Post-Translational Regulation of Cardiac Myosin Binding Protein-C: A Graphical Review

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Abstract

Cardiac myosin binding protein-C (cMyBP-C) is a fundamental component of the cardiac sarcomere involved in regulating systolic and diastolic activity, processes which must be tightly maintained to preserve cardiac function. Importantly, as a non-enzymatic protein, cMyBP-C relies solely on post-translational modifications and protein-protein interactions in order to modulate its function, and does so through phosphorylation, glutathionylation and acetylation amongst others. Although some are better understood than others, these modifications may represent novel therapeutic routes to modulate cMyBP-C function in the treatment of cardiac disease.

Introduction

Since its discovery as a contaminant of a skeletal myosin preparation in the 1970s, the past four decades have seen a significant amount of research dedicated to the sarcomeric protein myosin binding protein-C (MyBP-C)[1]. Named C-protein by its original discovery as a contaminant of a cardiac myosin preparation (with each contaminant given a letter based on their size compared to myosin heavy chain), MyBP-C can be found localised as distinctly spaced stripes of the C-zone of the A band, where due to its abundance it was initially considered a purely structural component of the sarcomere[1,2]. Although it does play a role in stability via C-terminal interactions with the light meromyosin (LMM) component of the myosin backbone[3] and giant sarcomeric protein Titin[4], cMyBP-C is now best known as an essential regulator of cardiac contractility. Additionally, it is a protein that is significantly influenced by a variety of post-translational modifications (PTMs), some of which are important clinically. This graphical review will briefly outline the functional role of cMyBP-C in the sarcomere before discussing the past and recent advancements in understanding its regulation by PTMs.

CMyBP-C in the Control of Cardiac Contractility

Following the advancement in biochemical sequencing, the C-protein identified in skeletal muscle was found to exist as three paralogues on three different genes, two in the skeletal muscle (fast skeletal, fsMyBP-C and slow skeletal, ssMyBP-C) and one exclusively expressed in the atria and ventricles of heart (cardiac, cMyBP-C)[5,6], which contains several specific cardiac modifications. Additionally, a smaller protein, MyBP-H, which shares similar domain structure has been identified in both skeletal and cardiac tissue (Figure 1)[7].

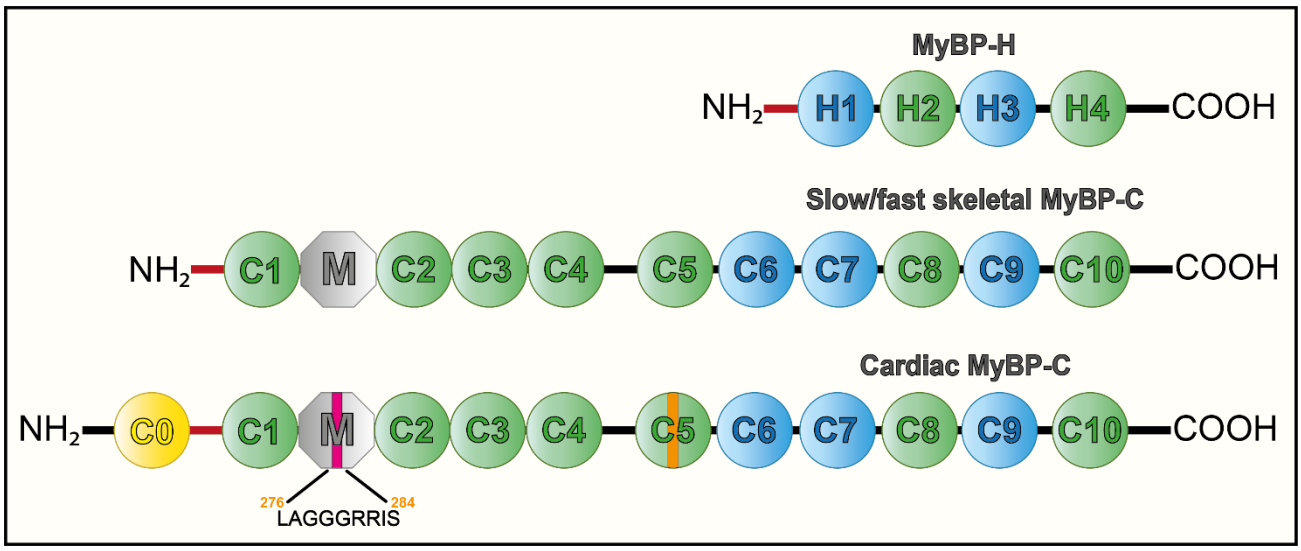

Figure 1: Isoforms of Myosin Binding Protein-C. Myosin binding protein-C (MyBP-C) exists as three paralogues, two in the skeletal muscle (fast and slow) which share high sequence homology (although there are up to 14 slow variants), and one in the cardiac tissue (cardiac, cMyBP-C) which shares 50-55% homology with the skeletal isoforms. Although the overall structure is shared between them, with each containing 7 immunoglobulin (green), 3 fibronectin-III (blue) domains, a MyBP-C specific M-domain and a proline/alanine rich linker sequence at the N terminal (red), cMyBP-C contains several specific cardiac modifications. These include an entire immunoglobulin domain (C0) at its N terminus and two cardiac specific sequence insertions in the M domain (LAGGGRRIS motif; pink) and central C5 domain (28 amino acid loop; orange). A smaller homolog known as MyBP-H, existing in both skeletal and cardiac tissue, shares a similar domain structure with the C-terminus of the MyBP-C isoforms. Adapted from Flashman, *et al.*[8]

Although important research has been conducted to understand the function of the skeletal isoforms (reviewed by McNamara and Sadayappan[9]), most studies have focussed on the regulation of cardiac isoform. This is largely due to a significant advance in the 1990s when two mutations in the MYBPC3 gene that encodes the protein were shown to cause hypertrophic cardiomyopathy (HCM)[10,11], a heritable condition resulting in left ventricular hypertrophy and diastolic dysfunction, that affects 1:200 in the population[12]. Since then it has been established that the majority of MYBPC3 mutations result in haploinsufficiency leading to trace levels of cMyBP-C in the sarcomere, indicating that cMyBP-C may play an important functional role. Importantly, the two sites of mutation originally identified in MYBPC3 occurred in the region associated with LMM binding and this led to a concerted effort to characterise the cMyBP-C-myosin functional relationship. It was subsequently revealed that the cMyBP-C N-terminal domains can extend from the myosin backbone and interact with the S2 region of myosin heavy chain, found C-terminal to the S1 motor head[13]. Functionally, knock-out models and ablation studies show that cMyBP-C has a minimal role in maintaining sarcomere stability but a significant role in the modulation of contraction, with its absence resulting in accelerated cross-bridge formation, impaired diastolic function and reduced myofilament calcium sensitivity[14–16]. This hypercontractility could be reversed by introducing the C1-M-C2 domains of cMyBP-C involved in the interaction with S2[17], and importantly, in-vivo loss of the sites on myosin proposed to bind cMyBP-C, resulted in cardiac hypertrophy[18]. CMyBP-C is now considered to have an overall inhibitory effect on myosin, doing so partly through sequestering of myosin heads and limiting the pool available for cross-bridge cycling[19,20].

Additionally, the role cMyBP-C plays in regulating cardiac contractility is in part due to with its interaction with actin, which was first described in skeletal muscle in 1978[21] with follow up studies demonstrating that the N-terminal regions shown to interact with the S2 of myosin, also interact with the thin filament[22,23]. In-vitro motility assays initially revealed cMyBP-C inhibits actin motion[24], but addition of regulatory protein tropomyosin saw cMyBP-C increase actin motility at low calcium[25]. Current work suggests cMyBP-C has dual inhibitory and activating effects through its interaction with actin and tropomyosin, with the C0-C1 domains competing for actin binding with tropomyosin, thus shifting tropomyosin to its high calcium state at low calcium, leading to increased actin activation, enhanced calcium sensitivity and prolonged relaxation[26,27] (Figure 2).

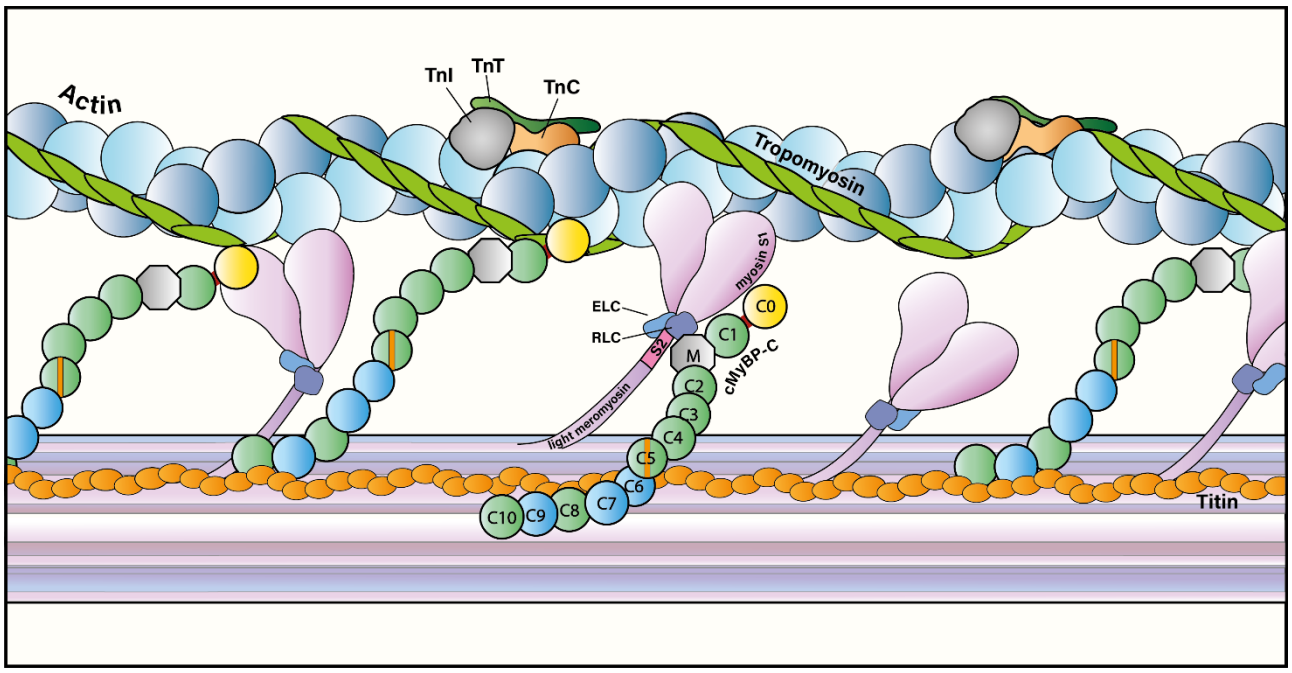

Figure 2: Cardiac Myosin Binding Protein-C in the Sarcomere. Cardiac myosin binding protein-C (cMyBP-C) is found at regular intervals (43nm apart) in the C-zone of the A band of the sarcomere where it is anchored through interactions of its C-terminal domains with the light meromyosin component of the myosin thick filament, as well as interactions with the giant sarcomeric protein Titin. Although it is still unclear how cMyBP-C organises itself in the sarcomere, the axial model proposes cMyBP-C N-terminal domains extend away from the thick filament where they can interact with the S2 component of the myosin heavy chain and potentially the motor domain (myosin S1). Myosin is decorated with myosin light chain elements, including the essential light chains (ELC) and regulatory light chains (RLC), the latter of which cMyBP-C is proposed to interact with via the C0 domain. The N-terminal domains also interact with the thin filament and it is this dynamic interaction that allows cMyBP-C to regulate acto-myosin cross-bridge formation, myofilament calcium sensitivity and influence on cardiac contractility. The thin filament is also regulated by the calcium sensing troponin complex formed of troponin-T (TnT), troponin-C (TnC) and Troponin-I (TnI) that when activated by calcium during systole, shift the tropomyosin molecule from its low to high calcium state exposing binding sites for myosin. CMyBP-C is also thought to interact with tropomyosin to prolong relaxation through activation of actin at low calcium. Adapted from McNamara and Sadayappan[9].

Phosphorylation and cMyBP-C

Although the detailed molecular mechanisms are still unclear, a credible body of research has cemented the idea that cMyBP-C acts as an inhibitor of acto-myosin cross-bridge formation and an overall negative regulator of cardiac contractility. However, influence from a variety of PTMs greatly complicates this picture. During times of increased cardiac demand, activation of β1 adrenoceptors leads to a signalling cascade resulting in protein kinase A (PKA) phosphorylation of downstream substrates, including sarcomeric proteins, and modulation of their activity in response[28]. CMyBP-C phosphorylation was first identified in isoproterenol treated frog cardiac tissue[29], and extensive research since has shown the profound effect cMyBP-C phosphorylation has on overall myocardial contractility. In fact, nearly all of the interactions described above are influenced by phosphorylation, leading to reduced binding of cMyBP-C to myosin S2[30] and actin[31], which results in increased cross-bridge formation, calcium sensitivity and overall enhanced inotropic and lusitropic response[32,33].

An initial study identified three serines in the cMyBP-C M-domain to be phosphorylated (Ser273, Ser282 and Ser302; mouse sequence)[30], with a comprehensive understanding of the importance of these phosphorylation sites coming from a series of transgenic animals expressing serine to aspartate (phospho-mimetic) or serine to alanine (phosho-null) versions of cMyBP-C. Phospho-null cMyBP-C showed significant changes in cardiac contractility and expression of phospho-null cMyBP-C could not rescue the hypertrophic phenotype of the cMyBP-C-null mouse, whereas wildtype and phospho-mimetic expression restored cardiac function[34,35]. Importantly, phospho-mimetic mice were protected against cardiac ischaemia/reperfusion injury[35] and it has been since shown that phosphorylation of cMyBP-C is significantly

reduced in both experimental and human heart failure[36,37]. Interestingly, a recent study has shown cMyBP-C phosphorylation in mice decreases with age, and phospho-mimetic mice show enhanced survival, with preserved cardiac function and ventricular wall thickness[38]. It has also been recently reported that cMyBP-C plays more of a role in regulating calcium homeostasis than initially thought, with reduced phosphorylation correlating with prolonged calcium transients and increased diastolic calcium levels, potentially through changes in the sodium-calcium exchanger function[39].

Phosphorylation of cMyBP-C is essential to maintain cardiac function, however the processes that govern this modification are complex. Additional work has further defined the roles of these sites, showing that phosphorylation of the M-domain sites occurs in a hierarchal manner, with Ser282, the site most dephosphorylated in heart failure[40], being required for subsequent phosphorylation of Ser302 *in vivo*[41]. In addition, strikingly different phenotypes in transgenic models were shown depending on which site is null for phosphorylation, again implicating S282 as the critical phosphorylation site[42]. (Figure 3).

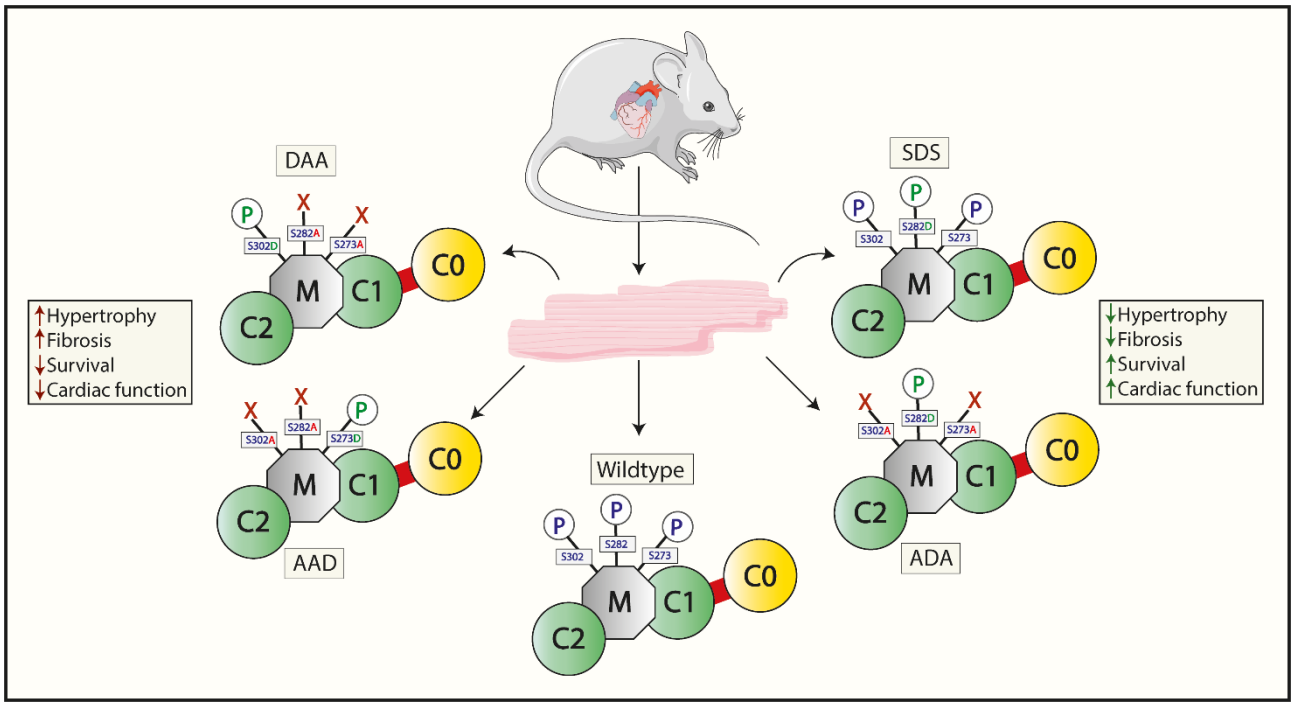

Figure 3: Phosphorylation Site Non-equivalency in Cardiac Myosin Binding Protein-C: Cardiac myosin binding protein-C (cMyBP-C) differs from other isoforms by having three phosphorylation sites in the M domain (S273, S282 and S302; mouse sequence) which are conserved in mammals. Several transgenic models have been created that show not all sites have equal function. Transgenic models where S282 is unphosphorylatable (alanine substitution, A) show significant cardiac dysfunction, increased hypertrophy and fibrosis, and reduced survival into adulthood. However, when S282 is constitutively phosphorylated (aspartate substitution, D), even when the other two sites are unphosphorylatable, transgenic mice have normal cardiac function compared to wildtype, and improved levels of hypertrophy, fibrosis and survival compared to S282A, implicating it as the critical phosphorylation site in the M domain. Figure adapted from Gupta and Robbins[43] using Servier Medical Art which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.

This non-equivalency of phosphorylation sites may be largely to do with the host of different kinases that have been identified to target N-terminal phospho-motifs. PKC, the effector enzyme from α-adrenoceptor signalling, targets two of the three identified sites (S273 and S302), with a preference for S302 in-vivo, and phosphorylation by PKC isoforms PKCε and PKCα lead to decreased myofibrillar ATPase activity and reduced calcium sensitivity[41,44]. PKD, activated through PKC directed phosphorylation, increases cross-bridge formation through phosphorylation of Ser302, analogous to PKA directed phosphorylation of the M-domain[45]. Interestingly, although known to be the critical phosphorylation site of cMyBP-C by PKA, mutation of Ser282 does not alter PKD dependent regulation[45]. M-domain phosphorylation produces distinct functional profiles in cMyBP-C, in terms of regulating the interaction with actin and myosin, and overall effect on cardiac contractility[46].

Aside from PKA, PKC and PKD regulation, the M-domain's Ser302 and Ser282 are phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII), and as a calcium-sensitive kinase, the extent to which phosphorylation occurs may be regulated by calcium concentration[41]. Functionally, phosphorylation of S302 by CAMKII may play a role in positive force-frequency relationship of cardiac tissue[47]. Phosphorylation by ribosomal S6 kinase (RSK) has been reported, whereby activation in response to mitogen-activated protein kinase pathway results in RSK phosphorylation at a single site (S282), leading to increased cross-bridge kinetics and reduced myofilament calcium sensitivity, and may be required for PKA mediated phosphorylation of other sites[48]. More recent study suggests the M-domain phosphorylation complexity allows cMyBP-C to direct particular affects over the thick and thin filaments in control of cross-bridge cycling, and that phosphorylation of a site by one kinase can positively or negatively impact the phosphorylation by additional kinases on other sites[46]. Understanding of cMyBP-C phosphorylation is expanding over time, with in-vivo mass spectrometry evidence suggesting there could be up to 24 phosphorylation sites present on cMyBP-C[49,50]. Of note, S307 has been implicated as a potential PKA phosphorylation site, although there was limited evidence of this in-vivo[51]. Additionally, S133, a site in the proline/alanine rich linker, is phosphorylated by glycogen synthase kinase 3β (GSK3β) but the functional consequences of this site have yet to be determined[52] (Figure 4).

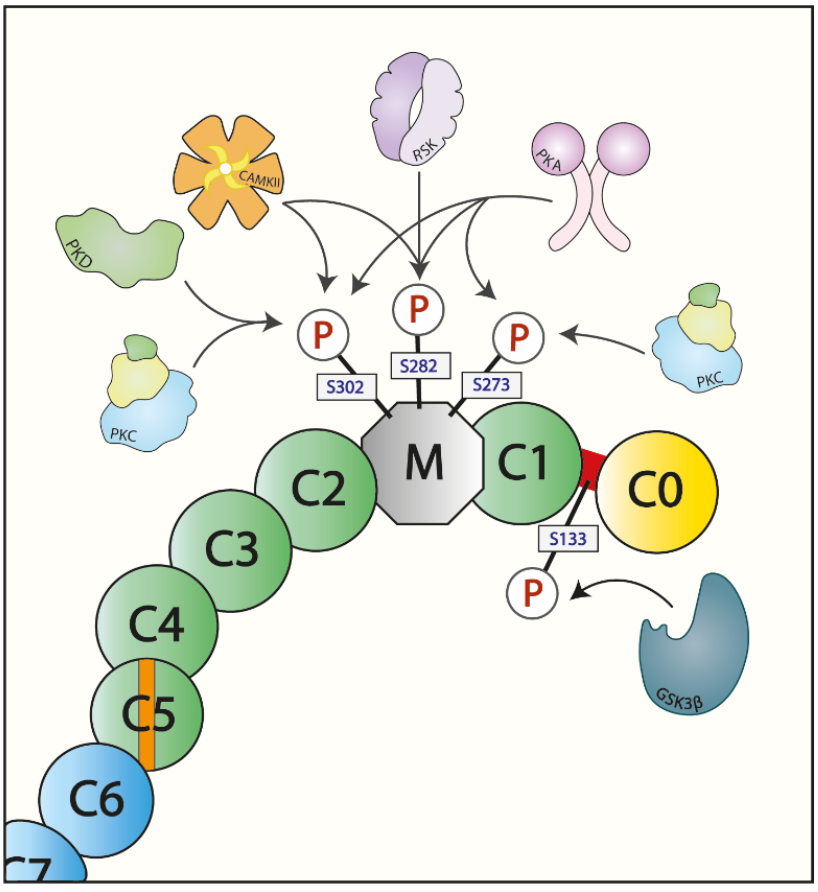

Figure 4: Kinase Targeting of Cardiac Myosin Binding Protein-C: The phosphorylation profile of cardiac myosin binding protein-C (cMyBP-C) is complicated by the fact the M-domain phosphorylation sites can be targeted by a host of different kinases. Protein kinase A (PKA), the main effector of β-adrenergic stimulated phosphorylation of cMyBP-C can target all three sites (S273, S282 and S302, mouse sequence) with S282 phosphorylation being required before the others can be phosphorylated. Protein kinase C (PKC) targets both S273 and S302 while protein kinase D only targets S302. Other phosphorylating kinases identified include Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII), which targets S282 and S302, ribosomal S6 kinase (RSK), which only targets S282, and glycogen synthase kinase 3β (GSK3β), which has not been reported to target any M-domain sites but instead phosphorylates S133 in the proline/alanine rich linker between C0 and C1.

Redox Modifications and cMyBP-C

Although phosphorylation has been the most widely studied modification, cMyBP-C is the substrate for a host of other PTMs, some demonstrating important functional and clinical relevance. Redox modifications, such as carbonylation, S-nitrosylation and S-glutathionylation are often enhanced in times of oxidative stress, a key mediator of cardiac dysfunction. Unlike phosphorylation, these modifications are generally non-enzymatic and driven by reactivity and availability of their amino acid side chains. Carbonylation, the irreversible oxidation of basic amino acid side chains, such as those of arginines and lysines, into carbonyl derivatives, often leads to protein degradation during oxidative stress. In the context of cardiac physiology, protein carbonylation is increased in the failing human heart[53] and has been studied in the functional regulation of the ryanodine receptor 2 (RYR2) and Na^+/Ca ATPase (SERCA)[54]. A proteomics screen revealed cMyBP-C carbonylation in response to Doxorubicin treatment, an anti-cancer agent with restricted use due to cardiotoxicity issues, and demonstrated cMyBP-C degradation through the proteasomal pathway and potential carbonylation-mediated dysregulation of actin binding. Although the functional consequences are not yet understood, this could implicate cMyBP-C and loss of binding to actin in the pathology of Doxorubicin-induced cardiotoxicity and warrants further study[55]. Similarly, S-nitrosylation, the reversible addition of a nitric oxide (NO) molecule to the thiol of a free cysteine residue (forming a protein SNO), has been observed in-vitro at C-terminal site C1260 (mouse sequence)[56]. S-nitrosylation has long been studied in cardiac (patho)physiology, where following oxidative stress, S-nitrosylation of cardiac proteins, including RYR2 and SERCA, is enhanced as a protective mechanism[57]. A number of myofilament proteins are found to be S-nitrosylated[58] and a recent study on S-nitrosylation of sarcomeric proteins, including cMyBP-C, showed decreased calcium sensitivity, decreased maximal isometric force and prolonged relaxation following S-nitrosylation[59]. This study also revealed potential further S-nitrosylation sites in the C-terminal which warrant further investigation as these sites lies in the key domains involved in LMM[60] and titin[61] binding.

Understanding how these modifications regulate cMyBP-C function and/or phosphorylation would improve our understanding of how to enhance cMyBP-C phosphorylation for potential therapeutic gain. The redox modification that has shown the most promise in this respect is the S-glutathionylation of cMyBP-C, involving the reversible addition of a glutathione (GSH) molecule to the thiol of a cysteine residue via a disulphide bond. This modification plays a number of roles including altering protein-protein interactions and is implicated in cardiac pathophysiology with overall S-glutathionylation being increased following ischaemia/reperfusion[62,63]. An initial proteomic study using a biotinylated pulldown approach from rat hearts revealed cMyBP-C to be one of eleven S-glutathionylated proteins identified[64]. Interest in its functional significance developed after one group showed enhanced glutathionylation of cMyBP-C in a DOCA-salt model of hypertensive stress, and reversal of the oxidative stress led to improved diastolic function and increased cross bridge formation[65,66]. A follow up study identified three S-glutathionylated cysteines in the lesser studied central C3-C5 region of the protein (C479, C627, C655; mouse sequence) and later an additional cluster of cysteines in the N-terminus of the protein were identified. Importantly, this studied demonstrated enhanced glutathionylation of cMyBP-C in human samples from ischaemic and dilated cardiomyopathy and showed that S-glutathionylation, at C249 in particular, negatively regulates cMyBP-C PKA and CAMKII mediated phosphorylation, leading to enhanced myosin S2 binding and reduced cross-bridge kinetics[63]. Although C249 was highlighted as a crucial S-glutathionylation site, this study revealed several other sites spread throughout the protein, the functional significance of which is yet to be determined.

Lesser Known Modifications and cMyBP-C

Aside from redox modifications, advances in mass spectrometry have led to the identification of a number of lesser known modifications of cMyBP-C. Acetylation, the reversible addition of an acetyl group to a lysine residue, controlled by histone acetyltransferase and deacetylases, has been shown to target several cardiac substrates. In terms of the myofilament, a seminal report identified sarcomere-associated acetylation enzymes and showed that enhanced acetylation increased myofilament contractility and calcium sensitivity[67]. A later study highlighted that inhibition of myosin heavy chain deacetylation led to enhanced

ATPase activity and contractility dynamics[68]. Acetylation of cMyBP-C was first observed following high-resolution mass spectrometry with sites later confirmed in vivo, including five sites located in the N-terminal region[69,70]. Interestingly, the cleaved cMyBP-C product, found following myocardial infarction and in heart failure, is heavily acetylated, however the functional consequences and whether acetylation is part of the pathogenic mechanism is yet to be determined[70]. A proteomic study has also led to the discovery of cMyBP-C R696 citrullination, the irreversible, enzymatic conversion of an arginine residue to citrulline, often leading to loss of protein structure and protein-protein interactions[71]. Subsequent investigation into the functional consequences of citrullination on actin, troponin, tropomyosin and myosin indicated that increased citrullination resulted in decreased acto-myosin activity and reduced calcium sensitivity in skinned cardiomyocytes, however cMyBP-C was not looked at specifically[72].

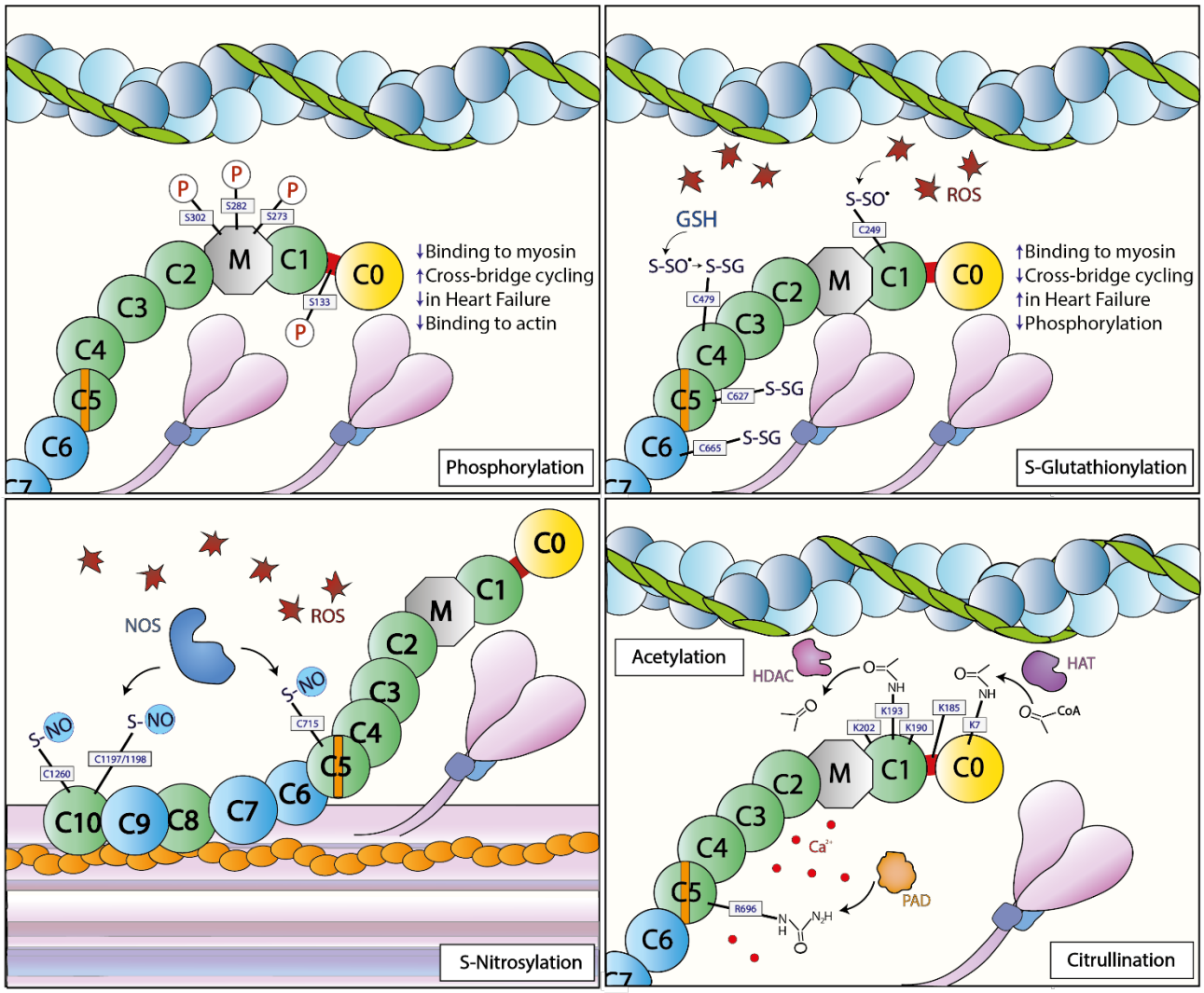

Figure 5: Post-translational Modification of Cardiac Myosin Binding Protein-C. Cardiac myosin binding protein-C (cMyBP-C) can undergo phosphorylation at three serines (S273, S282 and S302, mouse sequence) in the M-domain and one site in the proline/alanine rich linker, the overall effect of which is a reduction in the inhibition of myosin-actin cross-bridge formation by limiting cMyBP-C binding to both actin and myosin, allowing enhanced cross-bridge formation and positive inotropy. Phosphorylation of cMyBP-C is reduced in heart failure and preservation is thought to be beneficial. Reactive oxygen species (ROS) induced S-glutathionylation has been implicated at many sites throughout the protein, including three sites in the central region (C478, C627, C665) and one in the C1 domain (C249). S-glutathionylation increases binding to myosin S2 to reduce cross-bridge formation and is increased in heart failure and found to negatively regulate cMyBP-C phosphorylation. S-nitrosylation, a similar post-translational modification involving the addition of nitric oxide (NO) produced by nitric oxide synthase (NOS) to the thiol of a cysteine residue, has been reported at several sites in the C10 domain (highlighted are C1260 and C1197/1198) involved in light meromyosin and titin binding, and also in the C5 domain (C715), although these sites have not been confirmed in-vivo and the specific functional consequences are not understood. Acetylation, involving the addition of an acetyl group via a histone acetyltransferase (HAT), has been observed at several sites in cMyBP-C including a cluster in the C0-C1 region (K7, K185, K190, K193, K202). The modification is reversible by histone deacetylases (HDAC). Citrullination, the irreversible conversion of an arginine

to a citrulline mediated via peptidyl arginine deiminase (PAD) and calcium (Ca^{2+}), was identified at 696 in the C5 domain using mass spectrometry, but functionally remain to be investigated.

Future Perspectives

There is no doubt that cMyBP-C regulation by PTMs is complex, and the tools available to study these PTMs have been limited in recent years. Although fundamental for our understanding of phosphorylation site non-equivalency, a recent study suggests that serine to aspartate mutants, designed to represent constitutive phosphorylation, do not fully represent the effect of PKA phosphorylation in vivo[73]. However, transgenic animals such as these do allow a much more complete picture of the phosphorylation-dependent control of cMyBP-C compared to in vitro studies, which lack the ability to recapitulate the complex kinase profile of cMyBP-C and all the binding partners associated with its function. In that respect, a significant limitation in the study of cMyBP-C PTM function in general has been the disconnect between in-vitro studies, often using recombinant full length or particular domains of cMyBP-C, and these expensive in-vivo cMyBP-C transgenic models studying one site at a time. Recently, the Harris lab has engineered a transgenic "cut and paste" mouse model where C0-C7 domains of cMyBP-C can be selectively cleaved in permeabilise myocytes and replaced with recombinant C0-C7, modified in a number of ways. This powerful technique has already confirmed the functional role of the N-terminal domains in cross-bridge cycling and myofilament calcium sensitivity, as well as the role phosphorylation plays in these processes, and provides a unique system to study the effect of any modification in a more physiologically relevant system[74].

Now that significant advancements have been made in understanding cMyBP-C regulation in both healthy and diseased cardiac tissue, the question of its suitability as a target for treatment of cardiac diseases, such as heart failure, is at the forefront. By far the most progress has been in the study of HCM and viral replacement of cMyBP-C as a therapeutic strategy, with success in ameliorating the HCM phenotype and improving cardiac function in small animal models showing promising results[75,76]. In terms of non-heritable disease, there is now clear evidence of cMyBP-C dysregulation, particularly that of phosphorylation, in hearts of patients with heart failure[36,37], hypertension[77] and atrial fibrillation[78], and it is clear that preservation of phosphorylation in particular may represent a therapeutic strategy. Currently, work in small animal models shows good translational potential, for example the evidence that S282 (mouse sequence) is the most important site in animals and the site that displays the most decreased in phosphorylation in humans[40]. However, progress in modulating cMyBP-C phosphorylation therapeutically may be hindered by the complex phosphorylation profile of the protein. Additionally, targeting individual kinases and phosphatases clinically to treat cardiovascular disease may be limited due to off target effects.

As such, understanding potentially indirect routes to modify cMyBP-C phosphorylation through other PTMs may be beneficial in advancing therapeutics. A limitation that does remain in addressing PTM complexity is that producing single point mutants to study a modification in isolation may inadvertently impact other cMyBP-C PTMs. As has been demonstrated, there is clear correlation between cMyBP-C phosphorylation and S-glutathionylation which was not previously considered in early phosphorylation work[63], and further study has highlighted the co-ordinated action of the adrenergic and oxidative pathways in cardiac stress[79]. Additionally, there is a wealth of information emerging on how PTMs may influence each other, including the studies showing S-nitrosylation[80] and acetylation[81,82] cross-talk with phosphorylation in the regulation of cardiac substrates. This limitation is especially relevant for mutants of versatile amino acids, such as reactive cysteine thiols, that can undergo multiple modifications, such as S-glutathionylation, S-nitrosylation and S-palmitoylation, as well as forming inter- and intra- protein disulphides. To fully understand cMyBP-C PTM control, it is imperative that sites are confirmed in vivo to gain a more complete understanding of the functional significance of the modifications, and fortunately, the advancement in cMyBP-C and PTM mass spectrometry and imaging in recent years will help to build a more complete profile.

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Conflict of Interest

The authors declare no conflicts of interest.

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References

1. Starr, R. & Offer, G. Polypeptide chains of intermediate molecular weight in myosin preparations. *FEBS Lett.* **15**, 40–44 (1971).

2. Craig, R. & Offer, G. The location of C protein in rabbit skeletal muscle. *Proc. R. Soc. London - Biol. Sci.* **192**, 451–461 (1976).

3. Moos, C., Offer, G., Starr, R. & Bennett, P. Interaction of C-protein with myosin, myosin rod and light meromyosin. *J. Mol. Biol.* **97**, (1975).

4. Pfuhl, M. & Gautel, M. Structure, interactions and function of the N-terminus of cardiac myosin binding protein C (MyBP-C): Who does what, with what, and to whom? *J. Muscle Res. Cell Motil.* **33**, 83–94 (2012).

5. Weber, F. E., Vaughan, K. T., Reinach, F. C. & Fischman, D. A. Complete sequence of human fast-type and slow-type muscle myosin-binding-protein C (MyBP-C). Differential expression, conserved domain structure and chromosome assignment. *Eur. J. Biochem.* **216**, 661–669 (1993).

6. Fougerousse, F., Delezoide, A. L., Fiszman, M. Y., Schwartz, K., Beckmann, J. S. & Carrier, L. Cardiac myosin binding protein C gene is specifically expressed in heart during murine and human development. *Circ. Res.* **82**, 130–133 (1998).

7. Gilbert, R., Kelly, M. G., Mikawa, T. & Fischman, D. A. The carboxyl terminus of myosin binding protein C (MyBP-C, C-protein) specifies incorporation into the A-band of striated muscle. *J. Cell Sci.* **109**, 101–111 (1996).

8. Flashman, E., Redwood, C., Moolman-Smook, J. & Watkins, H. Cardiac myosin binding protein C: Its role in physiology and disease. *Circ. Res.* **94**, 1279–1289 (2004).

9. McNamara, J. W. & Sadayappan, S. Skeletal myosin binding protein-C: An increasingly important regulator of striated muscle physiology. *Arch. Biochem. Biophys.* **660**, 121–128 (2018).

10. Bonne, G., Carrier, L., Bercovici, J., Cruaud, C., Richard3, P., Hainque3, B., Gautel4, M., Labeit4, S., Jamess, M., Beckmann, J., Weissenbach, J., Vosberg, H.-P., Fiszman, M., Komajda, M. & Schwartz, K. *Cardiac myosin binding protein-C gene splice acceptor site mutation is associated with familial hypertrophic cardiomyopathy*. http://www.nature.com/naturegenetics (1995).

11. Volhard Klinik, F., Watkins, H., Conner, D., Thierfelder, L., Jarcho, J. A., MacRae, C., McKenna, W. J., MaronS, B. J., Seidman, J. & Seidman, C. E. Mutations in the cardiac myosin binding protein-C gene on chromosome 11 cause familial hypertrophic cardiomyopathy. (1995).

12. Semsarian, C., Ingles, J., Maron, M. S. & Maron, B. J. New perspectives on the prevalence of hypertrophic cardiomyopathy. *Journal of the American College of Cardiology* vol. 65 1249–1254 (2015).

13. Gruen, M. & Gautel, M. Mutations in β-myosin S2 that cause familial hypertrophic cardiomyopathy (FHC) abolish the interaction with the regulatory domain of myosin-binding protein-C. *J. Mol. Biol.*

286, 933–949 (1999).

14. Harris, S. P., Bartley, C. R., Hacker, T. A., McDonald, K. S., Douglas, P. S., Greaser, M. L., Powers, P. A. & Moss, R. L. Hypertrophic cardiomyopathy in cardiac myosin binding protein-C knockout mice. *Circ. Res.* **90**, 594–601 (2002).

15. Korte, F. S., McDonald, K. S., Harris, S. P. & Moss, R. L. Loaded Shortening, Power Output, and Rate of Force Redevelopment Are Increased With Knockout of Cardiac Myosin Binding Protein-C. *Circ. Res.* **93**, 752–758 (2003).

16. Stelzer, J. E., Fitzsimons, D. P. & Moss, R. L. Ablation of Myosin-Binding Protein-C Accelerates Force Development in Mouse Myocardium. *Biophys. J.* **90**, 4119–4127 (2006).

17. Harris, S. P., Rostkova, E., Gautel, M. & Moss, R. L. Binding of myosin binding protein-C to myosin subfragment S2 affects contractility independent of a tether mechanism. *Circ. Res.* **95**, 930–936 (2004).

18. Bhuiyan, M. S., McLendon, P., James, J., Osinska, H., Gulick, J., Bhandary, B., Lorenz, J. N. & Robbins, J. In vivo definition of cardiac myosin-binding protein C's critical interactions with myosin. *Pflügers Arch. - Eur. J. Physiol.* **468**, 1685–1695 (2016).

19. Kensler, R. W., Craig, R. & Moss, R. L. Phosphorylation of cardiac myosin binding protein C releases myosin heads from the surface of cardiac thick filaments. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E1355–E1364 (2017).

20. McNamara, J. W., Singh, R. R. & Sadayappan, S. Cardiac myosin binding protein-C phosphorylation regulates the super-relaxed state of myosin. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 11731–11736 (2019).

21. Moos, C., Mason, C. M., Besterman, J. M., Feng, I. N. M. & Dubin, J. H. The binding of skeletal muscle C-protein to F-actin, and its relation to the interaction of actin with myosin subfragment-1. *J. Mol. Biol.* **124**, 571–586 (1978).

22. Orlova, A., Galkin, V. E., Jeffries, C. M. J., Egelman, E. H. & Trewhella, J. The N-terminal domains of myosin binding protein C can bind polymorphically to F-actin. *J. Mol. Biol.* **412**, 379–386 (2011).

23. Kensler, R. W., Shaffer, J. F. & Harris, S. P. Binding of the N-terminal fragment C0-C2 of cardiac MyBP-C to cardiac F-actin. *J. Struct. Biol.* **174**, 44–51 (2011).

24. Previs, M. J., Beck Previs, S., Gulick, J., Robbins, J. & Warshaw, D. M. Molecular mechanics of cardiac myosin-binding protein C in native thick filaments. *Science (80-.).* **337**, 1215–1218 (2012).

25. Razumova, M. V., Shaffer, J. F., Tu, A. Y., Flint, G. V., Regnier, M. & Harris, S. P. Effects of the N-terminal domains of myosin binding protein-C in an in vitro motility assay: Evidence for long-lived cross-bridges. *J. Biol. Chem.* **281**, 35846–35854 (2006).

26. Mun, J. Y., Previs, M. J., Yu, H. Y., Gulick, J., Tobacman, L. S., Previs, S. B., Robbins, J., Warshaw, D. M. & Craig, R. Myosin-binding protein C displaces tropomyosin to activate cardiac thin filaments and governs their speed by an independent mechanism. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 2170–2175 (2014).

27. van Dijk, S. J., Kooiker, K. B., Napierski, N. C., Touma, K. D., Mazzalupo, S. & Harris, S. P. Point mutations in the tri-helix bundle of the M-domain of cardiac myosin binding protein-C influence systolic duration and delay cardiac relaxation. *J. Mol. Cell. Cardiol.* **119**, 116–124 (2018).

28. Solaro, R. J. Multiplex kinase signaling modifies cardiac function at the level of sarcomeric proteins. *Journal of Biological Chemistry* vol. 283 26829–26833 (2008).

29. Hartzell, H. C. & Titus, L. Effects of cholinergic and adrenergic agonists on phosphorylation of a 165,000-dalton myofibrillar protein in intact cardiac muscle. *J. Biol. Chem.* **257**, 2111–2120 (1982).

30. Gautel, M., Zuffardi, O., Freiburg, A. & Labeit, S. Phosphorylation switches specific for the cardiac

isoform of myosin binding protein-C: a modulator of cardiac contraction? *EMBO J.* **14**, 1952–1960 (1995).

31. Shaffer, J. F., Kensler, R. W. & Harris, S. P. The myosin-binding protein C motif binds to F-actin in a phosphorylation-sensitive manner. *J. Biol. Chem.* **284**, 12318–12327 (2009).

32. Tong, C. W., Nair, N. A., Doersch, K. M., Liu, Y. & Rosas, P. C. Cardiac myosin-binding protein-C is a critical mediator of diastolic function. *Pflugers Arch. Eur. J. Physiol.* **466**, 451–457 (2014).

33. Colson, B. A., Patel, J. R., Chen, P. P., Bekyarova, T., Abdalla, M. I., Tong, C. W., Fitzsimons, D. P., Irving, T. C. & Moss, R. L. Myosin binding protein-C phosphorylation is the principal mediator of protein kinase A effects on thick filament structure in myocardium. *J. Mol. Cell. Cardiol.* **53**, 609–616 (2012).

34. Sadayappan, S., Gulick, J., Osinska, H., Martin, L. A., Hahn, H. S., Dorn, G. W., Klevitsky, R., Seidman, C. E., Seidman, J. G. & Robbins, J. Cardiac Myosin-Binding Protein-C Phosphorylation and Cardiac Function. *Circ. Res.* **97**, 1156–1163 (2005).

35. Sadayappan, S., Osinska, H., Klevitsky, R., Lorenz, J. N., Sargent, M., Molkentin, J. D., Seidman, C. E., Seidman, J. G. & Robbins, J. Cardiac myosin binding protein C phosphorylation is cardioprotective. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 16918–23 (2006).

36. El-Armouche, A., Pohlmann, L., Schlossarek, S., Starbatty, J., Yeh, Y.-H., Nattel, S., Dobrev, D., Eschenhagen, T. & Carrier, L. Decreased phosphorylation levels of cardiac myosin-binding protein-C in human and experimental heart failure. *J. Mol. Cell. Cardiol.* **43**, 223–229 (2007).

37. Decker, R. S., Decker, M. L., Kulikovskaya, I., Nakamura, S., Lee, D. C., Harris, K., Klocke, F. J. & Winegrad, S. Myosin-binding protein C phosphorylation, myofibril structure, and contractile function during low-flow ischemia. *Circulation* **111**, 906–912 (2005).

38. Rosas, P. C., Warren, C. M., Creed, H. A., Trzeciakowski, J. P., Solaro, R. J. & Tong, C. W. Cardiac Myosin Binding Protein-C Phosphorylation Mitigates Age-Related Cardiac Dysfunction: Hope for Better Aging? *JACC Basic to Transl. Sci.* **4**, 817–830 (2019).

39. Kumar, M., Haghighi, K., Kranias, E. G. & Sadayappan, S. Phosphorylation of cardiac myosin-binding protein-C contributes to calcium homeostasis. *J. Biol. Chem.* **295**, 11275–11291 (2020).

40. Copeland, O., Sadayappan, S., Messer, A. E., Steinen, G. J. M., Van der Velden, J. & Marston, S. B. Analysis of cardiac myosin binding protein-C phosphorylation in human heart muscle. *J. Mol. Cell. Cardiol.* **49**, 1003–1011 (2010).

41. Sadayappan, S., Gulick, J., Osinska, H., Barefield, D., Cuello, F., Avkiran, M., Lasko, V. M., Lorenz, J. N., Maillet, M., Martin, J. L., Brown, J. H., Bers, D. M., Molkentin, J. D., James, J. & Robbins, J. A Critical Function for Ser-282 in Cardiac Myosin Binding Protein-C Phosphorylation and Cardiac Function. *Circ. Res.* **109**, 141–150 (2011).

42. Gupta, M. K., Gulick, J., James, J., Osinska, H., Lorenz, J. N. & Robbins, J. Functional dissection of myosin binding protein C phosphorylation. *J. Mol. Cell. Cardiol.* **64**, 39–50 (2013).

43. Gupta, M. K. & Robbins, J. Post-Translational Control of Cardiac Hemodyanmics Through Myosin Binding Protein C. *Pflungers Arch.* **466**, 231–236 (2014).

44. Pyle, W. G., Chen, Y. & Hofmann, P. A. Cardioprotection through a PKC-dependent decrease in myofilament ATPase. *Am. J. Physiol. Circ. Physiol.* **285**, H1220–H1228 (2003).

45. Bardswell, S. C., Cuello, F., Rowland, A. J., Sadayappan, S., Robbins, J., Gautel, M., Walker, J. W., Kentish, J. C. & Avkiran, M. Distinct Sarcomeric Substrates Are Responsible for Protein Kinase D-mediated Regulation of Cardiac Myofilament Ca $^{2+}$ Sensitivity and Cross-bridge Cycling. *J. Biol. Chem.* **285**, 5674–5682 (2010).

46. Ponnam, S., Sevrieva, I., Sun, Y.-B., Irving, M. & Kampourakis, T. Site-specific phosphorylation of myosin binding protein-C coordinates thin and thick filament activation in cardiac muscle. *Proc. Natl. Acad. Sci.* **116**, 15485–15494 (2019).

47. Tong, C. W., Wu, X., Liu, Y., Rosas, P. C., Sadayappan, S., Hudmon, A., Muthuchamy, M., Powers, P. A., Valdivia, H. H. & Moss, R. L. Phosphoregulation of Cardiac Inotropy via Myosin Binding Protein-C During Increased Pacing Frequency or β_1 -Adrenergic Stimulation. *Circ. Hear. Fail.* **8**, 595–604 (2015).

48. Cuello, F., Bardswell, S. C., Haworth, R. S., Ehler, E., Sadayappan, S., Kentish, J. C. & Avkiran, M. Novel Role for p90 Ribosomal S6 Kinase in the Regulation of Cardiac Myofilament Phosphorylation. *J. Biol. Chem.* **286**, 5300–5310 (2011).

49. Kooij, V., Holewinski, R. J., Murphy, A. M. & Van Eyk, J. E. Characterization of the cardiac myosin binding protein-C phosphoproteome in healthy and failing human hearts. *J. Mol. Cell. Cardiol.* **60**, 116–20 (2013).

50. Lundby, A., Andersen, M. N., Steffensen, A. B., Horn, H., Kelstrup, C. D., Francavilla, C., Jensen, L. J., Schmitt, N., Thomsen, M. B. & Olsen, J. V. In vivo phosphoproteomics analysis reveals the cardiac targets of β-adrenergic receptor signaling. *Sci. Signal.* **6**, rs11–rs11 (2013).

51. Jia, W., Shaffer, J. F., Harris, S. P. & Leary, J. A. Identification of Novel Protein Kinase A Phosphorylation Sites in the M-domain of Human and Murine Cardiac Myosin Binding Protein-C Using Mass Spectrometry Analysis. *J. Proteome Res.* **9**, 1843–1853 (2010).

52. Kuster, D. W. D., Sequeira, V., Najafi, A., Boontje, N. M., Wijnker, P. J. M., Rosalie Witjas-Paalberends, E., Marston, S. B., Dos Remedios, C. G., Carrier, L., Demmers, J. A. A., Redwood, C., Sadayappan, S. & Van Der Velden, J. GSK3β phosphorylates newly identified site in the proline-alanine-rich region of cardiac myosin-binding protein C and alters cross-bridge cycling kinetics in human: Short communication. *Circ. Res.* **112**, 633–639 (2013).

53. Brioschi, M., Polvani, G., Fratto, P., Parolari, A., Agostoni, P., Tremoli, E. & Banfi, C. Redox proteomics identification of oxidatively modified myocardial proteins in human heart failure: Implications for protein function. *PLoS One* **7**, (2012).

54. Tian, C., Alomar, F., Moore, C. J., Shao, C. H., Kutty, S., Singh, J. & Bidasee, K. R. Reactive carbonyl species and their roles in sarcoplasmic reticulum Ca 2+ cycling defect in the diabetic heart. *Heart Fail. Rev.* **19**, 101–112 (2014).

55. Aryal, B., Jeong, J. & Rao, V. A. Doxorubicin-induced carbonylation and degradation of cardiac myosin binding protein C promote cardiotoxicity. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 2011–2016 (2014).

56. Kohr, M. J., Aponte, A. M., Sun, J., Wang, G., Murphy, E., Gucek, M. & Steenbergen, C. Characterization of potential S-nitrosylation sites in the myocardium. *Am. J. Physiol. Heart Circ. Physiol.* **300**, H1327-35 (2011).

57. Lima, B., Forrester, M. T., Hess, D. T. & Stamler, J. S. S-nitrosylation in cardiovascular signaling. *Circulation Research* vol. 106 633–646 (2010).

58. Horenberg, A. L., Houghton, A. M., Pandey, S., Seshadri, V. & Guilford, W. H. S-nitrosylation of cytoskeletal proteins. *Cytoskeleton* **76**, 243–253 (2019).

59. Figueiredo-Freitas, C., Dulce, R. A., Foster, M. W., Liang, J., Yamashita, A. M. S., Lima-Rosa, F. L., Thompson, J. W., Moseley, M. A., Hare, J. M., Nogueira, L., Sorenson, M. M. & Pinto, J. R. S-Nitrosylation of Sarcomeric Proteins Depresses Myofilament Ca2+ Sensitivity in Intact Cardiomyocytes. *Antioxidants Redox Signal.* **23**, 1017–1034 (2015).

60. Flashman, E., Watkins, H. & Redwood, C. Localization of the binding site of the C-terminal domain of

cardiac myosin-binding protein-C on the myosin rod. *Biochem. J.* **401**, 97–102 (2007).

61. Tonino, P., Kiss, B., Gohlke, J., Smith, J. E. & Granzier, H. Fine mapping titin's C-zone: Matching cardiac myosin-binding protein C stripes with titin's super-repeats. *J. Mol. Cell. Cardiol.* **133**, 47–56 (2019).

62. Eaton, P., Wright, N., Hearse, D. J. & Shattock, M. J. Glyceraldehyde phosphate dehydrogenase oxidation during cardiac ischemia and reperfusion. *J. Mol. Cell. Cardiol.* **34**, 1549–1560 (2002).

63. Stathopoulou, K., Wittig, I., Heidler, J., Piasecki, A., Richter, F., Diering, S., van der Velden, J., Buck, F., Donzelli, S., Schröder, E., Wijnker, P. J. M., Voigt, N., Dobrev, D., Sadayappan, S., Eschenhagen, T., Carrier, L., Eaton, P. & Cuello, F. S-glutathiolation impairs phosphoregulation and function of cardiac myosin-binding protein C in human heart failure. *FASEB J.* **30**, 1849–64 (2016).

64. Brennan, J. P., Miller, J. I. A., Fuller, W., Wait, R., Begum, S., Dunn, M. J. & Eaton, P. The utility of N,N-biotinyl glutathione disulfide in the study of protein S-glutathiolation. *Mol. Cell. Proteomics* **5**, 215–225 (2006).

65. Lovelock, J. D., Monasky, M. M., Jeong, E. M., Lardin, H. A., Liu, H., Patel, B. G., Taglieri, D. M., Gu, L., Kumar, P., Pokhrel, N., Zeng, D., Belardinelli, L., Sorescu, D., Solaro, R. J. & Dudley, S. C. Ranolazine improves cardiac diastolic dysfunction through modulation of myofilament calcium sensitivity. *Circ. Res.* **110**, 841–850 (2012).

66. Jeong, E. M., Monasky, M. M., Gu, L., Taglieri, D. M., Patel, B. G., Liu, H., Wang, Q., Greener, I., Dudley, S. C. & Solaro, R. J. Tetrahydrobiopterin improves diastolic dysfunction by reversing changes in myofilament properties. *J. Mol. Cell. Cardiol.* **56**, 44–54 (2013).

67. Gupta, M. P., Samant, S. A., Smith, S. H. & Shroff, S. G. HDAC4 and PCAF bind to cardiac sarcomeres and play a role in regulating myofilament contractile activity. *J. Biol. Chem.* **283**, 10135–10146 (2008).

68. Samant, S. A., Pillai, V. B., Sundaresan, N. R., Shroff, S. G. & Gupta, M. P. Histone deacetylase 3 (HDAC3)-dependent reversible lysine acetylation of cardiac myosin heavy chain isoforms modulates their enzymatic and motor activity. *J. Biol. Chem.* **290**, 15559–15569 (2015).

69. Ge, Y., Rybakova, I. N., Xu, Q. & Moss, R. L. Top-down high-resolution mass spectrometry of cardiac myosin binding protein C revealed that truncation alters protein phosphorylation state. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 12658–63 (2009).

70. Govindan, S., Sarkey, J., Ji, X., Sundaresan, N. R., Gupta, M. P., de Tombe, P. P. & Sadayappan, S. Pathogenic properties of the N-terminal region of cardiac myosin binding protein-C in vitro. *J. Muscle Res. Cell Motil.* **33**, 17–30 (2012).

71. Fert-Bober, J. & Sokolove, J. Proteomics of citrullination in cardiovascular disease. *PROTEOMICS - Clin. Appl.* **8**, 522–533 (2014).

72. Fert-Bober, J., Giles, J. T., Holewinski, R. J., Kirk, J. A., Uhrigshardt, H., Crowgey, E. L., Andrade, F., Bingham, C. O., Park, J. K., Halushka, M. K., Kass, D. A., Bathon, J. M., Van Eyk, J. E. & Eyk, J. E. Van. Citrullination of myofilament proteins in heart failure. *Cardiovasc. Res.* **108**, 232–42 (2015).

73. Kampourakis, T., Ponnam, S., Sun, Y. B., Sevrieva, I. & Irving, M. Structural and functional effects of myosin-binding protein-C phosphorylation in heart muscle are not mimicked by serine-to-aspartate substitutions. *J. Biol. Chem.* **293**, 14270–14275 (2018).

74. Napierski, N. C., Granger, K., Langlais, P. R., Moran, H. R., Strom, J., Touma, K. & Harris, S. P. A Novel "Cut and Paste" Method for In Situ Replacement of cMyBP-C Reveals a New Role for cMyBP-C in the Regulation of Contractile Oscillations. *Circ. Res.* **126**, 737–749 (2020).

75. Li, J., Mamidi, R., Doh, C. Y., Holmes, J. B., Bharambe, N., Ramachandran, R. & Stelzer, J. E. AAV9 gene transfer of cMyBPC N-terminal domains ameliorates cardiomyopathy in cMyBPC deficient

mice. *JCI Insight* **5**, (2020).

76. Merkulov, S., Chen, X., Chandler, M. P. & Stelzer, J. E. In vivo cardiac myosin binding protein C gene transfer rescues myofilament contractile dysfiunction in cardiac myosin binding protein C null mice. *Circ. Hear. Fail.* **5**, 635–644 (2012).

77. Donaldson, C., Palmer, B. M., Zile, M., Maughan, D. W., Ikonomidis, J. S., Granzier, H., Meyer, M., VanBuren, P. & LeWinter, M. M. Myosin cross-bridge dynamics in patients with hypertension and concentric left ventricular remodeling. *Circ. Hear. Fail.* **5**, 803–811 (2012).

78. El-Armouche, A., Boknik, P., Eschenhagen, T., Carrier, L., Knaut, M., Ravens, U. & Dobrev, D. Molecular determinants of altered Ca2+ handling in human chronic atrial fibrillation. *Circulation* **114**, 670–680 (2006).

79. Chakouri, N., Reboul, C., Boulghobra, D., Kleindienst, A., Nottin, S., Gayrard, S., Roubille, F., Matecki, S., Lacampagne, A. & Cazorla, O. Stress-induced protein S-glutathionylation and phosphorylation crosstalk in cardiac sarcomeric proteins - Impact on heart function. *Int. J. Cardiol.* **258**, 207–216 (2018).

80. Irie, T., Sips, P. Y., Kai, S., Kida, K., Ikeda, K., Hirai, S., Moazzami, K., Jiramongkolchai, P., Bloch, D. B., Doulias, P.-T., Armoundas, A. A., Kaneki, M., Ischiropoulos, H., Kranias, E., Bloch, K. D., Stamler, J. S. & Ichinose, F. S-Nitrosylation of Calcium-Handling Proteins in Cardiac Adrenergic Signaling and Hypertrophy. *Circ. Res.* **117**, 793–803 (2015).

81. Habibian, J. & Ferguson, B. S. The crosstalk between acetylation and phosphorylation: Emerging new roles for HDAC inhibitors in the heart. *International Journal of Molecular Sciences* vol. 20 (2019).

82. Parker, B. L., Shepherd, N. E., Trefely, S., Hoffman, N. J., White, M. Y., Engholm-Keller, K., Hambly, B. D., Larsen, M. R., James, D. E. & Cordwell, S. J. Structural basis for phosphorylation and lysine acetylation cross-talk in a kinase motif associated with myocardial ischemia and cardioprotection. *J. Biol. Chem.* **289**, 25890–25906 (2014).