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REVIEW

Post-translational modifications of cardiac myosin-binding protein-C: Mechanisms behind fine-tuning the sarcomere

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Since the discovery of the cardiac isoform of myosin-binding protein-C (cMyBP-C), there has been continued interest in how cMyBP-C impacts cardiac function in both health and disease. cMyBP-C is a regulatory protein in the sarcomere that controls beat-to-beat changes in contractility in response to dynamic environmental demands placed upon the heart. Changes in force production during the contractile cycle are modulated through interactions of cMyBP-C with myosin and actin. Post-translational modifications (PTMs) of cMyBP-C, of which phosphorylation has received the most attention, are critical to the function of cMyBP-C in the healthy heart and is affected in many disease states. While each of the PTMs that will be discussed in this review have known and often widespread effects on important cellular processes spanning transcriptional regulation, cell signaling, and metabolism, their impact on cMyBP-C function remains poorly understood and in some cases unverified. This Review focuses on the current understanding of cMyBP-C PTMs, namely phosphorylation, S-glutathionylation, S-nitrosylation, acetylation, citrullination, carbonylation, and O-GlcNAcylation. The potential for PTMs to exert wide ranging and likely nuanced effects may influence the range of cMyBP-C's response to varied conditions and may offer opportunities to identify novel therapeutic paradigms in the setting of disease.

Cardiac myosin-binding protein-C

Cardiac myosin-binding protein-C (cMyBP-C) encoded by the *MYBPC3* gene is a 140-kDa protein comprised of eight immunoglobulin-like domains and three fibronectin type-III domains. The domains are arranged from C0 through C10, with a disorganized fragment between C1 and C2 referred to as the regulatory motif (i.e., M-domain) (Fig. 1). The M-domain of the cardiac isoform contains the identified amino acid residues that undergo phosphorylation and dynamically regulate cardiac contractility (Sadayappan et al., 2005). cMyBP-C is localized to the C-zone of the sarcomere, where it is anchored to light meromyosin and can be visualized as a regular pattern of nine stripes 43 nm apart (Dutta et al., 2023; Lee et al., 2015; Tamborrini et al., 2023). It is well-established that cMyBP-C binds to myosin subfragment-1 and subfragment-2 (Gruen and Gautel, 1999; Kunst et al., 2000; Ratti et al., 2011; Ponnam et al., 2019), which is predicted to restrict myosin head movement and therefore limit the ability of myosin to form a crossbridge with actin (Kampourakis et al., 2014). This theory is supported by more recent evidence for cMyBP-C assisting in restraining myosin heads in the so-called interacting heads motif (IHM) conformation and the lower energy consuming biochemical conformation referred to as the super-relaxed (SRX) state (McNamara et al., 2016; Nag et al., 2017; Nelson et al., 2023). cMyBP-C has also been shown to bind to actin, shifting the position of tropomyosin and exposing myosin-binding sites on actin, independent of calcium-induced changes in the troponin complex (Inchingolo et al., 2019; Kensler et al., 2011; Mun et al., 2014; Mun et al., 2016; Whitten et al., 2008). cMyBP-C binding to actin is predicted to sensitize the thin filament to calcium and promote actin-myosin crossbridges, which may be particularly relevant at lower calcium concentrations (Inchingolo et al., 2019; Mun et al., 2014; Mun et al., 2016). The cMyBP-C–actin interactions are generally considered to be activating, but may also play an additional role in the sarcomere through viscoelastic drag on contractility (Colson, 2019; Sanematsu et al., 2021; Robinett et al., 2019). Overall, the consequences of losing functional cMyBP-C from the sarcomere reduces myofilament calcium sensitivity and accelerates crossbridge cycling kinetics during both contraction and relaxation (Napierski et al., 2020; Tong et al., 2008; Stelzer et al., 2006; Stelzer et al., 2007; de Lange et al., 2012; Korte et al., 2003; Palmer et al., 2004; Harris et al., 2002; Dvornikov and Harris, 2025). These data support the hypothesis that cMyBP-C modulates sarcomere function by both assisting in activating the thin filament and by serving as a break on the thick filament, i.e., increasing the proportion of the myosin heads in the non-crossbridge forming IHM or SRX conformation. How and when these different

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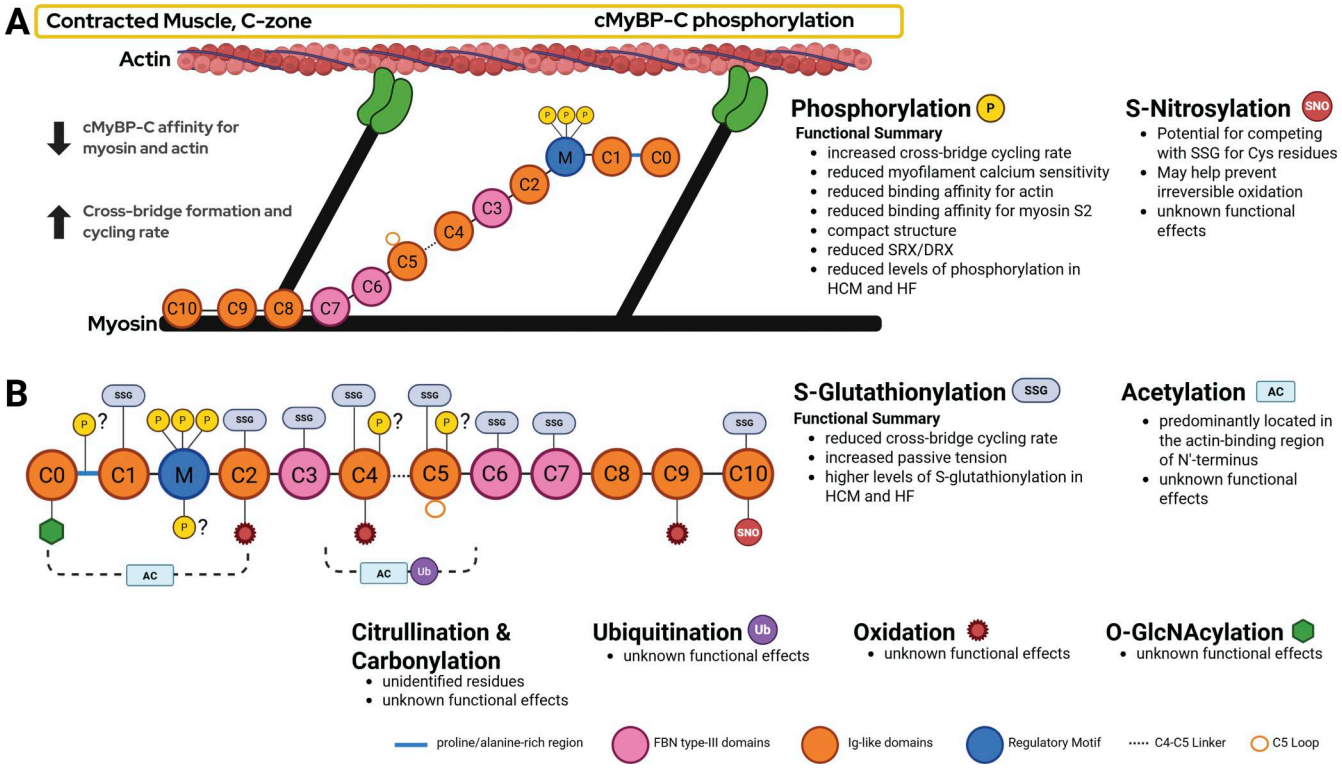

Figure 1. **Distribution of known post-translational modifications across cMyBP-C. (A)** Illustration demonstrating how cMyBP-C incorporates with myosin and actin, modulated by phosphorylation of the regulatory motif (M). **(B)** Visualization of the individual domains of cMyBP-C, highlighting where specific post-translational modifications occur across the protein. S-glutathionylation of cMyBP-C has been shown to reduce rates of crossbridge cycling and increase passive tension in permeabilized heart preparations. Elevated levels of cMyBP-C S-glutathionylation are found in hypertrophic cardiomyopathy (HCM) and heart failure (HF), whereas phosphorylation of cMyBP-C tends to be reduced under the same conditions, suggesting a potential for cross talk between these two modifications. Acetylation and S-nitrosylation alter sarcomere function, but it remains to be determined if these modifications of cMyBP-C are specifically responsible for altered heart function. Individual PTM sites have been discovered for oxidation, O-GlcNAcylation, ubiquitination, but with unknown functional consequences. Global cMyBP-C citrullination and carbonylation have been confirmed, but with unknown functional effects. ? demonstrates where uncertainty in the literature exists. Figure made using BioRender.

cMyBP-C interactions occur, particularly *in vivo*, are not well understood, but phosphorylation, and potentially other PTMs appear to be key regulators.

The conformation of cMyBP-C in the sarcomere is dynamic and dependent upon intra- and inter-domain interactions, which may be impacted by PTMs, in reversable or irreversible ways. In addition to the well-studied phosphorylation, data mainly derived from *in vitro* studies also suggest that S-glutathionylation, S-nitrosylation, acetylation, carbonylation, citrullination, O-GlcNAcylation, oxidation, and ubiquitination may be relevant (Fig. 1). These PTMs may play critical and quite varied roles in modifying cMyBP-C function during both health and disease.

Phosphorylation of cMyBP-C regulates myofilament function

Phosphorylation fundamentally alters protein structure and function through an enzymatic addition of a phosphate group, typically to serine (Ser), threonine (Thr), or tyrosine (Tyr) residues. This introduces a negatively charged moiety, which can disrupt existing electrostatic interactions, induce conformational changes, and modulate protein–protein interactions (Nishi et al., 2014). Phosphorylation of cMyBP-C has been extensively reviewed previously (Barefield and Sadayappan, 2010; Main et al., 2020); however, a brief summary of the effects of cMyBP-C phosphorylation on sarcomere function in health and disease is provided below.

Overview of cMyBP-C phosphorylation

The phosphorylation of cMyBP-C, first identified in the frog heart (Hartzell and Titus, 1982), reduces its binding affinity to both myosin and actin (Kunst et al., 2000; Shaffer et al., 2009), accelerates crossbridge kinetics (Tong et al., 2008; Tong et al., 2014; Colson et al., 2012), reduces the proportion of myosin heads in the SRX state (McNamara et al., 2019), and ultimately increases contractility (Moss et al., 2015). PKA phosphorylates cMyBP-C causing myosin heads to shift away from the thick filament backbone and closer to actin (Colson et al., 2008). These data led to the conclusion that PKA phosphorylation of cMyBP-C relieves its constraint on myosin, enhancing actin-myosin proximity and the probability of crossbridge formation (Moss et al., 2015).

There is current consensus that three main, or functionally relevant, phosphorylation sites on human cMyBP-C are located within the M-domain: Ser residues 275, 284, and 304 (corresponding to Ser273, Ser282, and Ser302 in mouse) (Barefield and Sadayappan, 2010; Gautel et al., 1995). While all three sites can

be phosphorylated by PKA, each site is also preferentially targeted by kinase and phosphatase enzymes variants, which may occur in a hierarchical manner (Sadayappan et al., 2011; Tong et al., 2015). The observed lack of equivalent effects on individual phospho-sites is likely multifactorial, but may reflect residue-specific influences on kinase and phosphatase activity (Main et al., 2020; Kampourakis et al., 2024). The phosphorylation of individual Ser residues in response to specific physiologic and pathophysiologic conditions plays an important role in modulating cMyBP-C function.

Role of cMyBP-C phosphorylation in cardiovascular health

The role of cMyBP-C phosphorylation in overall cardiac health has been identified through the study of mouse models with targeted amino acid substitutions to generate phosphomimetic (Ser to aspartate [Asp]) or phospho-ablated cMyBP-C (Ser to alanine [Ala]). In a phosphorylation-ablated mouse model, substituting cMyBP-C phosphorylation sites Ser275, Ser284, and Ser305 for Ala residues led to elevated stress markers in the transcriptome, altered sarcomere structure, and depressed contractility, leading to the conclusion that basal levels of cMyBP-C phosphorylation are critical to normal heart function (Sadayappan et al., 2005). Expression of phosphomimetic cMyBP-C led to a reduction in myosin–cMyBP-C interactions, normalization in fractional shortening, and protection from ischemic injury (Sadayappan et al., 2006). Cardiac muscle samples from human patients with heart failure and HCM reveal a reduction or absence of cMyBP-C phosphorylation, a finding also noted in rodent models of cardiac disease, suggesting that this PTM is important for normal sarcomere function (Barefield and Sadayappan, 2010; van Dijk et al., 2009; Copeland et al., 2010; El-Armouche et al., 2007; Jacques et al., 2008). Restoration of cMyBP-C phosphorylation with Ser304 peptides in papillary muscle preparations from failing hearts improved adenosine triphosphatase (ATPase) activity and contractility, suggesting that disrupting myosin inhibition by the phosphorylation of cMyBP-C could improve cardiac performance through increased crossbridge formation (Hou et al., 2022). Taken together, the studies in animal models and human heart tissue indicate that the phosphorylation of the M-domain is critical to normal cardiac function and may be a useful therapeutic target in states of disease.

Often neglected when considering the dynamic nature of cMyBP-C phosphorylation is the role of dephosphorylation. The two most prevalent phosphatases in the sarcomere are protein phosphatase 1 (PP1) and 2 (PP2) (Heijman et al., 2013). Both PP1 and PP2 dephosphorylated recombinant C1-M-C2 fragments of cMyBP-C, but at different rates (Kampourakis et al., 2024). Phosphorylation of Ser304 is the first to decline, followed by Ser275 and then Ser284, which is dephosphorylated at a significantly slower rate (Kampourakis et al., 2024). Notably, phosphatase activity increases in the failing heart (Pathak et al., 2005; Neumann et al., 1997) and may contribute to the observed decline in cMyBP-C phosphorylation in heart failure. One explanation may be due to less inhibition of PP1 by its inhibitor, phosphatase inhibitor-1, which is downregulated in heart failure (Grote-Wessels et al., 2008; Wittköpper et al., 2010; El-Armouche et al., 2004). Reducing phosphatase levels in murine models of heart failure has shown promise in preserving cardiac function (Belin de Chantemèle et al., 2009; Gogiraju et al., 2016; Gomez et al., 2012; Nicolaou et al., 2009). A role for oxidative stress through increases in reactive oxygen species (ROS) on PP1 and PP2 activity has been proposed, but remains controversial (Sommer et al., 2002; Pham et al., 2000). The role of ROS on cMyBP-C phosphorylation is discussed in detail below.

Potential other cMyBP-C phosphorylation sites

Additional residues on cMyBP-C can undergo phosphorylation, including Ser311 and Ser133, but their physiological relevance, if any, remains to be determined (Kuster et al., 2013; Kampourakis et al., 2024; Jia et al., 2010; Kooij et al., 2013). Recently, the middle domains of cMyBP-C were shown to be modified by phosphorylation *in vitro*. Treatment of recombinant C4–C5 fragments of cMyBP-C with six different kinases led to the identification of 13 different phosphorylation sites, including Ser690 that is near the 28-amino acid insertion in C5 known as the C5 loop (Doh et al., 2022b). Previous work has suggested a possible "hinge-and-latch" mechanism (Doh et al., 2022a), or just hinge (Greenman et al., 2025), between the C4 linker and C5 loop. The C4 linker, or hinge, is proposed to create a region that allows a great degree of flexibility between domains C4 and C5 that may shift the position of the N′-terminal domains toward or away from their myosin- and actin-binding partners (Greenman et al., 2025; Doh et al., 2022a). The C5 loop, or latch, was proposed to help stabilize the C4 linker into a bent confirmation (Doh et al., 2022a). The regulation of this potential dynamic control mechanism within the C4 and C5 domains merits further exploration, especially considering recent data indicating that this region can undergo PTMs through kinase activity (Doh et al., 2022b).

In summary, the phosphorylation status of cMyBP-C impacts its interactions with myosin and actin and impacts overall heart health. We suggest that cMyBP-C phosphorylation serves as a "rheostat" for modulating contractility, i.e., cMyBP-C phosphorylation can be turned "up" quickly to meet increased cardiac demand. In contrast, dysregulation of this rheostat role through hypo-phosphorylation of cMyBP-C is detrimental in heart disease.

S-glutathionylation of cMyBP-C and downstream functional effects

Overview of S-glutathionylation

S-glutathionylation is the reversible covalent attachment of reduced glutathione (GSH) to cysteine (Cys) thiol groups in proteins to form a disulfide bond (summarized in Fig. 2) (Rashdan et al., 2020). The most important physiological role of S-glutathionylation may be during periods of elevated ROS leading to the intracellular accumulation of unstable molecules, such as superoxide anions and hydrogen peroxide (H_2O_2). ROS are intrinsic to normal cell physiology (Sies and Jones, 2020), but an excess of oxidative and nitrosative stress results in cellular damage and disease (Moris et al., 2017; Juan et al., 2021). Oxidation of Cys thiol groups can generate sulfonic acid and lead to

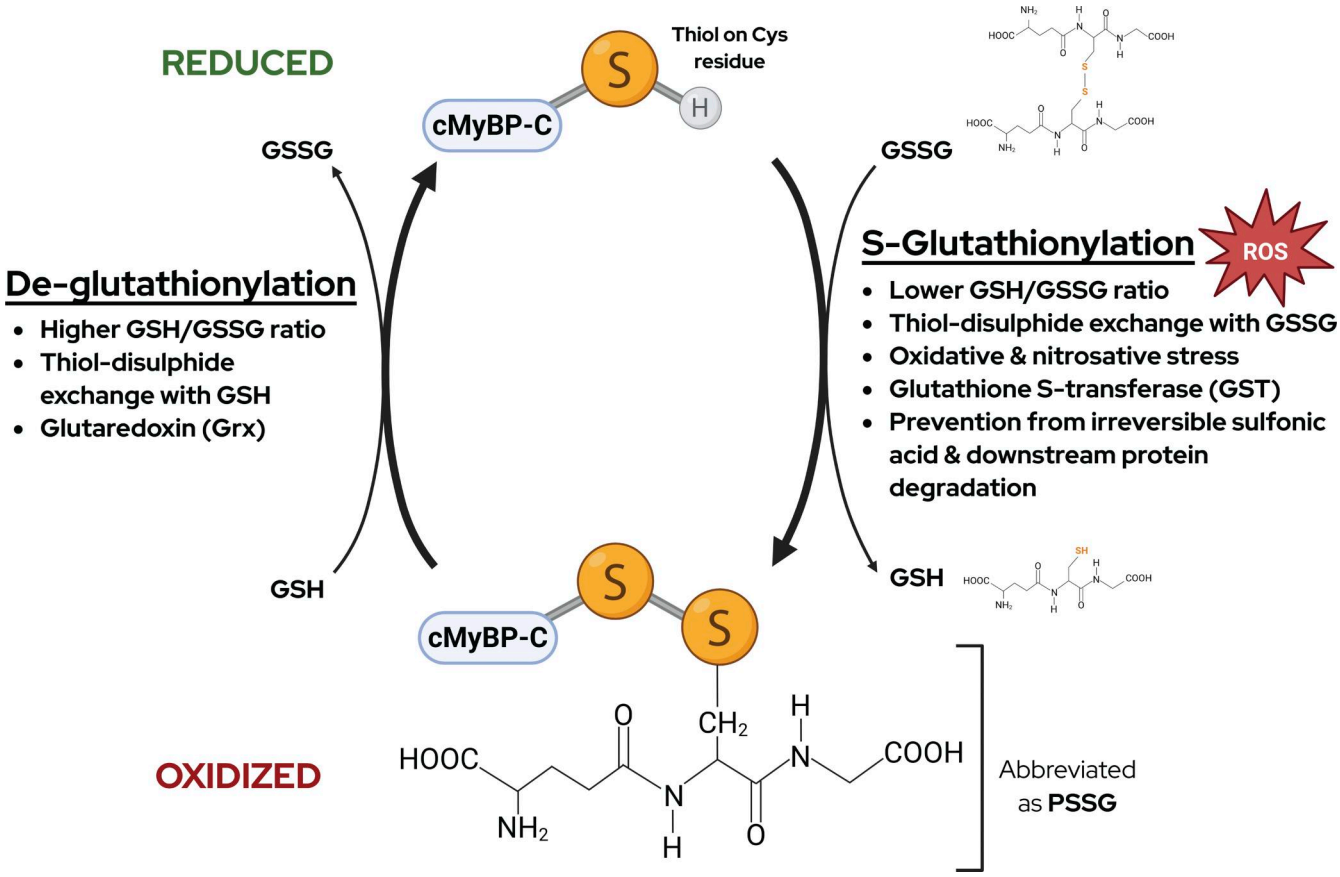

Figure 2. **An overview of the chemical basis for triggering the S-glutathionylation of proteins, including cMyBP-C.** S-glutathionylation is the forming a disulfide bond between a target protein and GSSG (PSSG). This reaction often occurs when the ratio of GSH/GSSG is lowered, secondary to increased ROS. The reaction can occur spontaneously with GSSG as a substrate or can be enzymatically catalyzed by GSH S-transferase (GST). The S-glutathionylation of proteins is an alternative pathway from the forming of sulfonic acid, which is also triggered during oxidative stress and leads to proteolysis. The reversal, or deglutathionylation of proteins, can occur spontaneously with the thiol-disulfide exchange of GSH or enzymatically with the help of Grx. Figure made using BioRender.

proteolysis (Chai and Mieyal, 2023; Grek et al., 2013); S-glutathionylation prevents this oxidative damage and protects proteins from proteolysis.

GSH is an abundant thiol with an intracellular concentration of 1–10 mM and functions as a scavenging antioxidant (Arthur, 2000; Meister, 1988). In high redox-activity states, such as in the setting of cancer and cardiovascular disease, GSH is oxidized to oxidized GSH (GSSG) (Rashdan et al., 2020). Typically, the ratio of GSH/GSSG is ~100/1, but, under highly oxidizing conditions, this ratio can drop as low as of 1/1 (Pallardó et al., 2009). The accumulation of GSSG favors the S-glutathionylation of target proteins (Fig. 2), recycling GSSG back into GSH, and the extrusion of GSSG from the cell (Tan et al., 2023; Ballatori et al., 2009). GSSG and GSH homeostasis is complex and remains to be fully determined, including in the context of cardiovascular disease. In the case of S-glutathionylation, the formation of the disulfide bond between a target protein (Cys residue) and GSSG can occur spontaneously or enzymatically, with the GSH S-transferase catalyzing the reaction (Townsend et al., 2009) and its reversal mediated by the ROS-detoxifying enzyme family glutaredoxins (Grx) and NAD phosphate (NADPH) (Chai and Mieyal, 2023; Mieyal et al., 2008). Grx1 and Grx2 are commonly found in the heart (Chai and Mieyal, 2023), and an overexpression of Grx1 may be cardioprotective (Adluri et al., 2012). Additionally, estrogen is known to regulate Grx1, making it plausible that sex differences may influence S-glutathionylation levels (Urata et al., 2006).

There are many known sarcomere protein targets of S-glutathionylation predicted by *in vitro* studies, including titin, actin, tropomyosin, myosin light chain I (MLCI), and cMyBP-C. The S-glutathionylation of titin in a model of myocardial infarction has been linked to cryptic sites that may alter the stiffness of titin (Alegre-Cebollada et al., 2014; Avner et al., 2012). The modification of Cys374 on actin led to decreases in cooperative tropomyosin-actin binding and reduced maximal developed force in trabeculae (Chen and Ogut, 2006). Tropomyosin has also been shown to be globally modified after treatment with GSSG (Patel et al., 2013), but the functional significance of tropomyosin S-glutathionylation remains unknown. MLCI has recently been shown to be a target of S-glutathionylation, although at a small magnitude and with undetermined functional implications (Chapman et al., 2025).

S-glutathionylation of cMyBP-C

The S-glutathionylation of cMyBP-C was first identified by an *in vitro* biotinylated pulldown approach in the rat heart (Brennan

et al., 2006b). S-glutathionylation of cMyBP-C was later identified after GSSG treatment in permeabilized multicellular preparations (Patel et al., 2013; Stathopoulou et al., 2016) and endogenously in several different disease states (Stathopoulou et al., 2016; Lovelock et al., 2012; Chakouri et al., 2020; Jeong et al., 2013; Cazorla et al., 2021; Budde et al., 2021). S-glutathionylation may serve dual purposes by protecting cMyBP-C from irreversible oxidative modifications and premature degradation, as well as to modulate its function, causing inotropic and lusitropic responses during periods of cardiac stress. Evidence for the physiologic role of S-glutathionylation of cMyBP-C was derived from experiments treating permeabilized cardiomyocytes with 1 mM GSSG, which produced a marginal increase in myofilament calcium sensitivity but also an increase in myofibril ATPase activity (Patel et al., 2013). However, conflicting data demonstrated a reduction in both crossbridge kinetics and maximal force in permeabilized tissue treated with 1 mM GSSG (Stathopoulou et al., 2016). Taken together, these data could suggest a high cost to generating muscle tension or that increased ATPase activity is a compensatory mechanism for slow crossbridge cycling when S-glutathionylation levels are elevated. The specific role of cMyBP-C S-glutathionylation was uncovered by using both wild-type and cMyBP-C knockout permeabilized cardiac tissue, leading to the conclusion that S-glutathionylation of cMyBP-C, and not neighboring sarcomere targets, explained the reduction in the rate of crossbridge cycling and an increase in passive force, but that cMyBP-C S-glutathionylation was not responsible for an observed reduction in maximal force (Stathopoulou et al., 2016). While the mechanism remains to be identified, the evidence suggests that S-glutathionylation of cMyBP-C may alter protein conformation to favor the inhibition of myosin heads and slow crossbridge kinetics. Further studies are needed to test this hypothesis and resolve the discrepancy in the available data.

Currently, there are 15 Cys residues on cMyBP-C that have been shown by mass spectrometry to be targeted by S-glutathionylation in the human heart (Stathopoulou et al., 2016). These sites span the entire protein and include Cys239, 249, 426, 436, 443, 475, 528, 566, 623, 651, 719, 788, 909, 913, and 1244 (Fig. 1). Notably, the majority of these Cys residues are located within the middle domains of cMyBP-C. Interestingly, non-failing heart samples revealed basal levels of cMyBP-C S-glutathionylation (Stathopoulou et al., 2016). While the functional implication of this remains to be identified, it is plausible that S-glutathionylation of cMyBP-C may protect the protein from premature degradation by preventing the formation of sulfonic acid and downstream proteolysis.

The role of cMyBP-C S-glutathionylation in heart disease

S-glutathionylation of cMyBP-C may impact diastolic function of the heart. In the deoxycorticosterone acetate-salt rat model of hypertension, the level of S-glutathionylation of cMyBP-C was elevated and correlated with diastolic dysfunction (Lovelock et al., 2012). In that model, treatment with tetrahydrobiopterin (BH4), a promising new therapy for ameliorating cell death that may regulate S-glutathionylation (Eichwald et al., 2023), significantly reduced cMyBP-C S-glutathionylation (Jeong et al., 2013). The reduction in cMyBP-C S-glutathionylation corresponded with increased maximal developed force, normalized the ATP consumed compared to the rise in muscle tension, and improved diastolic function (Jeong et al., 2013). It is, however, possible that the positive results from BH4 treatment could be an indirect effect of its ability to increase nitric oxide (NO) synthesis (Alp et al., 2003). Regardless, the potential therapeutic benefit is of clinical interest as there are few treatment options for diastolic dysfunction. Recently, circulating S-glutathionylated cMyBP-C was elevated in plasma taken from human patients, monkeys, and mice with diastolic dysfunction, suggesting a role as a possible biomarker of diastolic dysfunction (Zhou et al., 2022). The stability of this PTM, particularly in plasma, and its usefulness as a clinically relevant marker of diastolic dysfunction remain to be determined.

S-glutathionylation of cMyBP-C is also elevated in cardiomyopathies. S-glutathionylation of cMyBP-C was sixfold higher in mice with HCM compared to controls (Flenner et al., 2016). In dilated and ischemic cardiomyopathy patients with heart failure, several Cys residues had elevated cMyBP-C S-glutathionylation, including at Cys249, Cys426, Cys443, Cys475, Cys566, Cys651, and Cys719, compared to non-failing donor controls (Stathopoulou et al., 2016). As mentioned above, the elevation of cMyBP-C S-glutathionylation in the setting of heart disease might actually protect cMyBP-C from the detrimental effects of permanent oxidation. One group found three cMyBP-C residues to be oxidized in human failing heart samples, Cys443, Cys623, and Cys1124. Notably, Cys475 is a known S-glutathionylation site but was not modified by oxidation (Budde et al., 2021), suggestive that S-glutathionylation may regulate other aspects of cell biology beyond proteolysis. Future work should explore the possible protective effect S-glutathionylation may play, and if reversal of this PTM may be detrimental to long-term cardiovascular health.

GSH levels are consistently reduced in the failing heart (Al-Mubarak et al., 2025), thus increasing the ratio of GSH/GSSG in cardiomyocytes may be a therapeutic strategy. For instance, in human end-stage failing hearts, there was elevated cMyBP-C S-glutathionylation, reduced cMyBP-C and cardiac troponin I (cTnI) phosphorylation, and a reduction in the level of GSH. Myofilament dysfunction in these failing heart samples was rescued by GSH treatment (Budde et al., 2021). Others have found evidence that GSH supplementation may be beneficial in preclinical models (Budde et al., 2021; Golbidi et al., 2014), and there are promising results in clinical trials (Kalamkar et al., 2022; Sinha et al., 2018). However, it is possible that increasing the systemic levels of GSH could be detrimental by increasing the probability of increased GSSG levels and downstream S-glutathionylation during periods of oxidative stress. Future studies are needed to understand how the GSH/GSSG ratio and the effect of GSH addition itself impact heart function *in vivo*. In addition to GSH supplementation, currently available therapeutics appear to alter levels of GSH/GSSG and S-glutathionylation of sarcomere proteins. For instance, in a rabbit model of HCM, N-acetylcysteine (NAC) treatment restored the GSH/GSSG ratio, normalized α-actin S-glutathionylation, reversed cellular hypertrophy and interstitial fibrosis, while also preventing systolic dysfunction (Lombardi et al., 2009). Tadalafil treatment, a long-acting

PDE-5 inhibitor, significantly improved the GSH/GSSG ratio and the metabolic status in a mouse model of diabetes (Koka et al., 2012). The authors also found a reduction in the total protein expression of cMyBP-C in the diabetic mice, which recovered to wild-type levels post-Tadalafil treatment, but cMyBP-C PTMs were not explored. 6 wk of fingolimod (FTY720) treatment, an immunomodulatory drug currently approved for use in multiple sclerosis, reduced myofilament calcium sensitivity, improved left atrial size, and improved diastolic function in a mouse model of HCM when compared to vehicle (Ryba et al., 2019). The authors attributed these improvements in diastolic function to the downregulation in cMyBP-C S-glutathionylation due to reduced NADPH oxidase expression, an indicator of reduced oxidative stress. Additionally, mice on a high-fat diet for 8 wk had evidence of diastolic dysfunction, which was significantly improved by administration of MitoTEMPO, a mitochondrial antioxidant (Jeong et al., 2016). MitoTEMPO also reduced the level of cMyBP-C S-glutathionylation and led to recovery of relaxation kinetics in isolated cardiomyocytes, providing evidence that mitochondrial antioxidants may serve as a role in modulating the levels of cMyBP-C S-glutathionylation and the treatment of diastolic dysfunction (Jeong et al., 2016). These data indicate that currently available therapeutics alter S-glutathionylation, including at cMyBP-C, and may be responsible for, or contribute to, improvements in cardiac function. These results should, however, be treated with caution, as it remains to be determined whether these effects are truly causative rather than correlative.

Potential inverse correlation between S-glutathionylation and phosphorylation of cMyBP-C

Several studies showing elevated S-glutathionylation of cMyBP-C have also identified a decline in cMyBP-C phosphorylation, leading to the hypothesis that these two PTMs may act in a coordinated fashion. Incubating recombinant N′-terminal domains (C1, M, and C2) with GSSG led to an increase in cMyBP-C S-glutathionylation, but also reduced phosphorylation of cMyBP-C at Ser275, Ser284, and Ser304 directly supporting a direct relationship between cMyBP-C phosphorylation and S-glutathionylation (Stathopoulou et al., 2016). Mass spectrometry data indicated that Cys249 was highly modified by S-glutathionylation in recombinant protein, rodent hearts, and human tissue. This led the authors to explore the relationship of this S-glutathionylation site and the regulation of the C1-M-C2 phosphorylation status. Modification of the Cys249 to Ser249 followed by GSSG treatment resulted in no change in the level of phosphorylation of the C1–M–C2 fragment, suggesting a direct cross talk mechanism (Stathopoulou et al., 2016). To test if physiologically relevant, the authors explored the S-glutathionylation and phosphorylation status of cMyBP-C in the failing heart. Notably, S-glutathionylation of cMyBP-C was increased, while cMyBP-C phosphorylation was reduced in these same samples, as shown previously (van Dijk et al., 2009). Together, these data indicate that the reduction in cMyBP-C phosphorylation found in the failing heart might be due to an increase in S-glutathionylation potentially via altered electro-steric effects on neighboring cMyBP-C residues (Fig. 3). Additional studies are needed to tease out the potential for a direct cross talk relationship between phosphorylation and S-glutathionylation or if the inverse relationship is explained by unrelated, coinciding mechanisms that will be discussed in more detail below.

In summary, the modification of cMyBP-C Cys residues by S-glutathionylation appears to play an important role in regulating mechanisms of contraction and relaxation, impacting overall heart health. Further studies are needed to better understand when and how this PTM alters cMyBP-C function and if its reversal or prevention will prove to be cardioprotective.

S-nitrosylation of cMyBP-C

S-nitrosylation is the covalent addition of NO to Cys thiol groups. Through an effect on the redox state of Cys residues, S-nitrosylation acts as a molecular switch, influencing processes such as signal transduction, calcium signaling, and apoptosis. S-nitrosylation is also known to contribute to vasodilation, which improves coronary blood flow and protects against ischemia-reperfusion injury (Mao et al., 2025). S-nitrosylation plays a vital role in cellular signaling pathways, particularly in cardiovascular and neurological systems through a mechanism of rapid and reversible responses to changes in NO availability (Sun and Murphy, 2010).

Through modification of key proteins involved in cardiac muscle contraction, calcium handling, and signal transduction, S-nitrosylation modulates heart rate, contractility, and relaxation. Specifically, S-nitrosylation modifies proteins that control intracellular calcium dynamics, including ryanodine receptors, sarco-endoplasmic reticulum calcium ATPase, and phospholamban (Lima et al., 2010; Irie et al., 2015). Based on *in vitro* studies, cMyBP-C can be modified by S-nitrosylation at Cys1260 located within its C′-terminal region (Kohr et al., 2011). Given the location of this S-nitrosylation site, it might affect cMyBP-C anchoring to the thick filament, but this hypothesis has not been tested. While S-nitrosylation of numerous myofilament proteins leads to an overall decrease in myofilament calcium sensitivity, reduced maximal developed force, and prolonged relaxation (Figueiredo-Freitas et al., 2015), the specific functional effects of cMyBP-C S-nitrosylation are unknown. In end-stage failing human heart samples, NO availability is reduced in the failing heart compared to donors, suggesting that the S-nitrosylation of cMyBP-C may also be reduced in the failing heart (Budde et al., 2021).

Acetylation of cMyBP-C

Acetylation is the transfer of an acetyl group most commonly to lysine (Lys) residues, neutralizing its positive charge. This PTM can influence a broad range of cellular processes, including gene transcription, signal transduction, and metabolic pathways, through the varied effects upon protein stability, localization, and activity. This dynamic process, controlled by acetyltransferases and deacetylases (HDACs), allows cells to rapidly and reversibly adjust protein function in response to diverse stimuli. Pathologic acetylation is associated with hypertension,

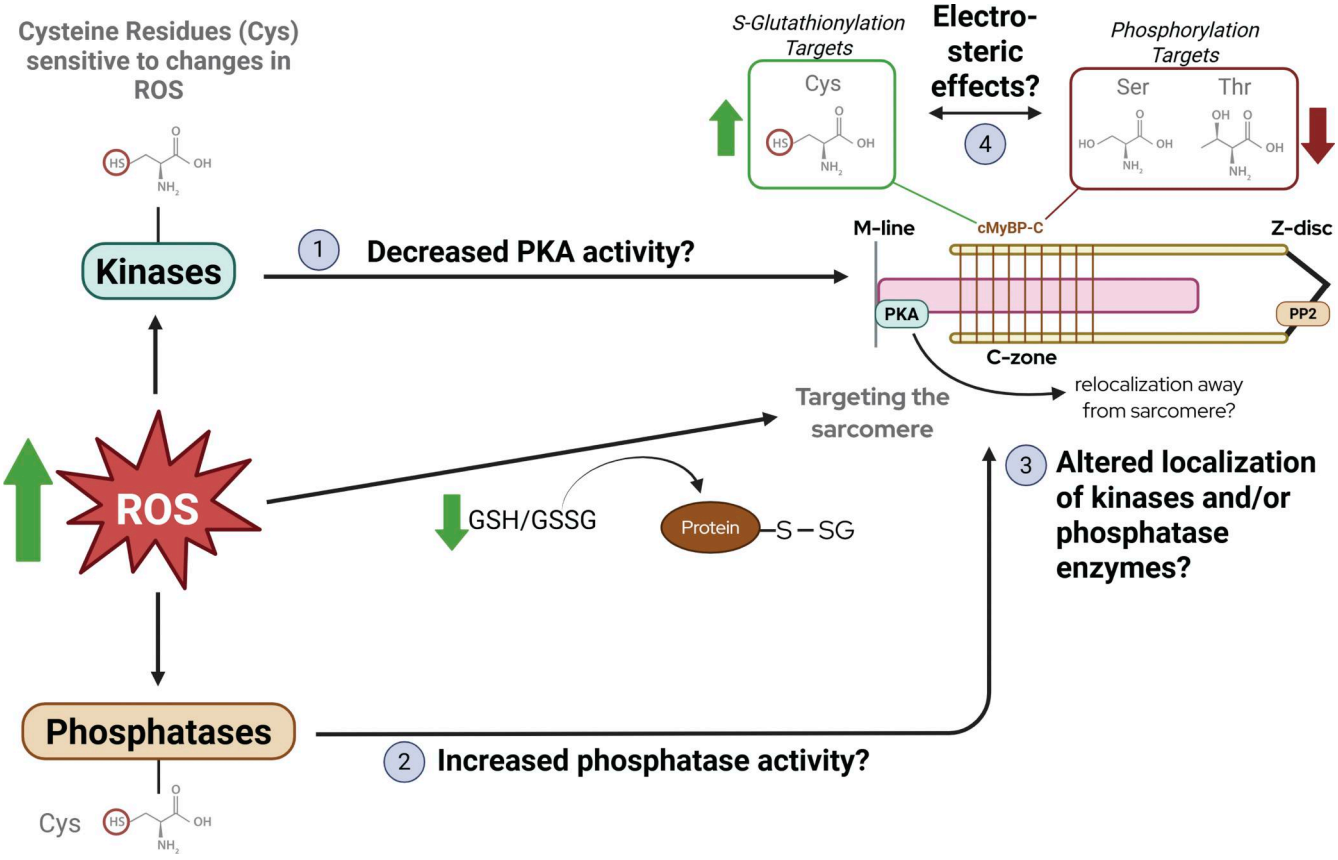

Figure 3. **Potential mechanisms behind the inverse relationship of phosphorylation and S-glutathionylation at cMyBP-C seen in cardiovascular disease.** PKA might have reduced activity in cardiovascular disease due to increased oxidation of Cys residues (mechanism 1), ultimately leading to a reduction in cMyBP-C phosphorylation. Additionally, or alternatively, protein phosphatase, such as PP1 and PP2, might have increased activity with increased ROS (mechanism 2) and led to cMyBP-C hypo-phosphorylation seen in cardiovascular disease. In addition to altered activity of PKA and phosphatase enzymes, localization of these enzymes has been shown to change in response to other oxidative-related pathways and, thus, may play a role (mechanism 3). More directly, elevated ROS causes a decreased ratio of GSH/GSSG, which in turn favors S-glutathionylation and electro-sterically hinders the phosphorylation of cMyBP-C (mechanism 4). Figure made using BioRender.

vascular disease, arrhythmias, angiogenesis, and heart failure (Li et al., 2020). Changes in cellular metabolism, particularly the availability of acetyl group donors (acetyl CoA), significantly impacts acetylation levels (Cai et al., 2011; Weinert et al., 2014). Pathophysiologic states that alter glucose metabolism can increase acetyl-CoA production, promoting acetylation (Yucel et al., 2019; Everetts et al., 2013; Wellen et al., 2009; Friis et al., 2009; Kosanam et al., 2014).

The myofilament is a target of acetylation. In one example, treating skinned cardiomyocytes with an HDAC inhibitor led to an increase in myofilament calcium sensitivity (Gupta et al., 2008). Whereas thin filaments reconstituted with Lys132Gln cTnI (i.e., substitution of Lys for glutamine [Gln]), a known acetylation site (Lin et al., 2020; Lundby et al., 2012), had reduced myofilament calcium sensitivity and accelerated relaxation kinetics compared to wild-type controls (Lin et al., 2020), suggesting that acetylation may play an important role in regulating myofilament relaxation parameters.

Site-specific acetylation of cMyBP-C was first identified by mass spectroscopy. N′-terminal cMyBP-C calpain-degradation fragments were confirmed to be susceptible to acetylation *in vivo*, with several predominant acetyl sites located within the actin-binding region (Lys185, Lys190, Lys193, and Lys202) (Ge et al., 2009; Govindan et al., 2012). Additionally, in an ischemia-reperfusion mouse model, there were eight sites found to be highly targeted by acetylation: Lys7, Lys185, Lys190, Lys193, Lys202, Lys442, Lys935, and Lys962 (Govindan et al., 2012). More recently, there have been additional sites uncovered in the middle domains of recombinant cMyBP-C: Lys540, Lys555, Lys561, Lys662, Lys681, and Lys711 (Doh et al., 2022b). Given the specific location of cMyBP-C acetylation, it is possible that that acetylation of cMyBP-C may affect the ability of cMyBP-C to interact with actin and myosin. *In vivo* validation of cMyBP-C acetylation and any potential role this plays in cardiac physiology and disease, particularly in the setting of elevated acetyl CoA (e.g., diabetes), remains to be explored.

Citrullination of cMyBP-C

Citrullination is an irreversible, calcium dependent, enzymatic conversion of a positively charged arginine (Arg) to a negatively charged citrulline, which can disrupt existing hydrogen bonds

and electrostatic intra- and inter-protein interactions (Fert-Bober and Sokolove, 2014). Citrullination plays a role in both normal physiological processes like cell death and differentiation and pathological conditions such as rheumatoid arthritis, multiple sclerosis, cancer, and COVID-19 infection, where the PTM contributes to inflammation and autoimmunity (Darrah and Andrade, 2018; Ciesielski et al., 2022).

The reaction is catalyzed by a family of enzymes called peptidyl-Arg deiminases (PADs) in a calcium-dependent manner (Anzilotti et al., 2010). Notably, PAD2 treatment in cardiomyocytes resulted in a reduction in myofilament calcium sensitivity (Fert-Bober et al., 2015). PAD2 is expressed in both the nuclear and myofibrillar compartments of cardiomyocytes and has been implicated in impaired diastolic function seen in women with heart failure (Shorthill et al., 2024). Additionally, extensive citrullination was found in the myocardium of individuals with rheumatoid arthritis and was associated with increased cardiovascular burden of rheumatoid arthritis patients (Fert-Bober and Sokolove, 2014). cMyBP-C was shown to be modified by citrullination at Arg696 in both non-failing and failing heart samples (Fert-Bober and Sokolove, 2014; Fert-Bober et al., 2011). Although citrullination has been shown to cause a reduction in both ATPase activity and myofilament calcium sensitivity in permeabilized cardiomyocytes (Fert-Bober et al., 2015), the functional relevance of cMyBP-C citrullination remains to be determined, as other sarcomere proteins, including actin, troponin, tropomyosin, and myosin are also citrullinated (Fert-Bober et al., 2015).

Carbonylation of cMyBP-C

Carbonylation is an irreversible oxidative modification of proteins by the addition of carbonyl groups. This process, primarily targeting Lys, Arg, proline, and Thr residues, introduces carbonyl moieties that can lead to significant conformational changes, including protein unfolding, aggregation, and fragmentation. By altering hydrophobicity and charge distribution, carbonylation can impair enzymatic activity, disrupt protein–protein interactions, and compromise cellular signaling pathways (Nyström, 2005). As a hallmark of oxidative stress and aging, carbonylation contributes to cellular dysfunction through an accumulation of damaged and nonfunctional proteins and is implicated in various pathological conditions, including neurodegenerative diseases, cancer, and cardiovascular diseases (Fert-Bober and Sokolove, 2014; Darrah and Andrade, 2018).

Treating skinned cardiomyocytes with myeloperoxidase, an enzyme responsible for carbonylation, and its substrate, H_2O_2, led to a reduced maximal force, increased passive tension, and reduced myofilament calcium sensitivity (Kalász et al., 2015). Dual myeloperoxidase and H_2O_2 treatment increased carbonylation of cMyBP-C and other sarcomere proteins (Kalász et al., 2015). H_2O_2 treatment alone did not change calcium sensitivity, but did increase the carbonylation of cMyBP-C globally (Kalász et al., 2015). Anthracycline doxorubicin (Dox), a chemotherapy drug that causes well-described cardiotoxicity, increased cMyBP-C carbonylation, and its degradation in Dox-treated rodents. These effects were, however, prevented by iron chelator treatment (Aryal et al., 2014). The authors concluded that cMyBP-C carbonylation serves as an important determinant of cardiotoxicity. Using molecular dynamic simulations, the chemotherapy toxicity affected the environment surrounding domains C0 and C1 of cMyBP-C, suggesting that the carbonylation-prone residues on cMyBP-C may interfere with actin-cMyBP-C binding (Bergonzo et al., 2023). Furthermore, both cMyBP-C and cardiac troponin T were highly carbonylated in a mouse model of Duchenne's muscular dystrophy. Hyper-carbonylation of these proteins was prevented by low intensity exercise training in dystrophic mice (Hyzewicz et al., 2015). While cMyBP-C appears to be modified by carbonylation *in vivo*, its full impact on muscle function and relevance to human disease remains to be determined.

O-GlcNAcylation of cMyBP-C

O-GlcNAcylation is the enzymatic addition of O-GlcNAc to Ser and Thr residues in target proteins. The addition of O-GlcNAc is catalyzed by the enzyme O-GlcNAc transferase (OGT) and reversed by O-GlcNAcase (OGA) (Zachara et al., 2022). The localization of OGT and O-GlcNAc are typically near the Z-line, whereas OGA is found within the A-band of the sarcomere, but these localizations can be disrupted by disease with an observed increase in the O-GlcNAcylation of myofilament proteins (Ramirez-Correa et al., 2015). O-GlcNAcylation is a major contributor to diabetic heart disease (Ritchie and Abel, 2020), and excessive O-GlcNAcylation causes cardiomyopathy in mice (Umapathi et al., 2021). The sarcomere proteins, myosin heavy chain, actin, MLCI, and MLCII were first identified to be targeted by O-GlcNAcylation in rat skeletal muscle (Hedou et al., 2007), but specific residues were not identified. Treating skinned trabecular preparations with GlcNAc reduced myofilament calcium sensitivity and led to the identification of 32 novel O-GlcNAcylated peptides, including those from myosin heavy chain, α-actin, MLCI, MLCII, and cTnI (Hedou et al., 2007). cMyBP-C was found to be a target of O-GlcNAcylation after treatment with an OGA analog, specifically at Ser47 (Ramirez-Correa et al., 2015).

Prolonged or excessive O-GlcNAcylation of cardiac proteins is associated with heart disease and is elevated in models of diabetes, hypertension, hypertrophy, and heart failure (Lunde et al., 2012; Clark et al., 2003; Erickson et al., 2013; McLarty et al., 2013; Umapathi et al., 2021; Gélinas et al., 2018). Genetically modified mice with OGT overexpression had a sixfold increase in cardiac O-GlcNAc levels, developed dilated cardiomyopathy, and sudden death (Umapathi et al., 2021). In the streptozotocin rodent model of type I diabetes, use of an OGA bacterial analog resulted in the removal of O-GlcNAc from myofilaments, ultimately restoring myofilament calcium sensitivity (Ramirez-Correa et al., 2015). It is worth noting that many known O-GlcNAcylation sites are located near phosphorylation sites and may compete for the same Ser or Thr residues (Butkinaree et al., 2010). Whether the O-GlcNAcylation of cMyBP-C plays a role in these pathophysiological states or may interfere with cMyBP-C phosphorylation is yet to be determined.

The effects of ROS on cMyBP-C PTMs

The accumulation of ROS is commonly associated with many forms of cardiovascular disease (Kim et al., 2016), and ROS interacts with several of the PTMs discussed in this review (e.g., S-glutathionylation). ROS are highly reactive forms of oxygen (e.g., H_2O_2) that are produced in the mitochondrial matrix during ATP production. Basal levels of ROS serve important physiological roles in the cell, but during oxidative stress, ROS accumulates to excessive levels within cells causing damage to both DNA and proteins. As discussed above, elevations in ROS lead to a reduction in the GSH/GSSG ratio, resulting in increased S-glutathionylation of target proteins (e.g., cMyBP-C) and protecting these proteins from degradation at the cost of altered muscle function. ROS directly affects the activity levels of enzymes essential to modify the phosphorylation of cMyBP-C (Foley and Kintner, 2005; Johnston et al., 2015; Rao and Clayton, 2002; Singh et al., 2018). The common finding that cMyBP-C phosphorylation negatively correlates with cMyBP-C S-glutathionylation could be due to cross talk (i.e., electric-steric effects). Alternatively, the inverse relationship may occur simultaneously due to the elevations in ROS affecting the overall PTM status of cMyBP-C by various mechanisms. The later suggests that reduced phosphorylation and increased S-glutathionylation of cMyBP-C may coincide and amplify the functional consequences of altered phosphorylation and S-glutathionylation of cMyBP-C, further slowing crossbridge kinetics. Kinases and phosphatases that modulate cMyBP-C phosphorylation are sensitive to ROS and, thus, might play a role in altering cMyBP-C phosphorylation in addition to the potential cross talk with S-glutathionylation.

PKA phosphorylates cMyBP-C at its well-established M-domain residues, Ser275, Ser284, and Ser304 (Sadayappan et al., 2011; Mohamed et al., 1998). Typically, PKA is activated by cAMP binding, freeing the catalytic subunits to phosphorylate its target substrates (Taylor et al., 1990). However, this pathway is altered by ROS (Brennan et al., 2006a; Cuello and Eaton, 2019), ultimately influencing cMyBP-C phosphorylation. Oxidation of PKA at Cys199 decreased PKA activity (Humphries et al., 2002). In heart failure samples from human tissue, there was a reduction in PKA activity that corresponds with a reduction in cMyBP-C phosphorylation and increased cMyBP-C S-glutathionylation (Budde et al., 2021). Treating adult rat ventricular myocytes with nitroxyl (HNO) donors, acting as a reducing agent, led to increased phosphorylation of the three M-domain sites (Ser275, Ser284, and Ser304) (Diering et al., 2020). The authors confirmed that HNO donors increased PKA activity and, more significantly, led to the co-localization of PKA to the myofilament compartment of cardiomyocytes (Diering et al., 2020). Interestingly, disulfide bond formation between the regulatory type I subunits of PKA was enough to relocate PKA (Diering et al., 2020). These data indicate that PKA activity may be reduced and that PKA may localize away from the myofilament proteins during states of elevated ROS and result in the reduction in cMyBP-C phosphorylation often seen in heart disease (Fig. 3, mechanism 1 and 3). Other kinases that target cMyBP-C, such as protein kinase C, protein kinase D, and calcium/calmodulin-dependent protein kinase II, have elevated activity when exposed to ROS (Avner et al., 2010; Gopalakrishna and Jaken, 2000; Korichneva et al., 2002; Rocco-Machado et al., 2022; Waldron et al., 2004; Waldron and Rozengurt, 2000), indicating that PKA is uniquely targeted by ROS and may outweigh the increased activity of other kinases, at least in the context of cMyBP-C.

Based on the available evidence, we suggest four different mechanisms that may underlie the inverse relationship of cMyBP-C phosphorylation and S-glutathionylation commonly seen in cardiovascular disease. First, a reduction in PKA activity due to elevations in ROS may explain reductions in cMyBP-C phosphorylation (mechanism 1). Second, ROS might increase phosphatase activity resulting in a downstream decrease in cMyBP-C phosphorylation (mechanism 2). Third, when oxidized, PKA is localized away from the myofilament compartment of cardiomyocytes, reducing cMyBP-C phosphorylation independent of its overall activity (mechanism 3). Lastly, elevations in ROS reduce the GSH/GSSG ratio in favor of the S-glutathionylation of cMyBP-C, which may have steric effects on neighboring phosphorylation residues resulting in hypo-phosphorylation. For instance, the well-established Cys249 S-glutathionylation site is adjacent to the functionally relevant M-domain phosphorylation sites, which may play a role in how the protein folds or the accessibility of phospho-residues for kinase binding (mechanism 4). Future work is needed to determine the relevance of these potential mechanisms. It is plausible that a better understanding of these mechanisms and potential cross talk may lead to new therapeutic targets that limit, or leverage, the S-glutathionylation of cMyBP-C and dial-up the rheostat of cMyBP-C phosphorylation. Additionally, given that other PTMs discussed in this review are sensitive to elevations in ROS, a full-spectrum analysis studying the effects of oxidative stress on the PTM-state of cMyBP-C is greatly needed.

Conclusions

This Review highlighted the numerous PTMs that can modify cMyBP-C and impact heart function in both health and disease. Some of these PTMs, like phosphorylation, are well documented to play a role *in vivo*, while others remain unproven to have a relevant role. Additional work is needed to define the relevance of the cMyBP-C PTMs, either in the preservation of cardiac homeostasis or in contributing to the pathology of specific cardiac diseases. These insights should foster a greater understanding of the dynamic role of cMyBP-C in the heart and suggest potential therapeutic insights.

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