

8.1 Summary, Conclusion and Future Perspectives

‘Sarcomeric function and protein
changes in human cardiomyopathy:
mutation or phenotype’

SUMMARY

The work described in this thesis was conducted to gain more insight in the complex pathophysiology of hypertrophic (HCM) and dilated (DCM) cardiomyopathy, with special emphasis on familial cardiomyopathies. Many mutations in genes encoding different sarcomeric proteins cause cardiomyopathies, although the roles of these proteins in sarcomere contractility are diverse. Furthermore, mutations in the same sarcomeric protein can give rise to either HCM or DCM.

We performed functional force measurements in single cardiomyocytes from HCM patients with mutations in *MYBPC3*, the gene encoding cardiac myosin binding protein C (cMyBP-C), from familial HCM patients without an identified mutation in the sarcomeric proteins (mutation negative) and from familial DCM patients. In addition, protein composition of the cardiac tissue was determined in these patients. By comparing sarcomeric properties of these patient groups to those of hearts from non-failing donors and from patients with end-stage failing idiopathic dilated cardiomyopathy we observed alterations in cardiomyocyte function and protein composition that could contribute to the human familial cardiomyopathy phenotypes. The main findings described in this thesis are summarized below and in figure 8.1. By combining information on protein composition with functional force measurements, we provided insight in the pathophysiological mechanisms by which an initial gene

mutation or defect may lead to cardiomyocyte dysfunction, cardiac remodelling and cardiomyopathy.

Chapter 1 – Cardiomyopathy and the sarcomere: general introduction

This chapter gives an introduction on possible causes and phenotypes of cardiomyopathies, focussed on HCM and DCM. It describes the components of the smallest functional contractile unit of the heart, the sarcomere and their role in cardiac contraction.

HCM and DCM can both be caused by mutations in genes encoding proteins of the sarcomere and cause cardiac failure, yet they have very distinct phenotypes. One of the most commonly affected sarcomeric proteins in HCM is cMyBP-C, a protein with structural and functional roles that are still poorly understood, in particular in the human heart. Many sarcomeric proteins are regulated via post-translational modifications, such as phosphorylation. An important regulatory system that increases cardiac output upon increased demand is the β -adrenergic receptor system. Upon activation of β -adrenergic receptors, the downstream effector protein kinase A (PKA) phosphorylates cMyBP-C, as well as sarcomeric proteins troponin I (cTnI) and titin.

Chapter 2 – Myocardial adaptations in the failing heart: Cause or consequence?

Many changes in morphology and cellular properties are reported in the failing heart. They may initially be compensatory, in order to maintain proper cardiac output, but are in the long run harmful to the myocardium and contribute to a negative spiral by worsening cardiac function.

Among the alterations observed in the failing heart is altered protein phosphorylation, in part caused by decreased β -adrenergic receptor signalling. The data provided in this invited review paper demonstrated lower phosphorylation of the β -adrenergic target protein cTnI in hearts from patients with end-stage idiopathic dilated cardiomyopathy (IDCM) and in myectomy samples (Morrow procedure) from patients with manifest HCM. The phosphorylation level of cMyBP-C was also decreased in cardiac samples from IDCM patients and to a lesser extent in HCM patients, indicating that especially cMyBP-C phosphorylation seems to depend on the specific cardiomyopathy. These results emphasize the need to unravel subtle sarcomere changes in different cardiomyopathies to clarify which pathophysiological processes are the primary

cause of human cardiomyopathy and which are a secondary consequence of altered cardiac performance.

Chapter 3 – A piece of the human heart: Variance of protein phosphorylation in left ventricular samples from end-stage primary cardiomyopathy patients

The availability of human cardiac tissue for research is limited. Often only small cardiac biopsies are available. However, regional differences in protein composition may exist in the healthy heart or may develop during disease progression. Moreover, protein modifications due to cardiomyopathies may not be uniform throughout the heart. To determine if cardiac biopsies are representative for the region they were taken from, we assessed if local differences in sarcomeric protein phosphorylation are more evident in manifest familial HCM and DCM than in non-failing Donor myocardium. Thereto, phosphorylation of the two main target proteins of the β -adrenergic receptor pathway, cTnI and cMyBP-C, was analysed by ProQ phospho stain in small, biopsy-sized pieces from different parts of the free left ventricular wall of end-stage failing HCM and DCM patients and Donor hearts obtained during transplant surgery.

Intra-subject variability in protein phosphorylation was comparable in Donor, HCM and DCM samples, indicating that, within the precision of the measurements, small left ventricular tissue samples are representative for the region they were taken from.

Chapter 4 – Protein kinase A treatment unmasks differences in myofilament Ca^{2+} -sensitivity between human familial hypertrophic and dilated cardiomyopathy

Familial HCM is characterized by often asymmetric thickening of the left ventricle and septum (concentric remodelling), while DCM is characterized by ventricular dilatation (eccentric remodelling). The pathomechanisms underlying or distinguishing these two cardiomyopathies are largely unknown. We compared sarcomeric protein composition and function in cardiomyocytes from end-stage failing familial HCM (FHCM) and DCM (FDCM) hearts obtained during transplant surgery in order to detect differences that might underlie diverse cardiac function and remodelling. Phosphorylation of the β -adrenergic target proteins cMyBP-C and cTnI was low in both FHCM and FDCM relative to non-failing Donor hearts (Figure 8.1). This was in accordance with an, on

average, increased myofilament Ca^{2+} -sensitivity in both patient groups compared to Donor. Force measurements were repeated after incubation with PKA, to establish if cardiomyocytes from FHCM and FDCM were still responsive to β -adrenergic receptor stimulation. After PKA incubation, Ca^{2+} -sensitivity of force was comparable between FHCM and Donor, but significantly lower in FDCM. A unique feature of cardiomyocytes from FHCM patients was decreased maximal force development; a characteristic which was not restored by PKA incubation.

The results suggest that some alterations in cardiomyocyte function that may contribute to impaired cardiac function are specific for FHCM (depressed maximal force development) or FDCM (decreased Ca^{2+} -sensitivity unmasked by PKA) and thus may underlie or at least contribute to the diverse pathophysiologic remodelling patterns in primary human cardiomyopathies. Differences in sarcomeric properties appeared to be masked at baseline by the activation status of β -adrenergic receptors in the end-stage failing hearts, highlighting the influence of post-translational modifications such as phosphorylation.

Chapter 5 – Cardiac myosin binding protein C mutations and hypertrophic cardiomyopathy: haploinsufficiency, deranged phosphorylation and cardiomyocyte dysfunction

Mutations in *MYBPC3* are a frequent cause of HCM, especially in the Netherlands where approximately 35% of the HCM patients have a founder mutation in *MYBPC3* (*MYBPC3*_{mut}). We compared cardiac myectomy and biopsy samples from *MYBPC3*_{mut} with truncating *MYBPC3* mutations (c.2373dupG or 2864_2865delCT) to non-failing Donor tissue. Messenger RNA (mRNA) analysis revealed the presence of about 20% mutant mRNA in the patient samples, indicating that the mutant allele is transcribed, though at a low level compared to the normal (non-mutant) allele. Specific antibodies directed against cMyBP-C revealed no truncated protein in *MYBPC3*_{mut} cardiac samples. The expression of full length cMyBP-C was reduced by 33% in *MYBPC3*_{mut} compared to Donor. The phosphorylation of cMyBP-C was comparable between *MYBPC3*_{mut} and Donor, whereas cTnI phosphorylation was significantly reduced (Figure 8.1). Antibodies against the PKA phosphorylation sites of cMyBP-C (Ser282) and cTnI (Ser23/24) confirmed this pattern of phosphorylation, indicating divergent phosphorylation of two main target proteins under β -adrenergic control.

Maximal force production was significantly depressed in *MYBPC3*_{mut}, while

myofilament Ca^{2+} -sensitivity was increased compared to Donor. Incubation with PKA lowered the Ca^{2+} -sensitivity and abolished the difference between $\text{MYBPC3}_{\text{mut}}$ and Donor. Maximal force production was not restored after PKA incubation.

In conclusion, truncating *MYBPC3* mutations caused haploinsufficiency, as can be concluded from the low expression level of cMyBPC and the absence of mutant cMyBPC in $\text{MYBPC3}_{\text{mut}}$. Cardiomyocyte dysfunction in $\text{MYBPC3}_{\text{mut}}$ was evident from depressed maximal force development and increased Ca^{2+} -sensitivity of myofilaments. The latter may be explained by low cTnI phosphorylation, which was restored after incubation with PKA.

Chapter 6 – Altered length-dependent activation independent of the mutant protein in human hypertrophic cardiomyopathy

HCM can be caused by mutations in different genes encoding sarcomeric proteins. In order to determine the specific contribution of *MYBPC3* mutations on cardiomyocyte dysfunction opposite to general adaptations in HCM, we compared properties of cardiac myectomy samples from $\text{MYBPC3}_{\text{mut}}$ to those of mutation negative HCM patients in which no mutation was detected upon screening of 9 genes (HCM_{mn}) and to non-failing Donor hearts. The reduced expression of cMyBP-C relative to Donor samples observed in $\text{MYBPC3}_{\text{mut}}$ (Chapter 5) was not found in HCM_{mn} . In addition, phosphorylation of cMyBP-C was decreased in HCM_{mn} but comparable to Donor in $\text{MYBPC3}_{\text{mut}}$. Phosphorylation of cTnI was decreased in both HCM patient groups (Figure 8.1) compared to Donor confirming the results in chapters 2, 4 and 5.

Sarcomeric dysfunction was comparable in $\text{MYBPC3}_{\text{mut}}$ and HCM_{mn} ; both groups had decreased maximal force development and increased myofilament Ca^{2+} -sensitivity compared to Donor cardiomyocytes. Furthermore, we measured the force response to the two mechanisms that are activated upon increased cardiac demand, i.e. the Frank-Starling mechanism (length-dependent activation) and β -adrenergic receptor signalling. Increasing sarcomere length from 1.8 μm to 2.2 μm increased Ca^{2+} -sensitivity of force less in both patient groups than in Donor, indicative of a blunted Frank-Starling mechanism. Incubation with PKA, to mimic β -adrenergic receptor stimulation, abolished the initial difference in myofilament Ca^{2+} -sensitivity between patients and Donor. Interestingly, the Frank-Starling response was also restored after PKA

incubation both in MYBPC3_{mut} and HCM_{mn}.

Our results demonstrate that alterations in protein composition appear to be, at least partly, related to mutations in *MYBPC3*. In contrast, cardiomyocyte dysfunction, as evident from decreased maximal force development, increased myofilament Ca²⁺-sensitivity and blunting of the Frank-Starling response, represents a general feature of HCM.

Chapter 7 – Preserved cross-bridge kinetics in human hypertrophic cardiomyopathy patients with a *MYBPC3* mutation

The role of cMyBP-C in regulating sarcomeric contraction is not fully understood yet, though it appears that cMyBP-C acts as a brake on cross-bridge cycling. As reported in chapter 5, mutations in *MYBPC3* lead to haploinsufficiency and decreased maximal force development in human. In chapter 7, we investigated if cMyBP-C haploinsufficiency altered cross-bridge kinetics in MYBPC3_{mut}. We measured the rate of force redevelopment (k_{tr}) after a quick shortening and re-stretch maneuver (slack test) and assessed the response to a 5% increase in length. Both in MYBPC3_{mut} and Donor cardiomyocytes, k_{tr} increased with increasing Ca²⁺-concentrations and was comparable in both groups. The rate of force relaxation (k_{rel}) in response to stretch did not differ between MYBPC3_{mut} and Donor. Also the next phase in the stretch activation response, delayed development of force, had a comparable rate constant (k_{df}) and amplitude in MYBPC3_{mut} and Donor. Incubation with PKA lowered k_{tr} slightly but not significantly, while k_{rel} was faster after PKA treatment. k_{df} was faster after PKA incubation only at maximal activation. The response to PKA incubation was similar in MYBPC3_{mut} and Donor, in line with comparable cMyBP-C phosphorylation between both groups.

Thus, we found no alterations in cross-bridge kinetics in MYBPC3_{mut} compared to Donor cardiomyocytes that could explain the low maximal force development in these patients. The reduced amount of cMyBP-C in the MYBPC3_{mut} patients appears to be sufficient to maintain proper cross-bridge kinetics. The ability to influence cross-bridge kinetics through β -adrenergic receptor activation appeared to be preserved in MYBPC3_{mut} patients.

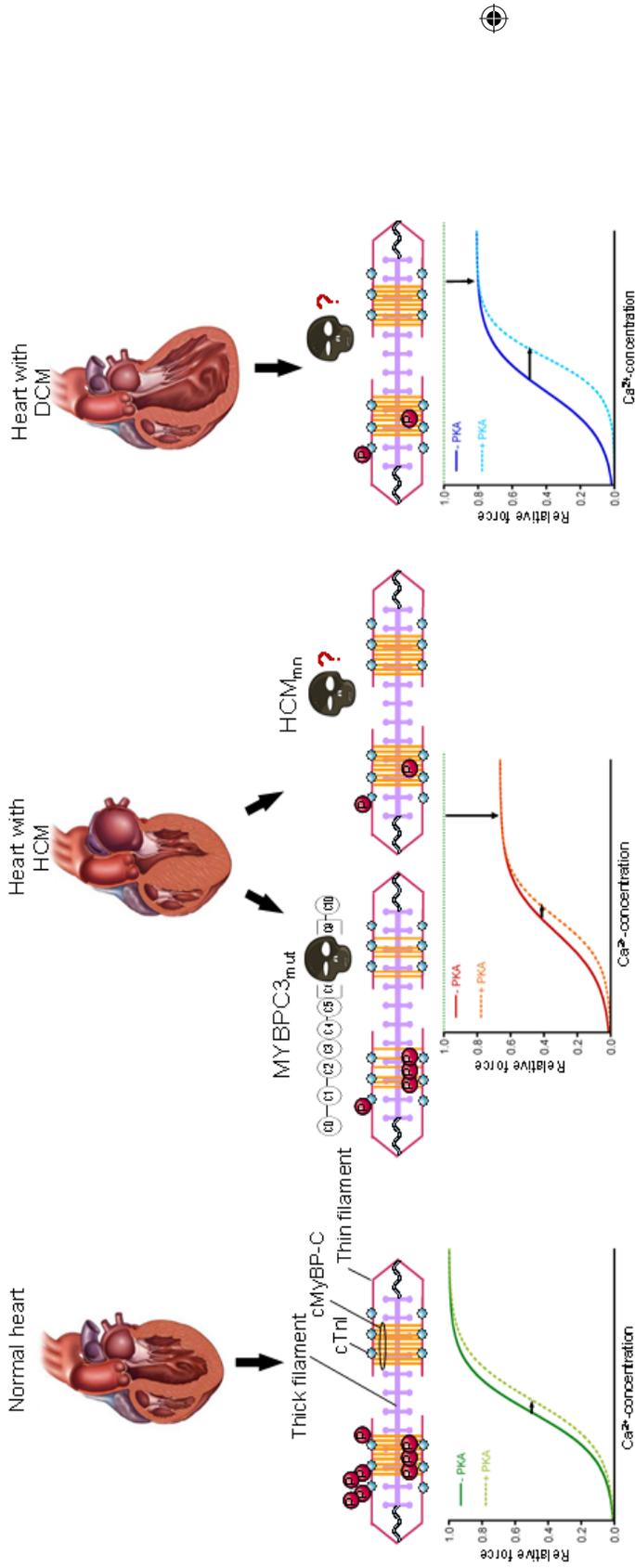


Figure 8.1. Overview of the main findings described in this thesis. In familial hypertrophic cardiomyopathy (FHCM) patients with a mutation in *MYBPC3* (*MYBPC3_{mut}*), the amount of cardiac myosin binding protein C (cMyBP-C) was decreased relative to normal hearts (middle part of the figure, less orange stripes). The phosphorylation level (represented by red dots) of cMyBP-C was comparable to Donor level. In FHCM patients with an unidentified mutation (mutation negative, HCM_{mn}), the expression level of cMyBP-C was comparable to Donor, but the phosphorylation was decreased. The phosphorylation of cardiac troponin I (cTnI; blue dots) was decreased in both FHCM groups. In end-stage familial dilated cardiomyopathy (FDCM) patients, the phosphorylation of both cMyBP-C and cTnI was decreased relative to the level in Donor cardiac tissue, which is in agreement with observations in end-stage failing idiopathic dilated cardiomyopathy hearts shown in chapter 2 of this thesis. Cardiac dysfunction (lower part of the figure) in FHCM patients was evident from a significant decrease in maximal force development (black arrow) and increased sensitivity to Ca²⁺ (leftward shift of the curve). Incubation with protein kinase A (PKA) lowered Ca²⁺-sensitivity to a level comparable to Donor. FDCM patients had an increased baseline Ca²⁺-sensitivity as well. Incubation with PKA lowered Ca²⁺-sensitivity remarkably, making the FDCM cardiomyocytes less sensitive to Ca²⁺ than donor cardiomyocytes.

CONCLUSION

As became evident from the studies described in this thesis, cardiomyocytes from FHCM patients have characteristics which are partly distinct from those measured in FDCM and described in other cardiomyopathies, such as ischemic and idiopathic dilated cardiomyopathy.^{70;92;94} The decreased phosphorylation level of cMyBP-C and cTnI in FDCM was comparable to IDCM (Chapter 2: Figure 2.1), as was the increased sensitivity to Ca^{2+} in these cardiomyocytes.^{70;92}

We have shown unique protein changes in cMyBP-C expression and phosphorylation in FHCM patients with a *MYBPC3* mutation which were not present in FHCM patients with no identified sarcomere mutation. Another protein alteration, decreased phosphorylation of cTnI, was common in all FHCM and FDCM patients (Figure 8.1) and likely represents a secondary adaptation in the development of cardiomyopathy. A specific feature of the FHCM patients studied was the decreased average maximal force development of cardiomyocytes, which in the case of *MYBPC3* mutations could not be explained by altered cross-bridge kinetics. Increased myofilament Ca^{2+} -sensitivity was seen in both FHCM and FDCM patient samples and appears to reflect a general property of cardiomyopathies that corresponds to the common reduction in cTnI phosphorylation. Furthermore, we demonstrated the importance of protein phosphorylation on cardiomyocyte dysfunction in cardiomyopathy patients.

Our studies suggest that FHCM causing mutations lead to specific (protein) alterations in the sarcomere, but that this eventually leads to common cardiomyocyte dysfunction. Cardiomyocyte dysfunction may partly contribute to impaired cardiac performance and be a substantial part of the pathophysiology of cardiomyopathies. Decreased force development of cardiomyocytes likely contributes to depressed contractility during systole. Although an increased Ca^{2+} -sensitivity of force in theory would be beneficial for cardiac contractility during systole (i.e. more force will develop at a given $[\text{Ca}^{2+}]$), our results indicate that this mechanism is insufficient to compensate for the decrease in F_{max} in FHCM patients (Chapter 4: Figure 4.7). During diastole an increased Ca^{2+} -sensitivity may even be hampering cardiac performance, as it limits cardiac relaxation needed for proper filling of the heart. Relaxation of the heart is further influenced by passive stiffness of the cardiomyocytes. Even though we

measured no increased F_{pas} in FHCM or FDCM cardiomyocytes, an increased passive stiffness could contribute to diastolic dysfunction by the inability of cells to adequately stretch during cardiac filling.

Furthermore, the timing of sarcomeric contraction is of pivotal importance to cardiac function. Altered cross-bridge kinetics, which we studied by measuring k_{tr} and the kinetics of stretch activation (Chapter 7), can affect systolic as well as diastolic function of the heart. When cross-bridge cycling is too fast, force may not be maintained long enough for systole. Too slow cross-bridge cycling would impair relaxation during diastole. We measured no significant differences in cross-bridge kinetics between MYBPC3_{mut} and Donor (Chapter 7), thus altered cross-bridge cycling appears not to underlie cardiac dysfunction in these HCM patients.

Finally, the cardiac response to increased demand depends on β -adrenergic receptor stimulation and the Frank-Starling mechanism. Stimulation of the β -adrenergic receptors exerts both positive inotropic and lusitropic effects to improve all phases of cardiac contraction during increased cardiac performance. The Frank-Starling mechanism matches cardiac output to increased cardiac filling during increased cardiac demand. The response to β -adrenergic receptor stimulation appeared to be preserved in FHCM and FDCM, as incubation with PKA lowered Ca^{2+} -sensitivity in FHCM to the same extent as in Donor, while the PKA effect in FDCM was even more pronounced (Chapters 4-7). We determined the Frank-Starling response in FHCM and Donor (Chapter 6). The length-dependent increase of Ca^{2+} -sensitivity was blunted in FHCM cardiomyocytes compared to Donor at baseline, but restored after PKA incubation. These results suggest that the mechanisms activated upon increased cardiac demand are still functional in FHCM and FDCM cardiomyocytes.

FUTURE PERSPECTIVES

Our studies are performed on cardiac tissue from overt cardiomyopathy patients. Therefore, the changes in cardiomyocyte function and sarcomeric protein composition represent the result of many adaptations within the cardiomyocyte over years of disease progression. We lack information on primary pathophysiologic alterations in human cardiomyocytes and disease development over time. Nonetheless, signs of cardiac dysfunction were observed

in individuals carrying a HCM mutation before clinical features of HCM were apparent,¹⁸⁻²⁰ suggesting intrinsic cardiomyocyte dysfunction independent of disease progression. Unfortunately, no specific treatments are available to prevent or reverse cardiac remodelling in cardiomyopathies. As such, it is pivotal to unravel primary pathophysiological defects so that eventually we can intervene in the disease progress before the heart remodels. FHCM has a high prevalence and, in comparison to FDCM, many disease causing mutations are identified. Together with the ability to obtain cardiac samples, especially from patients with left outflow tract obstruction that undergo a myectomy, FHCM patients are very appealing to further study primary pathophysiological defects in.

Functional characteristics of cardiomyocytes from FHCM patients were comparable irrespective of the disease causing mutation (Chapters 4-6), suggesting that shared hypertrophic processes are ongoing. Is the regulation of sarcomeric function by the diverse sarcomeric proteins so entwined that the mutant defect is of inferior importance to the development of FHCM? If so, it would be less relevant to unravel how hypertrophic pathways are activated by mutated proteins in FHCM as it would be sufficient to know which pathways are activated. However, decreased expression of cMyBP-C was exclusively observed in FHCM patients with *MYBPC3* mutations (Chapters 5 and 6). Also *in vitro* studies,¹⁰¹ animal models and human studies¹⁸⁰ showed distinct, sometimes even opposing, effects from different mutations.^{27;103} In conclusion, the disease causing mutations seem relevant for early cardiac dysfunction and the precise (detrimental) function of each sarcomere protein mutation might define how hypertrophic pathways are activated.

Mouse models for HCM provide unique opportunities to follow the time course of disease progression. To date, mouse models are available with many different HCM causing mutations, making it possible to discriminate consequences of defects in specific sarcomeric proteins. Despite the value of transgenic mouse models to study the effects of HCM causing mutations, not only on protein level but also at organ level, results should always be critically evaluated. The physiology of mice is different from human and adaptation processes in response to the transgenic modification might not resemble the human pathophysiological processes.⁷⁷ Promising new approaches to study the physiological consequences of HCM causing mutations in human tissue

are induced pluripotent stem (iPS) cells and engineered heart tissue (EHT). iPS cells are established by reprogramming adult somatic cells and can be differentiated in beating cardiomyocytes.^{181;182} As iPS cells could easily be derived from HCM patients, they closely represent the biological environment, including the HCM causing mutation, of each patient. Combining the iPS cell techniques with cardiac tissue engineering would increase their potential as a disease model even further. EHT makes it possible to conduct force measurements and study cardiomyocytes in a 3D structure. EHT reconstituted from neonatal rat cardiomyocytes and type I collagen displayed contractile characteristics of native myocardium, making it a very promising model to study cardiac function.¹⁸³

We demonstrated haploinsufficiency in HCM patients with a *MYBPC3* mutation evident from only 66% cMyBP-C expression in these patients compared to non-failing myocardium (Chapter 5). This reduced expression might cause cardiomyocyte dysfunction. Homozygous cMyBP-C knock out mice in which cMyBP-C was absent develop hypertrophy already at young age (3 weeks-4 months).^{32;33} In contrast, heterozygous cMyBP-C mice that express 75–90% cMyBP-C develop hypertrophy much later in life (at 30 weeks to 11 months of age).^{33;184} Surprisingly, 40% re-expression of cMyBP-C in cMyBP-C null mice was sufficient to restore systolic dysfunction.^{53;122} It should be noted that these measurements were performed in relative young mice (12-24 weeks) and thus might represent a pre-symptomatic stage of HCM. The stoichiometric proportion of cMyBP-C to myosin and its restricted location in the C-zone limits the number of myosin heads that are directly interfered by cMyBP-C.¹⁸⁵ Apparently cMyBP-C does not require 1:1 interaction to myosin heads to exert its regulatory role, but there is a lower threshold. Learning more on how cMyBP-C regulates cross-bridge cycling and what the minimal amount of cMyBP-C is to do so, might provide insight in the disease pathogenesis of HCM caused by *MYBPC3* mutations.

Cardiomyopathy related mutations are scattered throughout the *MYBPC3* gene. It is plausible that mutations within a certain gene can exert different detrimental effects due to their different locations within the protein. Part of the variation observed between cardiomyopathy patients might be explained by the location of mutations within genes. For several regions of cMyBP-C its interaction with other sarcomeric proteins is known, but the functional

relevance of different regions of cMyBP-C is subject of ongoing investigation. For example, *in vitro* experiments have already shown that the cMyBP-C N-terminus is able to activate cross-bridge cycling even in the absence of Ca^{2+} ,^{186;187} and accelerates the rate of tension redevelopment in skinned cardiomyocytes.^{163;164} However, the role of the N-terminus of cMyBP-C under physiological circumstances and in HCM remains to be investigated.

Next to further research into precise sarcomeric protein function in health and disease, it is important to clarify genetic and environmental factors that alter the disease course of cardiomyopathies. As FHCM and FDCM have no complete disease penetrance and individuals even within one family might present with different symptoms, modifying factors must have an effect on disease progression. Identification of these factors could give more insight in the development of human cardiomyopathy and present additional targets for treatment.