kassem et al., 2013).

Advances in molecular genetics underlying the causes of HCM have led to the identification of mutations in 11 genes for sarcomeric proteins, predominantly the β-myosin heavy chain (MYH7), cardiac myosin binding protein C (MYBPC3), cardiac troponin T (TNNT2), α-tropomyosin (TPM1), and cardiac troponin I (TNNI3) (summarized in Table 1) (Cirino and Ho, 2014; Roma-Rodrigues and Fernandes, 2014). The disease has also been linked to mutations in one of the genes that encode a protein from the Z-disc or calcium handling proteins (Marsiglia and Pereira, 2014).

The majority of HCM cases are generally heterozygous, although a small percentage of patients present with a severe form of HCM due to the compound effect of double heterogeneity or homogeneity (Gajendrarao et al., 2015), which usually is associated with a worsened prognosis (Schlossarek et al., 2011). HCM is considered a hereditary condition, as a result of almost 1,500 individual mutations in different sarcomeric genes. HCM can exhibit remarkable genetic, phenotypic, and clinical heterogeneity (Maron et al., 2014).

The unpredictability of HCM and the incoherent genotypic-phenotypic association renders HCM one of the most challenging, yet seemingly common, inherited cardiac disease. Therefore, a better understanding of different sarcomeric gene functions and the mutations that lead to HCM is critical in order to identify their potential clinical and therapeutic importance. This review discusses the implications of MYBPC3 in HCM and highlights the most recent areas of research in which mutations in MYBPC3 have proven valuable in the understanding of the disease mechanism and potential therapeutic opportunities.

2 The cardiac myosin binding protein C structure (cMyBP-C)

Myosin binding protein C (C-protein) is present in 3 isoforms encoded by the skeletal MYBPC1 & MYBPC2 and the cardiac MYBPC3 genes (Barefield and Sadayappan, 2010; Lin et al., 2013). The MYBPC3 cDNA (3.7 kpb) is composed of 35 exons, while the total length of the MYBPC3 gene is about 21 kbp (Figure 1a) (Behrens-Gawlik et al., 2014). The cMyBP-C consists of 7 immunoglobulin (IgI) domains, 3 fibronectin type III (FnIII) domains (C1-10), a 105-residue region between C1-C2 referred to as MyBP-C motif, and a prolin/alanine-rich (PA) region near the N-terminus (Figure 1b) (Barefield and Sadayappan, 2010). In addition to Ig domain (C0) at the N-terminus and 3 phosphorylation sites in the β-sheets rich MyBP-C motif, the domain C5 carries an added proline- rich 25 residue insertion (Luther and Vydyanath, 2011). Fluorescence, NMR and mass spectrometry techniques have provided evidence for the involvement of the C-terminal with the anchoring of the cMyBP-C with the thick filament (Finley and Cuperman, 2014), while the N-terminal is thought to interact mainly with actin and myosin in addition to allowing the MyBP-C motif to bind with myofilament-associated proteins (Finley and Cuperman, 2014).

Lu et al. have demonstrated that the C0 and C1 domains of the N-terminal can interact with both the S2 region of myosin and actin through a set of common binding bases, thereby postulating a phosphorylation-independent switching mechanism that allows the cMyBP-C to alternate between actin and myosin (Lu et al., 2011). In addition, it was shown that both calcium (Ca^{2+}) and calmodulin can interact with the C1C2 domains through a phosphorylation-independent manner

facilitated by the MyBP-C motif (Lu et al., 2012). On the contrary, others have discussed that the MyBP-C motif is thought to aid in the cMyBP-C/myosin S2 interaction in a phosphorylation-dependent manner (Figure 2) (Previs et al., 2012). In a recent study, Rybakova et al.indicated that cMyBP-C fragments lacking the N-terminal domains C0-C4 bonded with F-actin in an essentially similar manner to the full-length protein, suggesting that no apparent interaction of the N-terminal domains occurs with F-actin, and that the C-terminal end domains, C5-C10, bind to F-actin with high affinity (Rybakova et al., 2011). In spite of the controversy surrounding binding of actin to the C2 domain of cMyBP-C, it has been well established that the C-terminal end is critical for its interaction with the of myosin as well as titin (Behrens-Gawlik et al., 2014; Finley and Cuperman, 2014; Schlossarek et al., 2011; Sequeira et al., 2014), an association that appears to be critical for the cMyBP-C stability and sarcomere organization (Barefield and Sadayappan, 2010).

The cMyBP-C is thought to be involved in filament assembly and the regulation of cardiac muscle contractility via modification of the actin-myosin association as demonstrated by several recent studies (Finley and Cuperman, 2014; Luther and Vydyanath, 2011). Initially, it was thought that during β-adrenergic stimulation, the MyBP-C motif undergoes phosphorylation, thereby releasing the bound myosin S2 domain and accelerating the cross-bridge cycling rates and force generated (Finley and Cuperman, 2014). In contrast, data from constructs of cMyBP-C containing the MyBP-C motif suggest that calmodulin may serve as a structural conduit linking cMyBP-C with Ca^{2+} signaling pathways, thus aiding in the coordination of phosphorylation events and orchestrating different interactions between cMyBP-C, myosin, and actin during the process of cardiac muscle contraction (Lu et al., 2012). Phosphorylation of the cMyBP-C by the Ca^{2+}/calmodulin kinase II (CaMKII), protein kinase A (PKA) and protein kinase C (PKC) has been suggested to be essential for the regulation of actin-myosin association and sarcomeric contractility (Barefield and Sadayappan, 2010). A reduction in the phosphorylation of c-MyBP-C, however, is thought to degrade the protein and has been modeled in a number of patients presenting with HCM (Copeland et al., 2010; van Dijk et al., 2009).

The evidence presented highlights the importance of c-MyBP-C phosphorylation for normal cardiac function and suggests that lower protein phosphorylation could appear to be a common feature for the clinical HCM phenotype. Moreover, a recent study involving bioinformatics molecular modeling has suggested that disease-causing mutations Arg177His, Ala216Thr and Glu258Lys (Kassem et al., 2013) can affect the structural properties of the protein's C1 domain, thus altering its structural integrity and interaction with both actin and myosin (Gajendrarao et al., 2013). This association between the structural integrity of c-MyBP-C and the different molecular mechanisms affected is critical for adequately defining the phenotype of HCM. Further studies are necessary in order to accelerate the current molecular understanding of the structure of the cMyBP-C in order to uncover the variable disease phenotypes and prognoses with different MYBPC3 gene mutations (Lu et al., 2012).

3 MYBPC3 Mutations in Hypertrophic Cardiomyopathy

Mutations in 11 different sarcomeric genes account for HCM, up to 40% of which have been mapped to the MYBPC3 gene (Behrens-Gawlik et al., 2014). Nearly 197 HCM mutations have been identified in MYBPC3 ranging from missense mutations, nonsense mutations, splice site donor/acceptor mutations, insertions, and deletions (Mamidi et al., 2013; Vignier et al., 2009). However, ~57% of the established mutations occur as insertions and deletions, which contributes to the production of frameshift and null alleles while the remaining ~43% carry nonsense mutations (Sequeira et al., 2014). These mutations lead to the generation of a premature termination codon (PTC)-bearing mRNAs, which have been shown to produce truncated cMyBP-C proteins lacking the myosin and/or titin binding sites (Behrens-Gawlik et al., 2014; Schlossarek et al., 2011), binding domains involved in targeting cMyBP-C to the thick filament. On the other hand, missense alleles can lead to the production of dominant negative stable poison peptides, which, when incorporated into myosin filaments, can hinder the normal mechanical functions of the sarcomere and disrupt the electrophysiological properties of myocytes (Tian et al., 2013).

Two major quality control systems are thought to be responsible for minimizing mutant MYBPC3 and contributing to low levels of mutated cMyBP-C protein, the non-mediated mRNA decay (NMD) and ubiquitin-proteasome system (UPS) systems (Figure 3) (Schlossarek et al., 2011). At the mRNA level, the NMD system has been shown to become active in mouse models of HCM expressing mutant MYBPC3 (Marston et al., 2012). The NMD appears to target PTC-containing transcripts and swiftly degrade them, thereby protecting its host from the deleterious effects of any C-terminal truncated proteins that may have been generated (Carrier et al., 2010). On the protein level, the UPS, a form proteolysis that is highly specific and selective to the degradation of cytosolic, nuclear and myofibrillar proteins (Schlossarek et al., 2013), protects against the production of poison peptides (Behrens-Gawlik et al., 2014; Carrier et al., 2010; Vignier et al., 2009). Preventing the accumulation of damaged, misfolded and mutant proteins is one of the main functions of the UPS (Carrier et al., 2010; Schlossarek et al., 2012a). Therefore, degradation of truncated proteins through the NMD *and/or* UPS is crucial to effectively eliminate mutant proteins and avoid the resultant insufficiency in full-length, functional cMyBP-C, which is considered the basis for haploinsufficiency in HCM.

Over the last ten years, several mouse models have been designed to specifically determine whether the ablation of MYBPC3 or minute levels of truncated cMyBP-C (haploinsufficiency) were responsible for the induction of HCM and how MYBPC3 mutations contribute to the pathogenesis of this disease (Barefield and Sadayappan, 2010). In an HCM model of MYBPC3 knock-in (KI) mice, cardiac dysfunction and interstitial fibrosis accompanying eccentric LVH was associated with a significant reduction in MYBPC3, in the absence of truncated cMyBP-C (Stohr et al., 2013; Vignier et al., 2009). However, recent studies have strongly indicated that haploinsufficiency (Marston et al., 2012) serves as a key pathogenic determinant of HCM in both patients and animal models (Behrens-Gawlik et al., 2014; Schlossarek et al., 2011). Using the HCM model of MYBPC3 (KI) mice carrying a point mutation, Vignier et al. demonstrated a 90% haploinsufficiency in the KI mice, as well as a 21% in the heterozygous (HET-KI) breed (Vignier et al., 2009). In patients with

known HCM-causing MYBPC3 mutations, van Dijk et al. also identified haploinsufficiency associated with deranged contractile protein phosphorylation, as the culprit of reduced cardiac contractility (van Dijk et al., 2009; Vignier et al., 2009). Interestingly, in a later study, the authors demonstrated that despite the higher myofilament Ca^{2+} sensitivity detected in the MYBPC3 mutant carriers, there were no significant differences between the phosphorylation states of cMyBP-C in mutant carriers and non-failing donors (van Dijk et al., 2012). Whether the altered state of the sarcomeric function is attributed to hypophosphorylation-induced Ca^{2+} hypersensitivity, or enhanced Ca^{2+} sensitivity regardless of the phosphorylation state of the cMyBP-C is yet to be determined. Nevertheless, the evidence presented seems to indicate that haploinsufficiency in HCM-associated MYBPC3 mutations increases Ca^{2+} sensitivity and thus alters sarcomeric cardiac contractility.

Familial HCM patients with MYBPC3 frameshift mutations had a reduced cMyBP-C protein level and a contractile dysfunction supporting that the patho-mechanism involves haploinsufficiency rather than a poison peptide. In addition, mutant cMyBP-C mRNA levels were lower than the WT mRNA, suggesting that degradation of the mutant mRNA might be through the NMD (van Dijk et al., 2009). This study is consistent with that of Marston et al. which found that HCM patients with missense mutation and premature termination in domain C3 had no detectable truncated MyBP-C protein, arguing strongly that haploinsufficiency, as the HCM mechanism, and a reduction in mutant mRNA are possibly caused by NMD (Marston et al., 2009) On the basis of the former data, the UPS system could act as parallel quality control systems with the NMD to eliminate mutant cMyBP-C mRNA and protein (Marston et al., 2009; van Dijk et al., 2009; van Dijk et al., 2012).

A dysfunction in the UPS has also been suggested to underlie the pathogenesis of HCM and the degradation of truncated cMyBP-C protein. Sarikas et al. had indicated that truncated cMyBP-C protein was rapidly degraded by the UPS in neonatal rat cardiac myocytes transfected with human truncated cMyBP-C mutants. Additionally, truncated cMyBP-C mutant M7t resulted in the

formation of ubiquitin-positive aggregates that impaired the function of the UPS system (Sarikas et al., 2005). Schlossarek et al. have demonstrated that both KI and KO mice models exhibited low levels of mutant cMyBP-C protein due to their degradation via the UPS. A1-year-old HCM mouse KI model exhibited a UPS impairment (Schlossarek et al., 2012a).

The exact mechanism through which UPS dysfunction occurs during HCM has been attributed to the saturation of the UPS following the chronic degradation of mutant cMyBP-C, which appeared to be the case in adult MYBPC3 KI mice (Schlossarek et al., 2012a). Coupled with external stress, such as adrenergic stimulation, the altered cardiac phenotype in MYBPC3-KI mice could also exacerbate the impairment of the UPS (Schlossarek et al., 2013). It has also been suggested that mutant cMyBP-C competes with other factors for UPS-mediated degradation, such as the hypertrophic transcription factor GATA4 or the anti-apoptotic factor p35, thus contributing to the pathogenesis of HCM (Carrier et al., 2010).

In cardiomyocytes, the UPS impairment has also been shown to contribute to the pathophysiology of HCM (Schlossarek et al., 2012b). UPS impairment could result in increased levels of pro-hypertrophic and pro-apoptotic factors, accumulation of unfolded or misfolded proteins resulting in ER stress and alterations of Ca^{2+} handling as shown in an *in vitro* model of missense MYBPC3 mutation and in patients with adult-onset HCM, leading to cardiac dysfunction (Schlossarek et al., 2013). Interestingly, expression of the missense MYBPC3 mutant protein in cells led to a significant reduction in proteasome activity, indicating UPS impairment, and significantly increased the proapoptotic/antiapoptotic protein ratio, enhancing apoptosis (Bahrudin et al., 2008). Similarly, the novel missense MYBPC3 mutation E334K detected in Japanese patients induced proteo-toxicity and severe cardiac hypertrophy through a dysfunction in the UPS. E334K mutant cMyBP-C stable transfectants Cos-7 cells showed a decreased level of 20 S proteasome activity, increased ratio of proapoptotic/antiapoptotic regulating protein, increased apoptosis and consequent accumulation of truncated cMyBP-C protein (Bahrudin et al., 2008). Even though levels of truncated cMyBP-C are virtually undetectable in HCM patients, the incorporation of

truncated protein into the sarcomere could dysregulate sarcomeric function and promote myocyte hypertrophy (Olivotto et al., 2012), suggesting that the "poison peptide" mechanism could actually worsen the HCM phenotype when the UPS is impaired. Nevertheless, further studies are necessary in order to determine whether targeting the UPS could serve as a potential treatment for HCM.

In cardiac dysfunction, the alterations of the UPS and the autophagy-lysosyme pathway (ALP) that regulates autophagy suggests that UPS proteolytic function can become inadequate or impaired following severe cellular stress (Zheng and Wang, 2010). Under this circumstance ALP may be increasingly activated to help ameliorate the cellular stress. Autophagy plays a dual role in the pathogenesis of cardiac diseases. Autophagy is activated to protect cells against cellular stress, while excessive autophagy promotes cell death (Zheng and Wang, 2010). Nakai A et al. found that inhibition of autophagy by autophagy-related 5 protein Atg5 knockdown induced morphological and biochemical features of cardiomyocyte hypertrophy (Nakai et al., 2007), suggesting a role for the ALP system in heart disease.

Although the general assumption is that the UPS and ALP systems act separately, it was found that various proteins, including p62, NBR1, HDAC6, and co-chaperones, play an important role in synchronizing the consortium formed by the UPS and ALP systems to remove mutant cMyBP-C in HCM (Zheng and Wang, 2010). In fact, genetically engineered HCM KI mouse model showed UPS impairment, reduced belcin-1 degradation, and reduced lysosomal-mediated LC3 degradation leading to ALP impairment as a common disease mechanism. Schlossarek et al. attributed this impairment to the obstruction of the fusion occurring between autophagosome and lysosome, which thereby inhibits the development of the autophagolysosome and subsequent degradation of the autophagosome product (Schlossarek et al., 2012a). Additionally, Cao et al. supported that autophagy plays an important role in the regression of cardiac hypertrophy and that FoxO family proteins are critical regulators of autophagy after unloading the hypertrophic stimuli (Cao et al., 2013).

In order to develop novel and effective therapeutics for cardiac hypertrophy, it is necessary to identify the cellular and molecular mechanisms underlying regression. Regression of cardiac hypertrophy could be induced by the down-regulation of protein synthesis or the up-regulation of protein degradation.

4 Molecular modeling of HCM-causing mutations

Studying the structure-function relationships of cMyBP-C is extremely useful for understanding the pathogenesis of HCM. Determination of the 3D data of c-MyBP-C has been challenging for structural studies such as X-ray crystallography and NMR for decades due to the conformational plasticity of the protein. As a result, only 5 domain structures have been determined [C0 (Ratti et al., 2011), C1 (Ababou et al., 2008; Fisher et al., 2008; Ratti et al., 2011), C2 (Govada et al., 2008), C3 (Ababou et al., 2007) and C5 (Zhang et al., 2014)] out of 11 domains of cMyBP-C. Molecular modeling is an effective tool to model 3D structures of the protein, and it has been used to model HCM-causing mutations to study their structural consequences (Idowu et al., 2003). Molecular dynamics simulation is one of the advanced techniques in modeling, which can provide a dynamical behavior (stability, energy, and interactions) of molecular systems.

Recently, mutational modeling studies have been carried out on cMyBP-C in domain C1 (Gajendrarao et al., 2013) and in a molecular complex of domains C1-motif-C2 (Gajendrarao et al., 2015). In the C1 study, three disease-causing mutations (found in Egypt and Italy) were modeled (Figure 4). It provided possible explanations for the initiation of the malfunction of the protein at the molecular level from C1 and potential interference of cMyBP-C binding with actin and myosin. Interestingly, it was reported that these three mutations follow different mechanisms to affect the structural integrity of the domain C1. However, this study requires further experimental validations.

In the study of C1-motif-C2, double mutations (E258K and E441K from domains C1 and C2, respectively) were studied in a patient with severe end-stage HCM phenotype (Gajendrarao et al., 2015). This is the first study to represent a full-length 3D model of the c-MyBP-C motif (Figure

4a), which was modeled from a partial structure of motif from mouse (Howarth et al., 2012). It also presented a complex model of C1-motif-C2. This study described the fact that the double mutations in isolation can predominantly affect the native domain and the nearby motif via conformational changes, resulting in an additive effect when they coexist.

The molecular changes described in the modeling studies propose that it could distract binding of the cMyBP-C molecule to actin or myosin and thus interfere with both contractile and electrical functions. These molecular events can be responsible for at least some of the pathological changes observed in HCM. Although the modeling studies of cMyBp-C can prioritize essential models for understating the molecular mechanisms of HCM, it requires experimental support from bio-molecular and animal studies to progress towards designing potential drugs for treating HCM.

5 Models of MYBPC3 mutations

With the remarkable opportunities provided by the availability of human heart tissue samples come inevitable limitations, including the variable methods of sample collection, the heterogeneity of clinical characteristics, and restricted time frames in the course of diseases. These factors are important to recognize and acknowledge when interpreting data from such studies, and they highlight the importance of developing animal models as a means to further understand the pathogenesis of HCM (Day, 2013).

One of the valuable models of MYBPC3-induced HCM is the MYBPC3 KI mice initially developed by Vignier et al. Both homozygotes (KI) and HET-KI mice carried a single nucleotide transition at exon 6. Only the homozygotes KI mice expressed the missense mRNA and nonsense mRNA in the urea fraction of the protein and developed the overt-HCM phenotype. This alteration between both lines suggests an impairment of the NMD and a saturation of the UPS (Vignier et al., 2009). Furthermore, both lines exhibited impairment of both UPS and ALP activation. Both proteolytic systems would be responsible for minimizing mutant MYBPC3 mRNA and mutated cMyBP-C protein as the general pathology associated with the marked cardiac hypertrophy

presented by MYBPC3 KI and KO genetically-engineered cardiomyopathic mice (Schlossarek et al., 2012a).

Although in mice models of HCM, the homozygous line tends to display a cardiac hypertrophic phenotype, in human patients, the phenotype is typically heterogeneous (Olivotto et al., 2012; Schlossarek et al., 2012a; Schlossarek et al., 2012b; Vignier et al., 2009). Barefield et al. have demonstrated that in an HCM model of heterozygous KI *MYBPC3* truncation mutation (HET $^{+/t}$), the HET $^{+/t}$ mice developed diastolic dysfunction, as indicated by a reduced maximal force generation of skinned cardiomyocytes and altered cardiac function (Barefield et al., 2014). However, when compared to WT mice, the HET $^{+/t}$ mice did not express an apparent hypertrophic phenotype and preserved cMyBP-C levels (Barefield et al., 2014).

To further examine whether hemodynamic stress could unmask a cardiac hypertrophic phenotype in the heterozygous MYBPC3 KI mice (HET-KI), Schlossarek et al. investigated morphological changes to the heart in HET-KI mice exposed to adrenergic stimulation. When compared to the heterozygous MYBPC3 KO mice (HET-KO), HET-KI mice treated with a combination of isoprenaline and phenylephrine for 1 week exhibited significant septal hypertrophy as well as a UPS impairment, suggesting that the HET-KI model can mimic the genetic profile of HCM patients carrying only one mutant MYBPC3 allele (Schlossarek et al., 2012b).

In a more recent study, Barefield et al. investigated the effects of inducing cardiac stress on the impact of hypertrophic remodeling in HET mice carrying an *MYBPC3* truncating mutation (Barefield et al., 2015). The study revealed that systolic function, ejection fraction and cMyBP-C levels and were significantly reduced following transverse aortic constriction-induced pressure overload indicating that HET mice exposed to cardiac stress develop a magnified HCM phenotype compared to WT controls (Barefield et al., 2015). These findings stromgly indicate that while heterozygous MYBPC3 mutant carriers may appear asymptomatic, they suffer functional impairment on single cardiomyocyte and whole heart levels, an effect that is exacerbated and leads to an overt HCM phenotype upon hemodynamic and cardiac stress.

In order to verify the phenotypes observed in both HET-KI and homozygous MYBPC3 KI mice, Stöhr et al. generated engineered heart tissue (EHT) (Stohr et al., 2013; Wijnker et al., 2016) derived from the genetically defined and characterized mouse model of HCM (Vignier et al., 2009). This model appears to be more reliable than tissue samples extracted from animal models or HCM patients since some of the pathophysiological defects associated with MYBPC3 mutations may be secondary changes in response to treatment protocols of HCM patients or even the experimental protocol used to induce HCM disease model (Wijnker et al., 2016). Although the ability of EHTs to exhibit a disease phenotype was questionable, both models showed definite functional abnormalities, with marked differences in the sensitivity to external Ca^{2+} concentrations and the contractile response to drugs. These results highlight the importance of using induced pluripotent stem cell (iPSC) mediated disease modeling of human HCM to shed light on potential therapeutic targets for a more personalized approach to therapy.

The use of iPSC-mediated disease modeling of human HCM was recently demonstrated by Ojala et al. using HCM cardiomyocytes isolated from patient-specific human induced pluripotent stem cells (hiPSCs) (Ojala et al., 2016). hiPSCs carrying either a MYBPC3 *(MYBPC3-Gln1061X)* or α-tropomyosin (*TPM1-Asp175Asn)* mutation were examined for differences in Ca^{2+} handling, gene expression profiles, cellular size and electrophysiological properties. While the hiPSCs demonstrated a similar pathological phenotype of HCM, the results clearly indicated differences in cellular enlargement between the two HCM mutations, as well as Ca^{2+} sensitivity and arrhythmogenic events (Ojala et al., 2016). Taken together, the studies exploiting the use of iPSC-derived cell models, provide novel means to further understand the pathophysiological mechanisms of HCM and to possibly personalize the current treatments in mutation-specific direction.

In order to differentiate between the functional and structural phenotypes of cMyBP-C ablation, Chen et al. developed a tamoxifen-inducible cMyBP-C conditional knockout (cMyBP-C-cKO) mouse model of HCM. Using this approach, the authors were able to acutely abolish cMyBP-C expression in the myocardium of 12-week-old cMyBP-C-cKO mice, showing that cardiac

dysfunction preceded both LV dilation and cardiac hypertrophy in the cMyBP-C-cKO mice. Once subjected to transaortic constriction, the model developed progressive LV dilation and further LV hypertrophy. These findings demonstrate that in reality additional factors such as hemodynamic stress can entail the clinical expression of HCM (Chen et al., 2012).

In the precipitation of systolic and diastolic dysfunction in MYBPC3-associated HCM, little is known. Vignier et al. sought to investigate the involvement of the renin-angiotensin system in the heterozygous MYBPC3 KO mouse (HET-KO) model of HCM. Concurrent with the development of LV hypertrophy, ACE-1 gene expression appeared to be significantly elevated in HET-KO mice than in WT mice. Interestingly, both angiotensin II type 1 receptor (Agtr1) and MYPC3 gene expression were significantly increased in HET-KO mice treated with Agtr1 blocker irbesartan, revealing that FH1 (four-and-a-half LIM domains) is a regulator of cMyBP-C activity and is involved in the sarcomere assembly. These results suggest a predominant role for the renin-angiotensin system and FH1 in MYBPC3-mediated HCM, thus highlighting a potential therapeutic target that could prevent the development of LV hypertrophy and the progression of HCM (Vignier et al., 2014).

A major concern of using the rodent model of HCM is the vast differences in the presentation of HCM and sarcomeric proteins in rodents compared to humans (Chen et al., 2013; Marian et al., 2001). Such variations could even limit the use of transgenic rodent models to decipher the pathogenesis of HCM in humans. Zebrafish (*Danio rerio*), a tropical freshwater fish, has recently emerged as an ideal vertebrate animal system for investigating mechanisms of cardiac development and human CHDs (Poon and Brand, 2013). The ability of zebrafish mutant embryos to survive without a functional circulation system for several days further emphasizes the feasibility of carrying out gain-of-function and loss-of-function studies in zebrafish (Yang et al., 2014).

Chen et al. recently established the first model of MYBPC3 knockdown-induced cardiac hypertrophy and diastolic heart failure in zebrafish. The authors confirmed a sequence homology between human and zebrafish MYBPC3 and demonstrated that at 72 hpf, morpholino-mediated loss

of MYBPC3 induces ventricular wall thickening and the expression of hypertrophic sarcomeric proteins and cardiac myosin light chain 2, features that closely resemble human ventricular hypertrophy and HCM (Chen et al., 2013). The knockdown of the MYBPC3 in zebrafish showed LVH and contractile dysfunction; interestingly, that was also observed in FHCM patients carrying the MYBPC3 mutation (van Dijk et al., 2009).

Hodatsu et al. recently recapitulated the MYBPC3 mutations of HCM patients in a zebrafish model. The authors concluded that double MYBPC3 mutation (Arg815Gln and Ala745Asp) could lead to a worsened HCM phenotype, as indicated by the significantly elevated ventricular dimensions and heart rate. Interestingly, the phenotype/genotype association in the zebrafish model was exacerbated when compared to human carriers of young age, suggesting that the zebrafish model can provide a valuable insight into the clinical presentation of HCM prior to its overt onset in human carriers (Hodatsu et al., 2014).

6 Potential Therapeutic Targets for MYBPC3-associated HCM

The current therapeutic options for HCM revolve around the use of pharmacological agents or surgical interventions for symptomatic relief and the prevention of SCD (Schlossarek et al., 2011). However, these approaches fail to address the sarcomeric gene mutations. Recent studies have demonstrated that targeted mutated gene and RNA-based therapies could become realistic options for the treatment of HCM (Figure 5). Mearini et al. have revealed that the single dose administration of adeno-associated virus (AAV9) with the full-length MYBPC3 prevented the disease phenotype in the KI mice. At 34 weeks, KI treated mice had increased Mypbc3 mRNA and cMyBP-C protein and suppressed the accumulation of the mutant mRNA (Mearini et al., 2014). The phenotype difference between homozygous and het MYBPC3-KI mice supported the fact that the full-length (semi-functional) protein acted as a compensatory mechanism in an attempt to prevent cardiac dysfunction and protect against LVH (Vignier et al., 2009).

Gedicke-Hornung et al. demonstrated that cardiomyopathy was rescued through U7snRNA-mediated exon skipping. This approach was evaluated for that first time for cardiac diseases. Antisense oligoribonucleotides significantly reduce aberrant MYBPC3 *in vivo* in the MYBPC3-KI mice and *in vitro* in the transfected neonatal mouse cardiac myocytes. Despite the promising results observed, the therapeutic effects were only transient, lasting only 48 hours in cardiomyocytes and 55 days in newborn KI mice (Gedicke-Hornung et al., 2013; Vignier et al., 2009). Consequently, the 5'-trans-splicing strategy was sought out by Mearini et al. as an alternative approach to correct MYBPC3-associated HCM. The induction of 5'-trans-splicing between the mutant endogenous MYBPC3 pre-mRNA and an engineered pre-trans-splicing molecule (PTM) carrying WT MYBPC3 cDNA in the MYBPC3-KI mice significantly repaired the cMyBP-C protein and mediated its successful incorporation into the sarcomere, both in cardiomyocytes and in the MYBPC3-KI mice hearts (Mearini et al., 2013). These studies highlighted some of the novel gene and pre-mRNA based therapeutic options currently under investigation for the treatment of MYBPC3-associated HCM.

Since it remains somewhat unclear whether functional phenotypes in these mice reflect primary responses of the heart caused by removal of cMyBP-C or secondary responses of the heart caused by activation of compensatory mechanisms after the removal of cMyBP-C, further studies are necessary in order to determine the early changes that occur specifically in response to loss of MYBPC3 as demonstrated by the Chen et al. Thus, understanding the changes that occur in response to the loss or mutation of MYBPC3 will serve as a breakthrough, as it will provide bases to determine whether the direct loss of MYBPC3 drives the cardiac functional abnormalities observed in MYBPC3 KO or whether the development of cardiac hypertrophy and LV dilation induces diastolic and systolic dysfunction.

7 Conclusion

The necessity of cMyBP-C for normal cardiac physiology is shown by its involvement as a key

regulator of contraction and relaxation in both healthy and diseased hearts. Recent studies using different animal models have clearly demonstrated that mutations in the MYBPC3 gene are one of the most frequent triggers of HCM. The involvement of the NMD, UPS and possibly, the ALP as different quality control systems that could be acting independently or conjointly in an attempt to eliminate or reduce the amount of mutant cMyBP-C have also been shown. Yet despite the application of genome-wide association studies technology, the exact mechanism leading to the manifestation of the HCM phenotype is still somewhat poorly understood. Developing new models using HCM-iPSC; whether EHTs or cardiomyocytes, appears to advance the high-throughput investigation of the pathophysiological mechanisms and potential molecular therapy for MYBPC3-induced HCM. The understanding of detailed mechanisms underlying this inherited autosomal dominant condition, new therapeutic targets will definitely uncover in the not-too-distant future.

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Table 1
Most frequently occurring hypertrophic cardiomyopathy-associated proteins, genes and number of mutations described for each gene [adapted from (Cirino and Ho, 2014; Marsiglia and Pereira, 2014)]

Gene	Protein	Frequency
Myofilament genes		
MYH7	β-myosin heavy chain	15-25%
MYL2	Myosin regulatory light chain	<2%
MYL3	Myosin essential light chain	<1%
MYBPC3	Myosin binding protein C	15-25%
TNNT2	Troponin T	<5%
TNNI3	Troponin I	<5%
TPM1	α-tropomyosin	<5%
ACTC	Alpha actin cardiac	<1%
TNNC1	Troponin C	<1%
Z-Disc genes		
CSRP3	Muscular LIM protein	<1%
TCAP	Telethonin	<1%
ACTN2	α-actin	<1%
MYOZ2	Myozenin	<1%
NEXN	Nexilin	<1%
Calcium handling genes		
CACNA1C	Calcium Voltage-Gated Channel Subunit Alpha1 C	<1%
PLN	Phospholamban	<1%

Figures:

Figure 1. Schematic representation of the cardiac sarcomere and localization of the cMyBP-C protein. As a thick filament protein, cMyBP-C is localized in the inner two-thirds of the A-band, in the C-zone shown, and is aligned perpendicularly to the myosin filaments and titin. The thin filament of actin monomers, troponins and α-Tropomycin, is connected to nebulin in the I-bands. Structural representation of cMyBP-C protein and MYBPC3 cDNA. cMyBP-C is composed of 11 domains (C0–C10). It consists of 8 immunoglobulin (IgI) domains (rectangles), 3 fibronectin type III (FnIII) domains (hexagons), a 105-residue region between C1-C2 referred to as MyBP-C motif (S2), along with the four specific phosphorylation sites. The regions of interaction with other sarcomeric proteins (actin, myosin and titin) is indicated with solid black lines. Correspondence between domains of cMyBP-C and the 35 exons of MYBPC3 cDNA is indicated by the dashed lines.

Figure 2. Proposed mechanism of phosphorylation-dependent regulation of thick and thin filaments interaction with the cMyBP-C protein. Upon phosphorylation of cMyBP-C is, the M domain releases its interaction with myosin-S2 and actin rendering the myosin heads (myosin-S1) ordered and lengthened to interact with the actin thin filaments. This results in strong actin–myosin interactions, which appears to be crucial for cMyBP-C stability and sarcomere organization

Figure 3. Implication of MYBPC3 mutations in hypertrophic cardiomyopathy. In an effort to reduce the level of potential toxic cMyBP-C mutants, expression of the mutant MYBPC3 cDNA is regulated by the nonsense-mediated mRNA decay (NMD), which degrades nonsense mRNAs, and the ubiquitin–proteasome system (UPS) and autophagy-lysosome pathway (ALP), which are activated if mutant proteins are being produced. This leads to haploinsufficiency of wild-type (WT) cMyBP-C in the sarcomere and the expression of the overt hypertrophic cardiomyopathy (HCM) phenotype. Impairment of the quality control systems also causes the remaining mutant proteins to act as poison peptides, which ultimately leads to cardiac hypertrophy and heart failure.

Figure 4. Molecular model of the complex C1-Motif-C2. a) The 3D surface model of the C1-Motif-C2 coloured in ivory, blue and red respectively. Here, the residues, E258 and E441 are shown in green and yellow. (b) The cartoon 3D models of domain C1 and (c) C2 with the positions of the mutations are represented in sticks.

Figure 5. Putative RNA-based approaches to rescue the function of the MYBPC3 gene and subsequent cMyBP-C protein activity. Grey arrow indicates single exon skipping targets, double ended red arrow highlights exons that could be skipped solely together, using antisense oligonucleotides (AONs). Adeno-associated viral delivery of full-length MYBPC3 (AAV-MYBPC3) could serve as a means to prevent the HCM disease phenotype

Figure 1

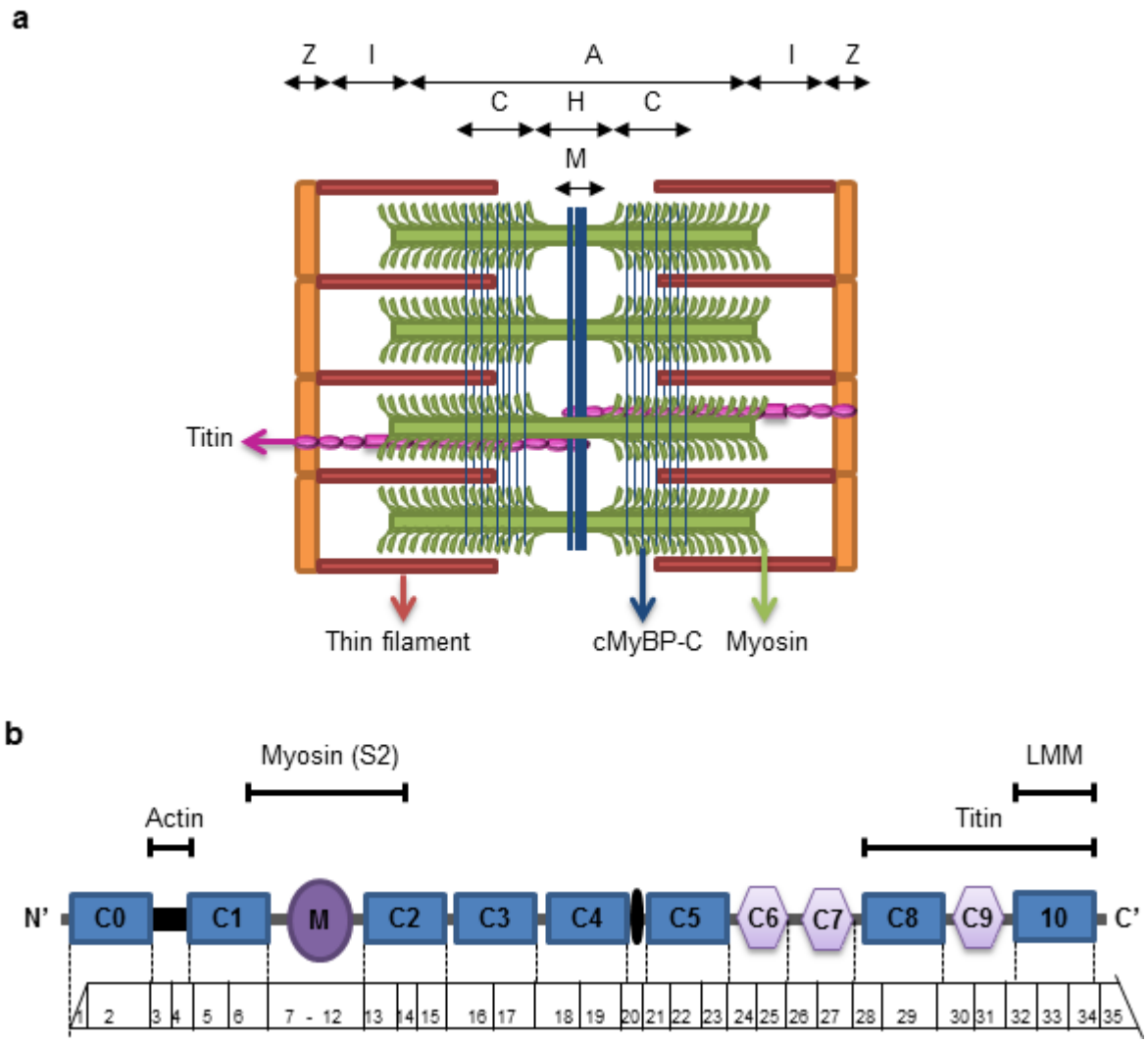

Figure 2

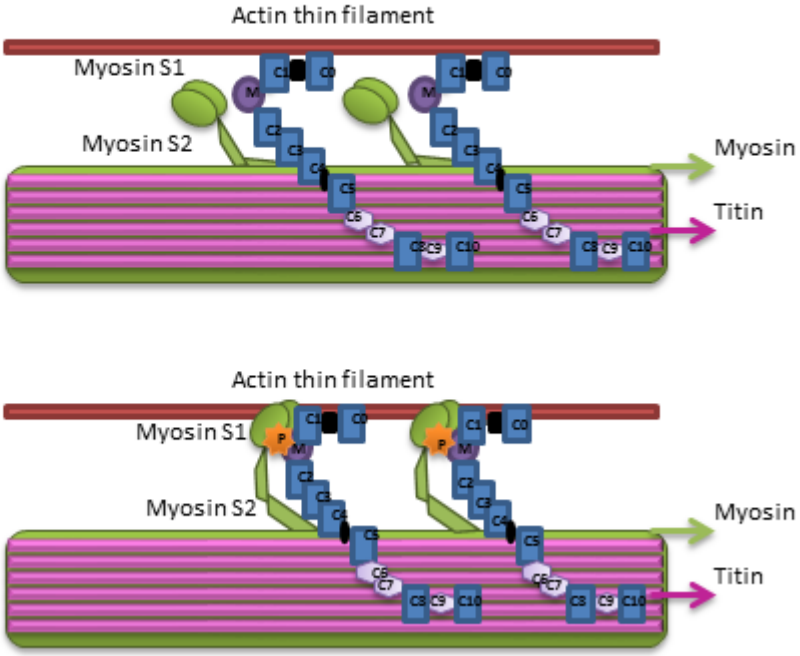

Figure 3

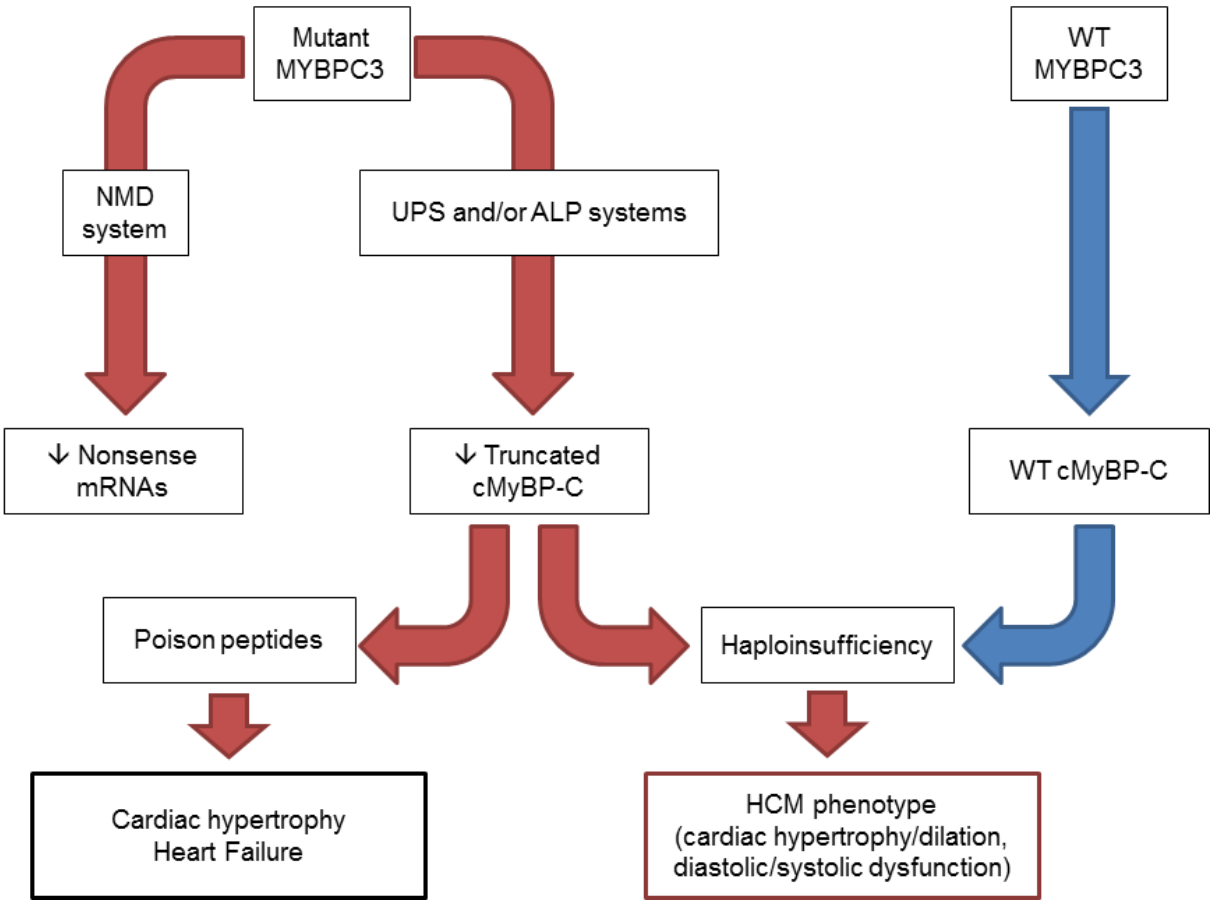

Figure 4

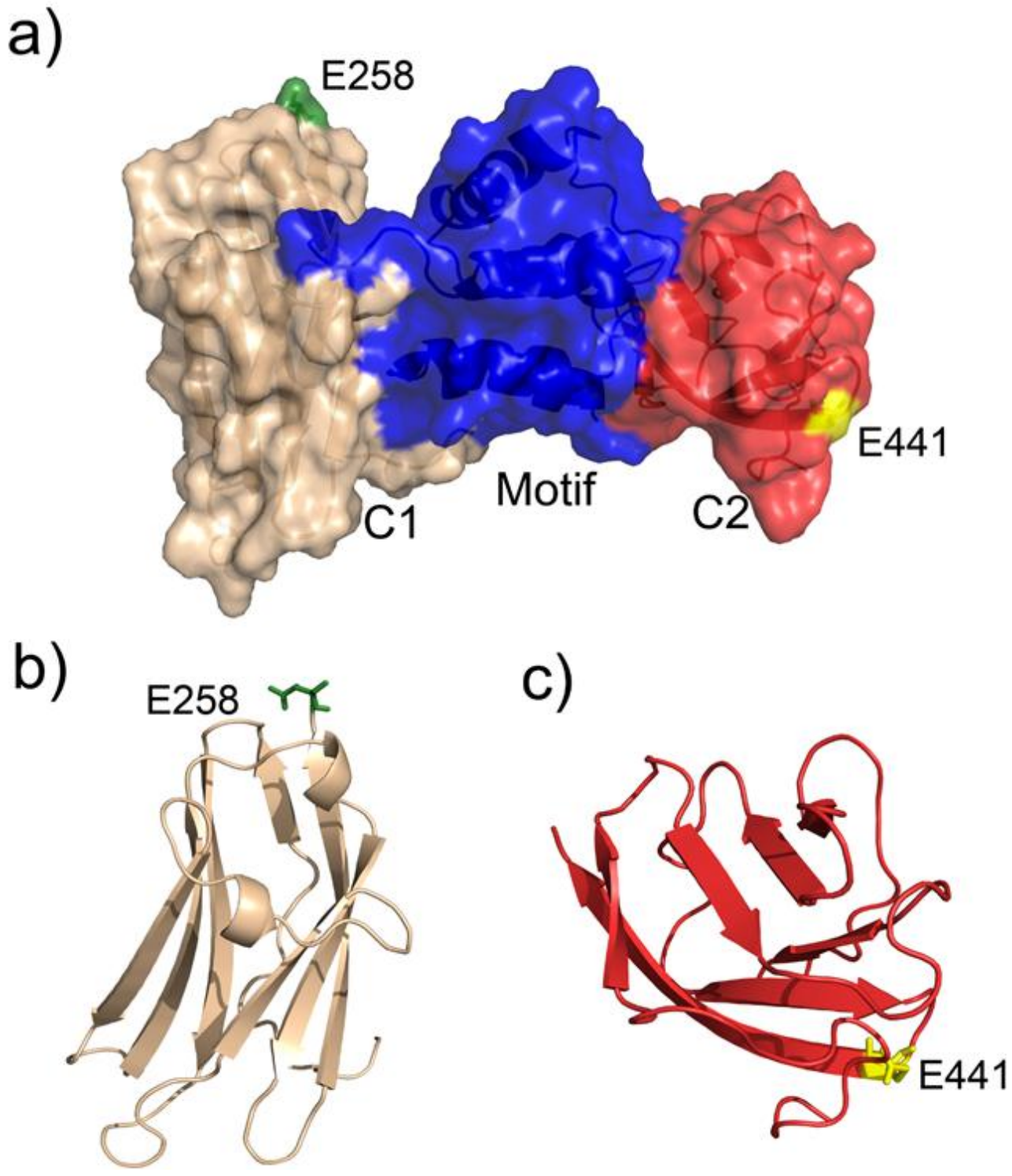

Figure 5