JGP Journal of General Physiology

COMMENTARY

Myofilament Function 2022

Is haploinsufficiency a sufficient mechanism for *MYBPC3* truncating mutations?

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Reduced expression of *MYBPC3* causes early dysfunction in human cell culture models prior to reduced cMyBP-C levels.

Cardiac myosin binding protein-C (cMyBP-C) is a myofilament regulatory protein with a growing number of roles. It promotes crossbridge formation, it inhibits crossbridge formation, it binds to thick and thin filament proteins, and its functions are tuned by differential posttranslational modifications. Importantly, when cMyBP-C goes missing, these regulations are lost, causing ventricular hypercontractility that leads to hypertrophic cardiomyopathy (HCM; Barefield and Sadayappan, 2010). The work by De Lange et al. (2023) demonstrates that heterozygous *MYBPC3* null cardiomyocyte cell models express normal amounts of cMyBP-C, but three-dimensional engineered cardiac tissue using these cells show haploinsufficiency. While this demonstrates that increased protein demand unmasks haploinsufficiency, the authors also provide data showing defects in calcium transient duration and tension development in two-dimensional culture with normal quantities of cMyBP-C. While haploinsufficiency of cMyBP-C has been previously demonstrated (Helms et al., 2014; Barefield et al., 2015; Glazier et al., 2019), the necessity and sufficiency of simply reducing protein levels to cause all the observed phenotypic changes remains unclear. The findings of this current study provide clues to the connection between loss of an *MYBPC3* allele and the early pathological changes that lead to hypertrophic cardiomyopathy.

HCM causing mutations in *MYBPC3* are typically inherited in an autosomal dominant manner, albeit with a significant variability of penetrance (Ho et al., 2018). The first *MYBPC3* mutations linked with HCM were identified in the early 1990's (Bonne et al., 1995; Watkins et al., 1995). As the number of identified mutations grew, it became clear that most of them caused premature truncations of the encoded protein. Mechanisms studied for these truncation mutations include loss of functionally sufficient protein quantity (haploinsufficiency), production of aberrant protein fragments that directly dysregulate myofilament function, or other indirect mechanisms of dysfunction (van Dijk et al., 2009; Schlossarek et al., 2012; Fig. 1).

The recent consensus is that *MYBPC3*-truncating mutations result in either no translation or rapid degradation of the truncated protein, ruling out a pathogenic truncated protein (Barefield et al., 2014; Helms et al., 2014; Barefield et al., 2015; Glazier et al., 2019). It is known that cMyBP-C requires nearly all its residues to correctly localize to the myosin thick filament, with deletion or replacement within the penultimate 50–60 amino acids sufficient for preventing proper localization (Welikson and Fischman, 2002; Kuster et al., 2019). It is fortuitous that most *MYBPC3* mutations that cause HCM likely share the same mechanism of action; namely, truncating alleles fail to produce any protein, and disease arises due to insufficiency of a single allele to express an appropriate amount of protein. However, it turns out there is more to it than that.

Analysis of human heart samples from symptomatic carriers of *MYBPC3*-truncating mutations showed minimal expression of mutant mRNA and an ∼30% reduction in total full-length cMyBP-C (van Dijk et al., 2009). This supports the hypothesis that haploinsufficiency is at play, but as it is virtually impossible to assess protein content from asymptomatic carriers, it is unknown whether protein reduction occurs before, concomitantly, or following ventricular hypertrophy. The first truncating or knockout *Mybpc3* mouse models showed total loss of cMyBP-C in the homozygous state, little to no expression of mutant protein, and fulminant cardiomyopathy (McConnell et al., 1999; Harris et al., 2002; Carrier et al., 2004; Palmer et al., 2004). However, while heterozygous *Mybpc3*-truncation mutant mice show normal levels of cMyBP-C, they have significantly decreased maximal isometric force development and diastolic dysfunction with

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This work is part of a special issue on Myofilament Function 2022.

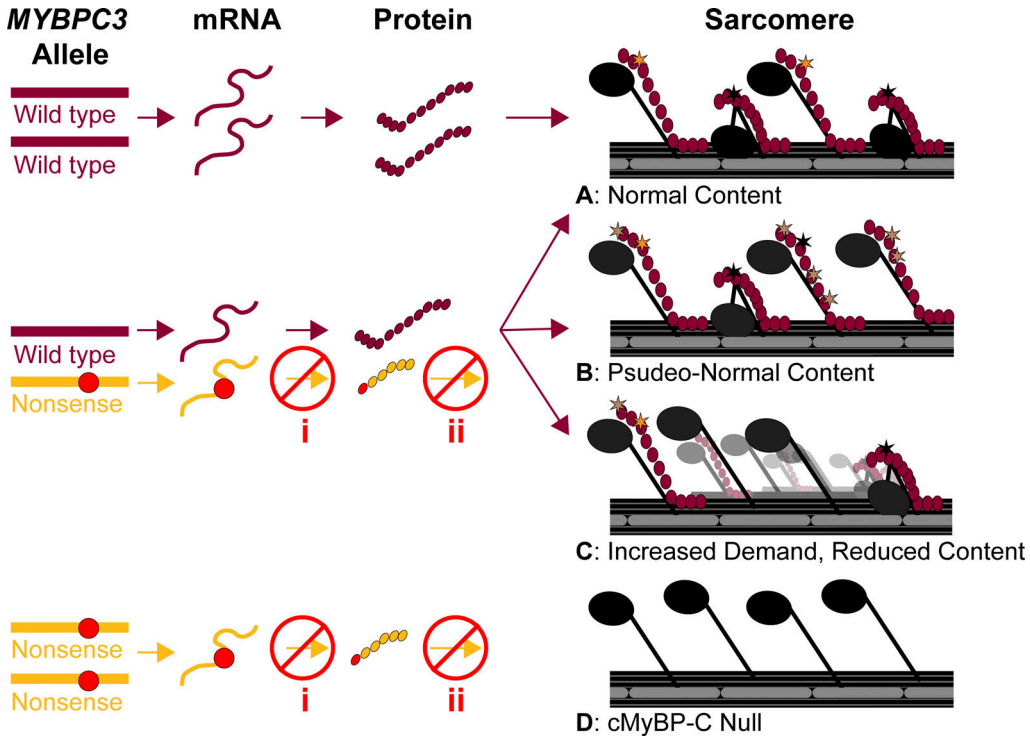

Figure 1. **Schematic illustrating a MYBPC3 allele carrying a nonsense mutation that encodes for a truncated cMyBP-C.** The alleles carried in the homozygous state fail to produce protein, either by nonsense-mediated decay of the transcript (i) or rapid degradation of the protein (ii). This results in a sarcomere with no cMyBP-C (D). The heterozygous nonsense allele is also eliminated. What occurs at the level of the sarcomere is reflected in data from different models. In sarcomere (A), there is a normal content of cMyBP-C, which may be possible with a single MYBPC3 allele. In sarcomere (B) there is a normal amount of protein, but the protein quality and function is impaired. This would be reflective of reduced protein expression and reduced protein turnover leading to longer-lived cMyBP-C. Sarcomere (C) illustrates the situation where increased demand for protein from hypertrophic remodeling or increased maturity of an engineered cardiac tissue results in insufficient protein expression to meet the demand.

an otherwise normal phenotype (Barefield et al., 2014). When challenged with hypertrophic stress, the levels of cMyBP-C fall below wild-type controls, and the hypertrophic phenotype is exacerbated (Barefield et al., 2015). These data from mouse models are in-line with the results from De Lange et al. (2023) and suggest that there is more going on here than a pure haploinsufficiency mechanism (i.e., a lack of a full complement of cMyBP-C is not required for some pathogenic processes; Fig. 1).

The use of human iPSC-derived cardiomyocytes (hiPSC-CMs) has provided mixed insights into the mechanism of *MYBPC3*-truncating mutants. As hiPSC-CMs have similarities to developing cardiomyocytes, they provide an excellent model to evaluate early changes that may occur due to a heterozygous *MYBPC3* truncation allele. Helms et al. (2020) reported on the onset of cellular dysfunction in the absence of overt cellular hypertrophy using homozygous and heterozygous *MYBPC3* null hiPSC-CM lines. They demonstrated that while total levels of cMyBP-C were normal in the *MYBPC3* heterozygous cardiomyocytes, with no obvious functional changes, the synthesis rate of cMyBP-C was slower. Additionally, the rate of degradation of cMyBP-C was also slower. They noted protein degradation pathways were downregulated in these cells and suggested that the reduced degradation allowed preservation of normal cMyBP-C levels. The reduced synthesis rate from heterozygous cells suggests there is a maximum amount of protein one allele can produce, and sometimes it's enough for a full complement of cMyBP-C. This agrees with data from *Mybpc3* heterozygous mice that show reduced total cMyBP-C levels only after hypertrophic stress (Barefield et al., 2015). It also agrees with data from De Lange et al. (2023) showing larger, three-dimensional tissue models require synthesis of more cMyBP-C than a single allele can satisfy.

There is an element of the concept that reduced rates of cMyBP-C synthesis and degradation that has not been systematically studied. Other data demonstrate the rate of cMyBP-C turnover is mediated by the synthesis rate of new protein, with stochastic degradation of new and old cMyBP-C as the proteins exchange in the myofilament (Wood et al., 2022). If the synthesis rate of full-length cMyBP-C is reduced, regardless of whether the total amount of cMyBP-C is maintained or reduced, the cMyBP-C in the sarcomeres would be longer lived. Sarcomere proteins normally have long half-lives, and further prolongation may cause the total cMyBP-C content to accumulate deleterious modifications. This mechanism fits with the current data from De Lange et al. (2023) demonstrating contractile dysfunction in the presence of normal cMyBP-C levels.

In their present work, the authors report data that shows the amount of mutant transcript is reduced in engineered cardiac tissues compared to the heterozygous cells grown in two-dimensional monolayers. They provide a rationale that nonsense-mediated mRNA decay plays a larger role in the engineered cardiac tissue compared to the monolayer and may contribute to the

haploinsufficiency. The possibility remains that the amount of wild-type transcript is more highly expressed in the engineered cardiac tissue, as shown previously (de Lange et al., 2021), and the increase in wild-type transcript is disproportionally higher than the mutant in these tissues. In either case, increased nonsense-mediated decay of the mutant transcript should have no direct effect on the ability of the wild-type allele to express protein, so this is unlikely to be a cause of the reduced total cMyBP-C levels in engineered cardiac tissues.

As in other investigations on this topic, the present study aims to provide a mechanistic link between the primary cellular defect and the emergent phenotype (Barefield et al., 2015; Farrell et al., 2018; Helms et al., 2020). The authors provide RNA-Seq data to identify changes in gene expression that may be indicative of early consequences of *MYBPC3* haploinsufficiency. They identify upregulated genes that play roles in metabolism, and a switch in calcium handling pathways that favor sarcolemma calcium and reduce the contribution of SR calcium release. The authors propose that higher energy use, stemming from hypercontractile sarcomeres due to reduced cMyBP-C, causes an increase in metabolic demand and inhibition of SERCA to reduce ATP consumption. These connections are correlative but do provide some specifically testable targets.

The body of work on *MYBPC3* haploinsufficiency suggests the primary defect in cMyBP-C levels has downstream consequences in a variety of cellular processes that precipitate further functional decline. This may be why *MYBPC3* haploinsufficiency has proven difficult to explain by one clear mechanism. The work by De Lange et al. (2023) provides a compelling link between *MYBPC3* haploinsufficiency and the emergence of dysfunction that is not directly related to the biophysical changes at the level of the sarcomere.

Acknowledgments

Henk L. Granzier served as editor.

Supported by National Institutes of Health grants R00HL141698 and R56HL165137.

The author declares no competing financial interests.

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