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Myosin ATPase inhibition relieves the energetic burden in skeletal myofibres of patients with heart failure with reduced ejection fraction

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What Is New?

- Skeletal muscle myosin dynamics are disrupted in HFrEF patients, leading to chronically elevated sarcomeric ATP consumption at rest.
- Skeletal muscle myosin in HFrEF patients show dysregulated acetyl-lysine modifications that predict to alter protein stability.
- *Ex vivo* treatment with the myosin inhibitor mavacamten restores biochemical function and improves ATP usage in skeletal muscle from HFrEF patients.

What are the Clinical Implications?

- Skeletal muscle myosin ATPase may represent a new therapeutic target for HFrEF patients.
- Incorporating pharmacological muscle-directed strategies into comprehensive HFrEF care could help alleviate symptoms.

Abstract

Background: Heart failure with reduced ejection fraction (HFrEF) affects millions worldwide and is characterized by chronic cardiac dysfunction, impaired perfusion, altered skeletal muscle energetics and, thus, exercise intolerance. Efficient therapeutic strategies reducing the burden of the impaired muscle metabolism in HFrEF are currently lacking. Hence, in the present study, we sought to determine whether myosin dynamics and its important role in ATP consumption can constitute a potent biochemical target to optimise skeletal muscle energy usage in HFrEF.

Methods: We used skeletal muscle tissue from eleven human HFrEF patients and ten controls with comparable age, sex and body mass index. We isolated individual myofibres and incubated them *ex vivo* with varying concentrations of a myosin inhibitor, mavacamten. We then performed Mant-ATP chase experiments, together with LC/MS-based proteomics profiling.

Results: We observed a distinct regulation of acetyl-lysine sites and higher myosin energy consumption in resting muscle fibres from HFrEF patients than controls. When exposed to mavacamten, we found a dose-dependent reduction of myosin ATP consumption in myofibres of HFrEF patients, reversing the pathological over-consumption.

Conclusions: Skeletal muscle myosin becomes inefficient in HFrEF. Pharmacological inhibition of myosin ATPase activity offers an inventive strategy to lower muscle energy demand and potentially address metabolic disturbances in HFrEF.

Key words: Heart failure; skeletal muscle; myosin; mavacamten; energetics

Non-standard Abbreviations and Acronyms

ATP: adenosine triphosphate

DRX: disordered-relaxed

EvoEF: EvoDesign physical Energy Function

GO: BP: Gene Ontology enrichment analysis for terms relating to Biological Processes

HFrEF: Heart failure with reduced ejection fraction

IHM: interacting-head-motif

LVEF: Left ventricular ejection fraction

Mant-ATP: 2'-(or-3')-O-(N-Methylanthraniloyl) adenosine 5'-triphosphate

PCA: Principal component analysis

PC1: First principal component

PC2: Second principal component

SRX: super-relaxed

6MWT = 6 minute-walk test

Introduction

Worldwide, more than 60 million people suffer from heart failure, and rates continue to increase, making this disease a major global public health issue.[1, 2] The most prevalent phenotype is heart failure with reduced ejection fraction (HFrEF), most commonly resulting from coronary artery diseases.[1] HFrEF is pathophysiologically defined by progressive left ventricular dilatation, maladaptive cardiac remodeling and a left ventricular ejection fraction (LVEF) < 40%.[1]

While impaired central hemodynamics remains the hallmark of HFrEF, the syndrome extends beyond the failing myocardium. It is increasingly understood as a multiorgan condition in which peripheral tissue alterations, particularly in skeletal muscles, play a critical role in disease progression and clinical expression.[3-5] Exercise intolerance is a cardinal symptom of HFrEF and a powerful predictor of prognosis.[6, 7] However, this cannot be explained solely by reduced cardiac output.[8] Rather, maladaptive changes in skeletal muscles contribute significantly to functional decline, diminished quality of life, and excess mortality.[3-5] Maladaptations in HFrEF include atrophy, changes in capillary density, high proportion of (non-oxidative) glycolytic fast-twitch muscle fibres, impaired mitochondrial number and function, and switch in substrate utilization.[9-13] These cellular and metabolic maladaptations mirror the chronic energy deficiency imposed by reduced perfusion and oxygen delivery. Importantly, they represent therapeutic targets. Interventions aimed at mitigating the effects of hypoperfusion not only by improving oxygen supply but also by lowering skeletal muscle ATP demand may offer novel strategies to improve functional capacity and outcomes in HFrEF.

While multiple processes consume ATP in skeletal muscle, recent evidence highlights myosin, the most abundant sarcomeric protein, myosin, as a key contributor to energy demand.[14, 15] Contraction requires large amounts of ATP hydrolyses to move myosin heads along actin filaments.[14] In parallel, maintaining a cellular resting state, is not energy free.[15] Indeed, myosin molecules can adopt at least two biochemical relaxed states. They can either be in a super-relaxed (SRX) state where ATP usage is very minimal or in a disordered-relaxed (DRX) state where ATP consumption increases ten-fold.[16] We have recently shown that resting skeletal muscle maintains an approximate 50:50 ratio between DRX and SRX states.[17]

Importantly, this balance is essential for skeletal muscle homeostasis[18, 19] and can be pharmacologically modified or rescued.[18, 20] Muscle myosin inhibitors such as mavacamten stabilise the SRX state, thereby lowering ATP turnover and reducing the energetic burden in cardiomyocytes and slow twitch skeletal myofibres that express cardiac β/slow myosin heavy chain.[18, 20] Since human skeletal muscles display heterogeneous fibre type composition and myosin isoform expression, this isoform specific action of mavacamten is directly relevant for interpreting its effects across fast- and slow-twitch skeletal fibres in HFrEF.

Other acquired conditions, such as critical illness and type 2 diabetes mellitus, where skeletal muscle metabolism is substantially altered, have been associated with increased proportions of myosin molecules in the DRX state due to aberrant post-translational modifications in these sarcomeric proteins.[17, 21] Hence, we hypothesized that the DRX-SRX equilibrium of myosin molecules in skeletal muscles is impaired in HFrEF and this relates to specific changes in post-translational modifications. Accordingly, pharmacological stabilization of the SRX state with myosin ATPase inhibitors could effectively reduce resting skeletal muscle ATP demand. The present study was therefore designed to test this hypothesis and to establish whether targeting skeletal muscle myosin ATPase could offer a mechanistically grounded strategy to alleviate skeletal muscle energy deficiency in HFrEF. While our experiments were performed under resting ex vivo conditions and do not directly assess contractile function or exercise performance,[22] they provide a framework to explore how altered myosin relaxation states may constrain the energetic reserve available for contraction.

Methods

Data availability

The physiological data supporting the findings of this study are available from the corresponding author upon reasonable request. Proteomics-related data are available via ProteomeXchange with identifier PXD068673.

Ethical approval

Our study used historical human tissue samples. It was approved by the Central Denmark Region Committee for Health Research Ethics (#1-10-72-218-16) and was performed in accordance with the latest version of the declaration of Helsinki. Written informed consent was received from all participants before inclusion.

Human muscle biopsy specimens

Vastus lateralis muscle biopsy specimens were obtained from male patients suffering from coronary artery disease and diagnosed with HFrEF (N = 11) and from male controls with no history of neuromuscular diseases, no signs/symptoms of cardiac diseases/heart failure and not engaged in any recreational physical activity (N = 10). Patients and controls were comparable in age, sex and body mass index as shown in Table 1.[23] Controls were required to be free of cardiac disease and were not engaged in regular recreational physical activity, but detailed objective physical activity or cardiorespiratory fitness measures were not available for either group. Additional baseline characteristics including medication, inclusion and exclusion criteria for the HFrEF patients are presented elsewhere.[23] All samples were flash-frozen and stored at -80°C until use. Full details of the subsequent analyses are provided within the Supplemental Material.

Sex as a biological variable

In the present study, we only used samples from human males, as shown in Table 1. This choice was driven by the design of the original clinical study from which tissue was obtained and by our intention to minimise additional variability arising from known sex related differences in skeletal muscle pathology and myosin regulation in HFrEF.[24] We acknowledge that this approach limits the generalisability of our findings to women.

Statistical analyses

Data are presented as means ± standard deviations. Graphs were prepared and analysed in GraphPad Prism v10.4. Statistical significance was set to $P < 0.05$. For each outcome, participant-level averages were calculated from all analysed fibres and used for between-group comparisons to account for the non-independence of repeated measurements within individuals. One-way ANOVAs were used to determine differences between groups (controls versus HFrEF patients) while taking fibre type (slow- versus fast-twitch) into account. Two-way repeated-measures ANOVAs were applied for experiments with within-participant factors, including fibre type and increasing concentrations of mavacamten (0, 1, 5 and 10 µM). Where appropriate, linear trends across mavacamten doses were evaluated. Data distribution was assessed by inspection of residuals and the Shapiro–Wilk test.

Results

Disrupted muscle myosin DRX to SRX ratio in HFrEF

To first verify our initial hypothesis of altered proportion of muscle myosin heads in either DRX or SRX biochemical states with heart failure, we isolated and membrane-permeabilised skeletal muscle fibres from eleven HFrEF patients and ten sex, age and activity-matched controls. In total, 322 individual myofibres were examined (≥ 15 fibers per subject), each standardized to a sarcomere length of 2.2 µm and analyzed using Mant-ATP chase experiments (Fig. 1A). In line with our expectations, the DRX state (P1) was significantly greater in both slow- and fast-twitch fibres from HFrEF patients than in controls (Fig. 1B). Conversely, the SRX state (P2) was significantly smaller across both fibre types in HFrEF patients than controls (Fig. 1C). The ATP turnover time of myosin molecules in the DRX state (T1) and the ATP turnover time of myosin heads in the SRX state (T2) were not statistically different between groups (Fig. 1D-E). Using these parameters (P1, P2, T1, and T2), we estimated the relative theoretical myosin ATP consumption in arbitrary units (a.u.) (equation provided in the Supplementary Material). Mant-ATP chase provides relative nucleotide kinetics, not absolute hydrolysis rates, as it does not quantify sarcomere volume, myosin head density, or non-myosin ATP use.[17, 25] Across individual participants, the relative theoretical myosin ATP consumption in slow twitch fibres ranged from 0.81 to 1.25 a.u. in controls and from 1.01 to 2.28 in HFrEF, whereas in fast twitch fibres values ranged from 0.55 to 1.29 in controls and 0.94 to 1.82 in HFrEF (Fig. 1F). The analysis revealed significantly higher resting myosin ATP demand in slow-twitch muscle fibres from HFrEF patients compared with controls (Fig. 1F), and in fast-twitch fibres, a similar trend towards elevated resting ATP demand was observed (Fig. 1F). This reflects energetic inefficiency at rest (increased ATP usage without enhanced mechanical output), narrowing the reserve available for contraction. Even though the estimated myosin ATP consumption did not correlate with the patients' left ventricular ejection fraction (Fig. S1), we unveiled a significant negative and linear relationship between the 6 minute walk test (6MWT, index of aerobic capacity and endurance) and the calculated myosin ATP consumption for both slow and fast twitch muscle fibres at rest in HFrEF patients (Fig. 1G; r = -0.7491, R^2 = 0.56, P = 0.008 for slow twitch fibres and r = -0.8176, R^2 = 0.66, P = 0.0021 for fast twitch myofibres), indicating a potential link between the extent of impaired myosin dynamics and exercise intolerance in HFrEF. 6MWT data were not systematically available for the control group, precluding

analogous analyses in controls or in the combined cohort. Note that our measurements were obtained in permeabilised fibres at rest and cannot quantify the impact on force generation, shortening velocity, or fatigue during dynamic contractions. The association with 6MWT distance therefore supports a mechanistic link between elevated resting myosin ATP demand and limited functional reserve, but interventional studies that concurrently assess myosin relaxation states and skeletal muscle contractile performance *in vivo* will be required to establish causality.

Altered muscle myosin acetyl-lysine sites in HFrEF

To provide molecular insights into the observed myosin maladaptation, we quantified both the abundance and post-translational modifications of skeletal muscle proteins, with particular attention to those implicated int the regulation of myosin DRX and SRX states.[15, 26] The subsequent proteomic and acetyl proteomic analyses were performed on whole muscle homogenates from vastus lateralis, which contain a heterogeneous mix of fibre types.

Principal component analysis (PCA) revealed clear separation of proteomic profiles between HFrEF patients and controls, primarily driven by the second principal component (PC2; Fig. 2A). Differential abundance analyses were defined using a permutation-based FDR < 0.01 and s0 = 1.0, as detailed in the Supplemental Methods. They confirmed significant proteomic dysregulation in muscle from HFrEF patients compared to controls (Fig. 2B). Although HFrEF samples displayed more proteins with increased abundance (159 upregulated and 116 downregulated), gene ontology enrichment analysis for terms relating to Biological Processes (GO:BP) identified significantly enriched biological processes only among downregulated proteins (Fig. 2C). Among the 275 differentially expressed proteins, entities belonging to metabolic homeostasis were prominent (e.g., gas transport, O2/CO2 transport; Fig. 2C) and confirmed the well-known hypoperfusion/skeletal muscle energetic remodelling happening in HFrEF.[9-13] Otherwise, 22 differentially expressed proteins were sarcomeric isoforms, of which the majority (15) showed reduced abundance (Table S1). Notably, myosin binding protein C (MYBPC) isoforms showed divergent regulations: the fast isoform (MYBPC2) was significantly downregulated, whereas the slow isoform (MYBPB1) upregulated in HFrEF patients (Table S1). Similarly, myosin light chains and related proteins were affected by heart failure (MYL1, MYLK and MYL11).

Following quantification of protein abundance, we investigated post translational modifications. Given the well described impairment of skeletal muscle metabolism in HFrEF,[24, 27] including altered substrate utilization that modifies acetyl CoA availability, we focused specifically on lysine acetylation as a post translational modification.[28, 29] Lysine acetylation is tightly linked to cellular metabolic state and can directly modulate sarcomeric protein structure, stability and function, making it a plausible regulatory mechanism for myosin relaxation states in this context. Unexpectedly, we identified only 73 acetyl-lysine sites across 53 proteins that were significantly altered between HFrEF patients and controls (15 up-regulated and 58 down-regulated; Tables S2 and S3 respectively). Gene ontology enrichment analyses (https://functionome.geneontology.org/) revealed that these acetylation changes in HFrEF patients primarily affected sarcomeric proteins of the contractile machinery and metabolic proteins involved in glycolysis, pyruvate metabolism, and ATP generation. Of particular interest for the present study, 15 acetyl-lysine sites were detected on myosin proteins: MYH7 (K34, K1528 and K1791 on cardiac-β/slow myosin heavy chain), MYH2 (K35, K562, K568, K1079 and K1266 on type IIa myosin heavy chain), and MYH1 (K205, K614, K829, K880, K914, K955 and K1655 on Type IIx myosin heavy chain) (Fig. 3A). These residues clustered in functionally critical regions involved in myosin head interactions and stabilizing the interacting-head motif (IHM) and are structurally associated with the energy-conserving SRX conformation.[30, 31] To assess the functional consequences of these unusual modifications, we introduced acetyl-mimicking mutations (lysine-to-glutamine substitutions, K→Q) and performed *in-silico* simulations using EvoDesign physical Energy Function (EvoEF).[25, 32, 33] Out of the fifteen mutations incorporated, fourteen led to increased ΔΔG Stabilities (Fig. 3B). This indicates that acetylations on MYH7, MYH2 and MYH1 lysine residues (that we found) are functionally relevant for myosin stability and essential for a normal physiological regulation. In further exploratory analyses, we examined correlations between the above myosin acetyl lysine sites and 6MWT distance; however, none of these were statistically correlated and therefore not shown/pursued here.

Mavacamten rescues muscle myosin biochemical dynamics in HFrEF

To further test our initial hypothesis that the disrupted balance of myosin in DRX and SRX states in HFrEF can be pharmacologically corrected, we evaluated the biochemical effects of

the clinically available mavacamten (also known as MYK-461 or Camzyos). This specific cardiac/β-slow myosin inhibitor stabilizes the SRX conformation, thereby reducing ATP consumption in myosin molecules of cardiomyocytes,[31, 34, 35] and skeletal muscle fibres.[18, 20] Additional experiments testing myosin inhibitors specific to type IIa or IIx isoforms were not pursued as none were available at the time of our study.[36] We then isolated and skinned muscle fibres from eight of the HFrEF patients and eight controls, yielding a total of 164 isolated myofibres (≥ 10 fibres per individual), standardized to a sarcomere length of 2.2 μm. Myofibres were incubated with increasing physiological concentrations of mavacamten (0, 1, 5 and 10 μM) and subjected to Mant-ATP chase experiments. In slow-twitch muscle fibres, mavacamten produced a marked biochemical effect. Indeed, in both HFrEF patients and controls, concentrations of 5 and 10 μM significantly decreased myosin DRX:SRX equilibrium (P1:P2 ratio; Fig. 4A). The ATP turnover time of myosin molecules in the DRX state (T1) and SRX state (T2) was also affected at the highest mavacamten concentration tested, 10 μM (Fig. S2). These changes resulted in a corresponding reduction in relative theoretical myosin ATP consumption, at 10 μM for controls, and at 1, 5, and 10 μM for HFrEF patients (Fig. 4B). In these slow twitch fibres, the absolute reduction in theoretical ATP consumption across the 0-10 μM range tended to be greater in HFrEF than in controls, consistent with a larger baseline energetic excess; however, the group by dose interaction did not reach statistical significance in our sample. In fast-twitch fibres, the effects of mavacamten were less pronounced. While 10 μM significantly lowered the DRX to SRX ratio (P1:P2 ratio; Fig. 4C), no meaningful changes in relative theoretical ATP consumption were detected (Fig. 4D), suggesting fibre type–specific responsiveness to SRX stabilization. Given the well described shift toward a higher proportion of type II fibres in HFrEF skeletal muscle,[9] these data imply that the aggregate effect of mavacamten on whole muscle ATP consumption in locomotor muscles may be attenuated compared with muscles with a higher type I fibre content.

Discussion

Here, we provide novel insights into skeletal muscle myosin maladaptation in HFrEF, demonstrated by the disrupted acetyl-lysine regulation and an altered DRX:SRX equilibrium, which together drive increased ATP consumption in sarcomeres/fibres. Importantly, the myosin-targeting small molecule mavacamten counteracts these maladaptations by stabilizing the SRX state and reducing the energy demand in a dose-dependent manner, suggesting a potential therapeutic strategy to address energy deficiency in HFrEF. Together, our data provide a basis for testable hypotheses regarding the contribution of skeletal muscle myosin energetics to exercise intolerance.

Muscle myosin dysfunction in HFrEF: Extending the clinical paradigm

The present study adds to the past evidence that exercise intolerance in HFrEF cannot be solely explained by the impaired cardiac output. Thus, we have identified a previously unrecognized molecular defect at the sarcomeric protein level, myosin. Specifically, the equilibrium between the biochemical DRX and SRX states is shifted towards a higher proportion of the ATP-consuming DRX conformation in both slow- and fast-twitch fibres from HFrEF patients than controls. ATP turnover time within each state did not differ between groups. This suggests that the higher basal energy consumption arises primarily from a shift in population occupancy rather than changes in intrinsic kinetics. Altogether, our findings indicate that skeletal muscle fibres in HFrEF patients exhibit energetic inefficiency at rest, imposing an elevated sarcomeric ATP demand that could limit the energetic reserve available for contraction characterized by elevated resting sarcomeric ATP demand (due to DRX shift) that diminishes the energetic reserve available for contraction. In turn, this may exacerbate overall metabolic stress and contribute to exercise intolerance, supporting a potential mechanistic link between elevated resting myosin ATP demand and reduced exercise capacity, though causality cannot be inferred from this correlation alone.[37] Our measurements quantified one component of resting sarcomeric ATP utilization and therefore contribute to understanding how energy is allocated at baseline, but they do not encompass total muscle ATP turnover, mitochondrial oxidative capacity or the cost of contraction across different workloads. Also, because our measurements were obtained under resting *ex vivo* conditions, these implications for contractile function and exercise bioenergetics should be viewed as hypothesis-generating and warrant confirmation in studies directly assessing *in*

vivo skeletal muscle performance. Alternatively, or in addition, the DRX:SRX imbalance in HFrEF patients may partially deprive other ATP-dependent processes that regulate fibre homeostasis and size, thereby contributing to cellular atrophy and skeletal muscle wasting, which in turn negatively affects exercise tolerance.[38]

Muscle myosin acetylation in HFrEF: Novel potential regulatory mechanism

Acetylation of lysine residues is a critical modulator of protein function and structural stability.[39] Our proteomic analysis identified 15 acetyl-lysine sites on myosin heavy chains that were dysregulated in HFrEF patients compared to controls, spanning cardiac-β/slow (MYH7), type IIa (MYH2) and Type IIx (MYH1) isoforms. Simulations and *in silico* studies suggest that many of these residues reside in functionally critical regions known to stabilize the interacting-heads motif (IHM), the structural basis of the energy-conserving SRX state.[30, 40] K34 and K35 are located in the head/mesa region and can form salt bridges, stabilizing IHM and SRX state.[30, 31] K562 and K568 are located at the interface of sub-fragment 1 and sub-fragment 2, and are involved in the dynamic folding and head-to-head contact.[31] K1079 and K1266 are located in the sub-fragment 2/tail region and are responsible for electrostatic tail-head contact, impacting energy conservation.[31] K1528 and K1791 are also located in the sub-fragment 2/tail region and anchor folding, stabilizing auto-inhibition and SRX state.[31] K205, K614, K829, K880, K914, K955 and K1655 are located in multiple interfaces and domains and are involved in head-to-head contact points, allowing shifts in SRX/DRX balance.[31] Taken together, these findings predict that altered lysine acetylation disrupts structural determinants of the SRX state, offering a plausible molecular mechanism for the heightened ATP consumption and energy imbalance observed in skeletal muscle of HFrEF patients. We did not perform acetyl proteomic profiling after ex vivo mavacamten treatment of skeletal muscle fibres. Our experiments were designed to probe the acute biochemical consequences of SRX stabilisation on myosin relaxation states and ATP consumption, and the time scale and conditions may not have been appropriate to elicit detectable changes in lysine acetylation. Whether chronic myosin ATPase inhibition can modulate myosin acetylation in vivo, and whether such changes contribute to sustained SRX stabilisation, remains an important question for future work.

Muscle myosin partners' isoform shifts in HFrEF: Another potential player

In addition to subtle but functionally relevant changes in myosin lysin acetylation, our proteomic analysis has also unveiled altered abundance of a key regulator of the SRX/DRX balance, MYBPC2.[15] MYBPC2, also known as fast skeletal myosin binding protein C, promotes formation of the myosin interacting-heads motif (IHM) and stabilization of the SRX state, thereby maintaining heads in the folded-back conformation and limiting ATPase activity.[41, 42] Consistent with impaired SRX stabilization, we observed reduced MYBPC2 expression in HFrEF muscle, accompanied by increased MYBPC1 expression (slow skeletal myosin-binding protein C). In parallel, our analyses indicated that the abundance of MYL1, MYL11 and MYLK (Ca^{2+}/calmodulin-dependent kinase that phosphorylates myosin regulatory light chains), were changed and may further affect the DRX and SRX conformations.[30, 40] Together, these changes provide an additional potential explanation for destabilization of the SRX state and enhanced myosin ATP consumption at rest in skeletal muscle of HFrEF patients.

Myosin ATPase inhibitors in HFrEF: Emerging therapeutic approach

Our results convincingly demonstrate the biochemical efficacy of mavacamten, a selective cardiac/β-slow myosin ATPase inhibitor, in shifting the myosin DRX to SRX ratio and lowering ATP consumption in a dose-dependent manner. The attenuation of the excessive resting energy demand due to SRX was most evident in slow-twitch muscle fibres, which predominantly express the cardiac/β-slow myosin heavy chain. In contrast, fast-twitch myofibres (type IIa and IIx myosin heavy chains isoforms), exhibited only blunted responses, as anticipated. In the context of HFrEF, where locomotor muscles frequently display a relative enrichment of type II fibres,[9] any systemic skeletal muscle benefit from a cardiac slow myosin selective inhibitor is therefore likely to be modest unless combined with strategies that also target fast twitch myosin isoforms or promote a shift toward a more oxidative phenotype.

Although mavacamten was originally developed to address hypertrophic cardiomyopathic pathologies,[36] our findings using an *ex vivo* approach provide a compelling rationale for targeting skeletal muscle myosin as therapeutic strategy to mitigate metabolic stress in HFrEF. Importantly, however, development of skeletal muscle-specific myosin inhibitors will be required to avoid risk of further lowering cardiac output in HFrEF patients (most frequently caused by coronary artery disease as was also the case for the patients included

in the present study).[36] As an example, in the EXPLORER trial investigating patients with hypertrophic obstructive cardiomyopathy and preserved LVEF, mavacamten had beneficial clinical effect in reducing left ventricular outflow gradients and symptoms. However, 10% of patients did experience a significant transient decrease in LVEF to < 50% to as low as 35%.[43] Future clinical studies should therefore assess the systemic effects of myosin ATPase inhibition on skeletal muscle energetics, function, and exercise performance in HFrEF. These cardiac findings mirror preclinical observations that stabilizing the SRX state can reduce contractile output in the myocardium,[44, 45] and they raise the possibility that excessive myosin inhibition in skeletal muscle could also compromise maximal force production or contribute to fatigue under high demand. Our ex vivo experiments, which were conducted under non contracting conditions, demonstrate biochemical normalization of myosin relaxation states and ATP utilization but do not address potential effects on force development or fatigability. Future work should therefore combine myosin targeted interventions with direct assessments of skeletal muscle function in both HFrEF and control populations.

From a therapeutic perspective, whether reducing resting skeletal muscle energy expenditure is desirable will depend on the extent to which this intervention preserves or compromises the capacity for force generation and power output during activity. Excessive stabilisation of the SRX state might theoretically blunt the contractile dynamics or alter fatigue development, especially in muscles already affected by atrophy and fibre-type shifts. Thus, future myosin-targeted strategies in HFrEF will need to balance improved resting energetics against the imperative to maintain adequate skeletal muscle strength and endurance, likely through the development of skeletal-specific agents with carefully titrated dosing regimens.

Limitations and considerations

Our study has primarily examined isolated permeabilized skeletal muscle fibres, which may not fully replicate *in vivo* conditions influenced by neural input, blood flow, and systemic metabolism.[14] The sample size, although robust for fibre-level biochemical assays, remains limited for broad population inferences and sex-specific effects, which have been reported in HFrEF muscle pathologies.[24] In addition, several clinical factors could have influenced our

findings. First, most patients with HFrEF are prescribed multiple cardiovascular medications (e.g., β-blockers, ACE inhibitors/ARNI, statins), which may independently modulate skeletal muscle metabolism, fibre composition, or exercise capacity.[46-49] As such, we cannot exclude the possibility that drug treatment contributed to some of the observed molecular or functional alterations. Second, although controls were age-, sex-, and BMI-matched, they were specifically selected to be free of cardiac disease and sedentary, which may not fully account for inter-individual differences in baseline exercise capacity, independent of HFrEF. We did not systematically quantify habitual physical activity or cardiorespiratory fitness in either group, and it is therefore possible that differences in daily locomotor loading contributed to the observed alterations in myosin relaxation states and ATP consumption in the vastus lateralis. Since this muscle is a primary locomotor muscle, these adaptations may differ from those in upper-limb muscles with distinct usage patterns and fibre-type distributions, and our findings should not be directly extrapolated to non-locomotor muscle groups without further investigation. These differences could partly confound the observed relationship between myosin ATP consumption and functional performance.

Conclusion

Our experiments identify a key maladaptation in skeletal muscle myosin dynamics in patients with HFrEF, defined by a shift toward the energy demanding DRX state that is consistent with, but does not by itself prove, a mechanistic contribution to skeletal muscle energy deficiency. Pharmacological stabilization of the energy-conserving SRX state by mavacamten corrected this defect and reduced myosin ATP consumption at rest. The findings extend the paradigm of HFrEF beyond cardiac dysfunction, providing molecular-level evidence that skeletal muscle energetics represent an actionable therapeutic target. Such strategies may ultimately enhance patient outcomes by addressing exercise intolerance and metabolic stress - limitations that remain insufficiently targeted by conventional cardiac-focused therapies.

Disclosure statement

The authors report no conflicts of interest. CTAL is now an employee at Novo Nordisk A/S. The work of this paper was performed prior to beginning this position and the conclusions drawn from this manuscript are in no way influenced by his current position.

Supplemental Materials

Supplemental methods

Tables S1-S3

Figures S1-S2

References 50-54

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Authors' contributions

HEB, KV and JO conceived the study; HEB and JO acquired funding; SP, RB, TSB, HEB, KV and JO supervised the work; JO managed the project; MA, CC, CZ, CTAL and RAES performed experiments; MA, CC, SP, RB, CZ, CTAL, TSB, RAES, HEB, KV and JO analyzed data and interpreted the results; all authors approved the manuscript.

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References

1. McDonagh TA, Metra M, Adamo M, Gardner RS, Baumbach A, Bohm M, Burri H, Butler J, Celutkiene J, Chioncel O, Cleland JGF, Coats AJS, Crespo-Leiro MG, Farmakis D, Gilard M, Heymans S, Hoes AW, Jaarsma T, Jankowska EA, Lainscak M, Lam CSP, Lyon AR, McMurray JJV, Mebazaa A, Mindham R, Muneretto C, Francesco Piepoli M, Price S, Rosano GMC, Ruschitzka F, Kathrine Skibelund A and Group ESCSD. 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure. *Eur Heart J*. 2021;42:3599-3726.
2. Savarese G, Becher PM, Lund LH, Seferovic P, Rosano GMC and Coats AJS. Global burden of heart failure: a comprehensive and updated review of epidemiology. *Cardiovasc Res*. 2023;118:3272-3287.
3. Zizola C and Schulze PC. Metabolic and structural impairment of skeletal muscle in heart failure. *Heart Fail Rev*. 2013;18:623-30.
4. Kinugawa S, Takada S, Matsushima S, Okita K and Tsutsui H. Skeletal Muscle Abnormalities in Heart Failure. *Int Heart J*. 2015;56:475-84.
5. Anker SD, Ponikowski P, Varney S, Chua TP, Clark AL, Webb-Peploe KM, Harrington D, Kox WJ, Poole-Wilson PA and Coats AJ. Wasting as independent risk factor for mortality in chronic heart failure. *Lancet*. 1997;349:1050-3.
6. Del Buono MG, Arena R, Borlaug BA, Carbone S, Canada JM, Kirkman DL, Garten R, Rodriguez-Miguelez P, Guazzi M, Lavie CJ and Abbate A. Exercise Intolerance in Patients With Heart Failure: JACC State-of-the-Art Review. *J Am Coll Cardiol*. 2019;73:2209-2225.
7. Wilson JR. Exercise intolerance in heart failure. Importance of skeletal muscle. *Circulation*. 1995;91:559-61.
8. Haykowsky MJ, Tomczak CR, Scott JM, Paterson DI and Kitzman DW. Determinants of exercise intolerance in patients with heart failure and reduced or preserved ejection fraction. *J Appl Physiol (1985)*. 2015;119:739-44.
9. Gallagher H, Hendrickse PW, Pereira MG and Bowen TS. Skeletal muscle atrophy, regeneration, and dysfunction in heart failure: Impact of exercise training. *J Sport Health Sci*. 2023;12:557-567.
10. Wiener DH, Fink LI, Maris J, Jones RA, Chance B and Wilson JR. Abnormal skeletal muscle bioenergetics during exercise in patients with heart failure: role of reduced muscle blood flow. *Circulation*. 1986;73:1127-36.
11. Wilson JR, Fink L, Maris J, Ferraro N, Power-Vanwart J, Eleff S and Chance B. Evaluation of energy metabolism in skeletal muscle of patients with heart failure with gated phosphorus-31 nuclear magnetic resonance. *Circulation*. 1985;71:57-62.
12. Miller MS, VanBuren P, LeWinter MM, Braddock JM, Ades PA, Maughan DW, Palmer BM and Toth MJ. Chronic heart failure decreases cross-bridge kinetics in single skeletal muscle fibres from humans. *J Physiol*. 2010;588:4039-53.
13. Miller MS, Vanburen P, Lewinter MM, Lecker SH, Selby DE, Palmer BM, Maughan DW, Ades PA and Toth MJ. Mechanisms underlying skeletal muscle weakness in human heart failure: alterations in single fiber myosin protein content and function. *Circ Heart Fail*. 2009;2:700-6.
14. Gordon AM, Homsher E and Regnier M. Regulation of contraction in striated muscle. *Physiol Rev*. 2000;80:853-924.
15. McNamara JW, Li A, Dos Remedios CG and Cooke R. The role of super-relaxed myosin in skeletal and cardiac muscle. *Biophys Rev*. 2015;7:5-14.
16. Stewart MA, Franks-Skiba K, Chen S and Cooke R. Myosin ATP turnover rate is a mechanism involved in thermogenesis in resting skeletal muscle fibers. *Proc Natl Acad Sci U S A*. 2010;107:430-5.
17. Lewis CTA, Moreno-Justicia R, Savoure L, Calvo E, Bak A, Laitila J, Seaborne RAE, Larsen S, Iwamoto H, Cefis M, Morais JA, Gouspillou G, Alegre-Cebollada J, Hawke TJ, Vazquez J, Adrover M, Marcangeli V, Hammad R, Granet J, Gaudreau P, Aubertin-Leheudre M, Belanger M, Robitaille R, Deshmukh AS and Ochala J. Dysregulated skeletal muscle

myosin super-relaxation and energetics in male participants with type 2 diabetes mellitus. *Diabetologia*. 2025; 68:1836-1850.
18. Buvoli M, Wilson GC, Buvoli A, Gugel JF, Hau A, Bonnemann CG, Paradas C, Ryba DM, Woulfe KC, Walker LA, Buvoli T, Ochala J and Leinwand LA. A Laing distal myopathy-associated proline substitution in the beta-myosin rod perturbs myosin cross-bridging activity. *J Clin Invest*. 2024;134(9):e172599.
19. Carrington G, Hau A, Kosta S, Dugdale HF, Muntoni F, D'Amico A, Van den Bergh P, Romero NB, Malfatti E, Vilchez JJ, Oldfors A, Pajusalu S, Ounap K, Giralt-Pujol M, Zanoteli E, Campbell KS, Iwamoto H, Peckham M and Ochala J. Human skeletal myopathy myosin mutations disrupt myosin head sequestration. *JCI Insight*. 2023;8 :e172322.
20. Laitila J, Seaborne RAE, Ranu N, Kolb JS, Wallgren-Pettersson C, Witting N, Vissing J, Vilchez JJ, Zanoteli E, Palmio J, Huovinen S, Granzier H and Ochala J. Myosin ATPase inhibition fails to rescue the metabolically dysregulated proteome of nebulin-deficient muscle. *J Physiol*. 2024;602:5229-5245.
21. van den Berg M, Shi Z, Claassen WJ, Hooijman P, Lewis CTA, Andersen JL, van der Pijl RJ, Bogaards SJP, Conijn S, Peters EL, Begthel LPL, Uijterwijk B, Lindqvist J, Langlais PR, Girbes ARJ, Stapel S, Granzier H, Campbell KS, Ma W, Irving T, Hwee DT, Hartman JJ, Malik FI, Paul M, Beishuizen A, Ochala J, Heunks L and Ottenheijm CAC. Super-relaxed myosins contribute to respiratory muscle hibernation in mechanically ventilated patients. *Sci Transl Med*. 2024;16:eadg3894.
22. Guyatt GH, Sullivan MJ, Thompson PJ, Fallen EL, Pugsley SO, Taylor DW and Berman LB. The 6-minute walk: a new measure of exercise capacity in patients with chronic heart failure. *Can Med Assoc J*. 1985;132:919-23.
23. Groennebaek T, Sieljacks P, Nielsen R, Pryds K, Jespersen NR, Wang J, Carlsen CR, Schmidt MR, de Paoli FV, Miller BF, Vissing K and Botker HE. Effect of Blood Flow Restricted Resistance Exercise and Remote Ischemic Conditioning on Functional Capacity and Myocellular Adaptations in Patients With Heart Failure. *Circ Heart Fail*. 2019;12:e006427.
24. Wood N, Critchlow A, Cheng CW, Straw S, Hendrickse PW, Pereira MG, Wheatcroft SB, Egginton S, Witte KK, Roberts LD and Bowen TS. Sex Differences in Skeletal Muscle Pathology in Patients With Heart Failure and Reduced Ejection Fraction. *Circ Heart Fail*. 2024;17:e011471.
25. Lewis CTA, Melhedegaard EG, Ognjanovic MM, Olsen MS, Laitila J, Seaborne RAE, Gronset M, Zhang C, Iwamoto H, Hessel AL, Kuehn MN, Merino C, Amigo N, Frobert O, Giroud S, Staples JF, Goropashnaya AV, Fedorov VB, Barnes B, Toien O, Drew K, Sprenger RJ and Ochala J. Remodeling of skeletal muscle myosin metabolic states in hibernating mammals. *Elife*. 2024; 13:RP94616.
26. Lewis CTA and Ochala J. Myosin Heavy Chain as a Novel Key Modulator of Striated Muscle Resting State br. *Physiology*. 2023;38:3-9.
27. Knuiman P, Straw S, Gierula J, Koshy A, Roberts LD, Witte KK, Ferguson C and Bowen TS. Quantifying the relationship and contribution of mitochondrial respiration to systemic exercise limitation in heart failure. *ESC Heart Fail*. 2021;8:898-907.
28. Renguet E, Bultot L, Beauloye C, Horman S and Bertrand L. The Regulation of Insulin-Stimulated Cardiac Glucose Transport via Protein Acetylation. *Front Cardiovasc Med*. 2018;5:70.
29. Tsuda M, Fukushima A, Matsumoto J, Takada S, Kakutani N, Nambu H, Yamanashi K, Furihata T, Yokota T, Okita K, Kinugawa S and Anzai T. Protein acetylation in skeletal muscle mitochondria is involved in impaired fatty acid oxidation and exercise intolerance in heart failure. *J Cachexia Sarcopenia Muscle*. 2018;9:844-859.
30. Alamo L, Ware JS, Pinto A, Gillilan RE, Seidman JG, Seidman CE and Padron R. Effects of myosin variants on interacting-heads motif explain distinct hypertrophic and dilated cardiomyopathy phenotypes. *Elife*. 2017;6:e24634.
31. Anderson RL, Trivedi DV, Sarkar SS, Henze M, Ma W, Gong H, Rogers CS, Gorham JM, Wong FL, Morck MM, Seidman JG, Ruppel KM, Irving TC, Cooke R, Green EM and Spudich JA. Deciphering the super relaxed state of human beta-cardiac myosin and the

591 mode of action of mavacamten from myosin molecules to muscle fibers. *Proc Natl Acad Sci*
592 *U S A*. 2018;115:E8143-E8152.
593 32. Lin YH, Schmidt W, Fritz KS, Jeong MY, Cammarato A, Foster DB, Biesiadecki BJ,
594 McKinsey TA and Woulfe KC. Site-specific acetyl-mimetic modification of cardiac troponin I
595 modulates myofilament relaxation and calcium sensitivity. *J Mol Cell Cardiol*. 2020;139:135-
596 147.
597 33. Van der Pijl RJ, Ma W, Lewis CTA, Haar L, Buhl A, Farman GP, Rhodehamel M, Jani
598 VP, Nelson OL, Zhang C, Granzier H and Ochala J. Increased cardiac myosin super-
599 relaxation as an energy saving mechanism in hibernating grizzly bears. *Mol Metab*.
600 2025;92:102084.
601 34. Gollapudi SK, Ma W, Chakravarthy S, Combs AC, Sa N, Langer S, Irving TC and
602 Nag S. Two Classes of Myosin Inhibitors, Para-nitroblebbistatin and Mavacamten, Stabilize
603 beta-Cardiac Myosin in Different Structural and Functional States. *J Mol Biol*.
604 2021;433:167295.
605 35. Olivotto I, Oreziak A, Barriales-Villa R, Abraham TP, Masri A, Garcia-Pavia P, Saberi
606 S, Lakdawala NK, Wheeler MT, Owens A, Kubanek M, Wojakowski W, Jensen MK, Gimeno-
607 Blanes J, Afshar K, Myers J, Hegde SM, Solomon SD, Sehnert AJ, Zhang D, Li W,
608 Bhattacharya M, Edelberg JM, Waldman CB, Lester SJ, Wang A, Ho CY, Jacoby D,
609 Bartunek J, Bondue A, Van Craenenbroeck E, Kubanek M, Zemanek D, Jensen M,
610 Mogensen J, Thune JJ, Charron P, Hagege A, Lairez O, Trochu J-N, Axthelm C, Duengen H-
611 D, Frey N, Mitrovic V, Preusch M, Schulz-Menger J, Seidler T, Arad M, Halabi M, Katz A,
612 Monakier D, Paz O, Viskin S, Zwas D, Olivotto I, Brunner-La Rocca HP, Michels M, Dudek
613 D, Oko-Sarnowska Z, Oreziak A, Wojakowski W, Cardim N, Pereira H, Barriales-Villa R,
614 García Pavia P, Gimeno Blanes J, Hidalgo Urbano R, Rincón Diaz LM, Elliott P, Yousef Z,
615 Abraham T, Afshar K, Alvarez P, Bach R, Becker R, Choudhury L, Fermin D, Jacoby D,
616 Jefferies J, Kramer C, Lakdawala N, Lester S, Marian A, Masri A, Maurer M, Nagueh S,
617 Owens A, Owens D, Rader F, Saberi S, Sherrid M, Shirani J, Symanski J, Turer A, Wang A,
618 Wever-Pinzon O, Wheeler M, Wong T and Yamani M. Mavacamten for treatment of
619 symptomatic obstructive hypertrophic cardiomyopathy (EXPLORER-HCM): a randomised,
620 double-blind, placebo-controlled, phase 3 trial. *The Lancet*. 2020;396:759-769.
621 36. Lehman SJ, Crocini C and Leinwand LA. Targeting the sarcomere in inherited
622 cardiomyopathies. *Nat Rev Cardiol*. 2022;19:353-363.
623 37. Poehlman ET, Scheffers J, Gottlieb SS, Fisher ML and Vaitekevicius P. Increased
624 resting metabolic rate in patients with congestive heart failure. *Ann Intern Med*.
625 1994;121:860-2.
626 38. Bowen TS, Schuler G and Adams V. Skeletal muscle wasting in cachexia and
627 sarcopenia: molecular pathophysiology and impact of exercise training. *J Cachexia*
628 *Sarcopenia Muscle*. 2015;6:197-207.
629 39. Yang M, Zhang Y and Ren J. Acetylation in cardiovascular diseases: Molecular
630 mechanisms and clinical implications. *Biochim Biophys Acta Mol Basis Dis*.
631 2020;1866:165836.
632 40. Alamo L, Qi D, Wriggers W, Pinto A, Zhu J, Bilbao A, Gillilan RE, Hu S and Padron R.
633 Conserved Intramolecular Interactions Maintain Myosin Interacting-Heads Motifs Explaining
634 Tarantula Muscle Super-Relaxed State Structural Basis. *J Mol Biol*. 2016;428:1142-1164.
635 41. Harris SP. Making waves: A proposed new role for myosin-binding protein C in
636 regulating oscillatory contractions in vertebrate striated muscle. *J Gen Physiol*. 2021;153.
637 42. McNamara JW, Song T, Alam P, Binek A, Singh RR, Nieman ML, Koch SE, Ivey MJ,
638 Lynch TL, Rubinstein J, Jin JP, Lorenz JN, Van Eyk JE, Kanisicak O and Sadayappan S.
639 Fast skeletal myosin binding protein-C expression exacerbates dysfunction in heart failure.
640 *bioRxiv*. 2024; 3:2024.04.30.591979.
641 43. Papadakis M, Basu J and Sharma S. Mavacamten: treatment aspirations in
642 hypertrophic cardiomyopathy. *Lancet*. 2020;396:736-737.
643 44. Toepfer CN, Garfinkel AC, Venturini G, Wakimoto H, Repetti G, Alamo L, Sharma A,
644 Agarwal R, Ewoldt JK, Cloonan P, Letendre J, Lun M, Olivotto I, Colan S, Ashley E, Jacoby
645 D, Michels M, Redwood CS, Watkins HC, Day SM, Staples JF, Padron R, Chopra A, Ho CY,

Chen CS, Pereira AC, Seidman JG and Seidman CE. Myosin Sequestration Regulates Sarcomere Function, Cardiomyocyte Energetics, and Metabolism, Informing the Pathogenesis of Hypertrophic Cardiomyopathy. *Circulation*. 2020;141:828-842.
45. Toepfer CN, Wakimoto H, Garfinkel AC, McDonough B, Liao D, Jiang J, Tai AC, Gorham JM, Lunde IG, Lun M, Lynch TLt, McNamara JW, Sadayappan S, Redwood CS, Watkins HC, Seidman JG and Seidman CE. Hypertrophic cardiomyopathy mutations in MYBPC3 dysregulate myosin. *Sci Transl Med*. 2019;11:eaat1199.
46. Parker BA and Thompson PD. Effect of statins on skeletal muscle: exercise, myopathy, and muscle outcomes. *Exerc Sport Sci Rev*. 2012;40:188-94.
47. Boschmann M, Engeli S, Adams F, Franke G, Luft FC, Sharma AM and Jordan J. Influences of AT1 receptor blockade on tissue metabolism in obese men. *Am J Physiol Regul Integr Comp Physiol*. 2006;290:R219-23.
48. Robinson MM, Bell C, Peelor FF, 3rd and Miller BF. beta-Adrenergic receptor blockade blunts postexercise skeletal muscle mitochondrial protein synthesis rates in humans. *Am J Physiol Regul Integr Comp Physiol*. 2011;301:R327-34.
49. Carter CS, Onder G, Kritchevsky SB and Pahor M. Angiotensin-converting enzyme inhibition intervention in elderly persons: effects on body composition and physical performance. *J Gerontol A Biol Sci Med Sci*. 2005;60:1437-46.
50. Jones STM, Paradine Cullup FE, Gollapudi SK, Melhedegaard EG, Steeples V, Schmid M, Psaras Y, Robinson P, Pena A, Barefield DY, Ochala J, Nag S and Toepfer CN. Assaying the myosin super-relaxed state across muscle types, cells and proteins for understanding muscle biology and use in drug discovery. *Nat Protoc*. 2025. In press.
51. Laitila J, Seaborne RAE, Ranu N, Kolb JS, Wallgren-Pettersson C, Witting N, Vissing J, Vilchez JJ, Zanoteli E, Palmio J, Huovinen S, Granzier H and Ochala J. Myosin ATPase inhibition fails to rescue the metabolically dysregulated proteome of nebulin-deficient muscle. *J Physiol*. 2024; 602:5229-5245.
52. Van der Pijl RJ, Ma W, Lewis CTA, Haar L, Buhl A, Farman GP, Rhodehamel M, Jani VP, Nelson OL, Zhang C, Granzier H and Ochala J. Increased cardiac myosin super-relaxation as an energy saving mechanism in hibernating grizzly bears. *Mol Metab*. 2025;92:102084.
53. Santos A, Colaco AR, Nielsen AB, Niu L, Strauss M, Geyer PE, Coscia F, Albrechtsen NJW, Mundt F, Jensen LJ and Mann M. A knowledge graph to interpret clinical proteomics data. *Nat Biotechnol*. 2022;40:692-702.
54. Ge SX, Jung D and Yao R. ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics*. 2020;36:2628-2629.

Tables

Table 1. Characteristics of the donors.

Data are presented as Mean ± Standard Deviation. BMI refers to body mass index; Ctrl = controls; HFrEF = patients diagnosed with HFrEF; NYHA = New York Heart Association classification system of chronic heart failure with 1 defined as no symptoms and no limitation in physical activity and 4 referring to inability to carry out any physical activity without discomfort; symptoms present even at rest. LVEF = left ventricular ejection fraction; 6MWT = 6-minute walk test to assess aerobic capacity and endurance (a previous meta-analysis indicates that for healthy control males aged between 60 and 69, the mean distance is 638 m).[22] Note out of the eleven HFrEF patients, five were NYHA class 1; four were class 2 and two were class 3. All the clinical details have previously been published.[23]

	Ctrl (N=10)	**HFrEF (N=11)**
Sex (% male)	100	100
Age (years)	65.2 ± 5.9	62.2 ± 10.0
BMI ($kg.m^{-2}$)	27.6 ± 4.1	26.5 ± 4.2
Diabetes (type 1 or 2) (%)	0	0
NYHA classification	-	1.8 ± 0.8
LVEF (%)	-	34.8 ± 6.3
6MWT (m)	-	553.3 ± 79.9

Figure legends

Figure 1. Muscle myosin dynamics in HFrEF.

Skeletal muscle fibres were dissected from patients with HFrEF (N = 11) and controls (Ctrl, N = 10). (A) displays typical Mant-ATP chase experiments, from two slow-twitch fibres of one Ctrl and one HFrEF patient, to estimate the proportions of myosin molecules in the DRX (B; P1) or SRX states (C; P2). DRX (D; T1) or SRX ATP turnover lifetimes (E; T2) and related theoretical myosin ATP consumptions (F). (G) shows clear significant linear correlations between myosin ATP consumption and exercise capacity defined as 6MWT (r = -0.7491, R^2 = 0.56, P = 0.008 for slow-twitch muscle fibres and r = -0.8176, R^2 = 0.66, P = 0.0021 for fast-twitch myofibres). As multiple measurements were done per individual (minimum of 15 single muscle fibres per subject), dots correspond to averages. Data were divided according to their types (slow = slow-twitch versus fast = fast-twitch). One-way ANOVAs were applied with $P < 0.05$ as level of significance. DRX = disordered-relaxed state; SRX = super-relaxed state; P1 = amplitude of the initial rapid decay approximating the DRX state; P2 = amplitude of the slower second decay approximating the SRX state; T1 = time constant for the DRX state; T2 = time constant for the SRX state; and 6MWT = 6-minute walk test (index of exercise of capacity).

Figure 2. Global proteome skeletal muscle profiling in HFrEF.

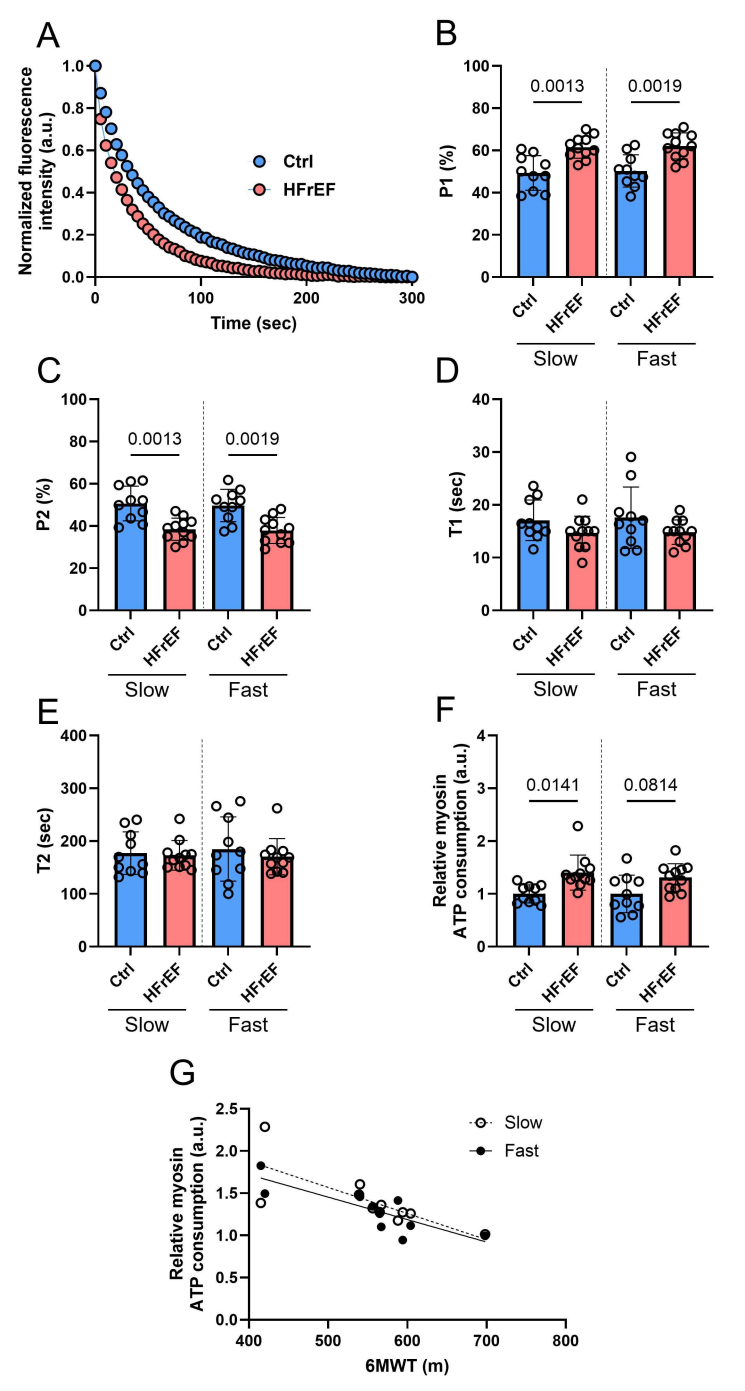
Global proteome of from patients with HFrEF (HFrEF; N = 11) and controls (Ctrl; N = 10). (A) Principal component analysis of patient skeletal muscle proteome displaying a clustering of samples influenced largely PC2 (= Second Principal Component) rather than PC1 (= First Principal Component). (B) Permutation (250) analysis revealed 275 differentially abundant proteins when comparing HFrEF to Ctrl patient (116 proteins upregulated, 159 downregulated), including several sarcomeric proteins related to myosin (see Table S1). (C). Enrichment analysis of biological processing Gene Ontology (GO:BP) terms revealed a list of significant terms (including those related to striated muscle and regulation of muscle contraction) only found in significantly downregulated proteins. No significantly enriched GO:BP terms were identified in the upregulated proteins.

Figure 3. Aberrant myosin acetylation levels in HFrEF.

(A) displays the three myosin heavy chain sequences, MYH7, MYH2 and MYH1 with their various regions (motor domain, lever arm and rod region). This panel also presents the various acetyl-lysine sites that are altered in HFrEF. (B) is the *in-silico* simulation (EvoEF) where ΔΔGStabilities were calculated for mutations chemically mimicking acetyl-lysines. Note that EvoDesign physical Energy Function (EvoEF) was employed to estimate the protein stability change upon mutation, expressed as ΔΔG. EvoEF was applied to compute the stabilities (= ΔΔG stabilities).

Figure 4. Muscle myosin dynamics with mavacamten.

Skeletal myofibres were isolated from patients with HFrEF (red, N = 8) and controls (Ctrl, blue, N = 8). (A and C) shows the myosin DRX to SRX ratio with varying concentrations of mavacamten (0, 1, 5 and 10 µM) while (B and D) represents the estimated related theoretical myosin ATP consumptions. (A) and (B) are data for slow-twitch muscle fibres. (C) and (D) are findings for fast-twitch fibres. As multiple measurements were done per individual (minimum of 10 myofibres per individual), dots correspond to averages. For each fibre type, two-way repeated-measures ANOVAs with factors group (Ctrl vs HFrEF) and dose (0, 1, 5, 10 µM) were used. P-values indicated in the panels correspond to within-group effects of increasing mavacamten concentration. DRX = disordered-relaxed state; SRX = super-relaxed state; P1 = amplitude of the initial rapid decay approximating the DRX state; P2 = amplitude of the slower second decay approximating the SRX state. The ATP turnover times of DRX (T1) and SRX (T2) are presented in Fig. S2. Note that these experiments were performed in a subset of participants (when compared to Fig. 1) with sufficient remaining tissue for multiple mavacamten concentrations, which explains the smaller baseline difference between groups at 0 µM.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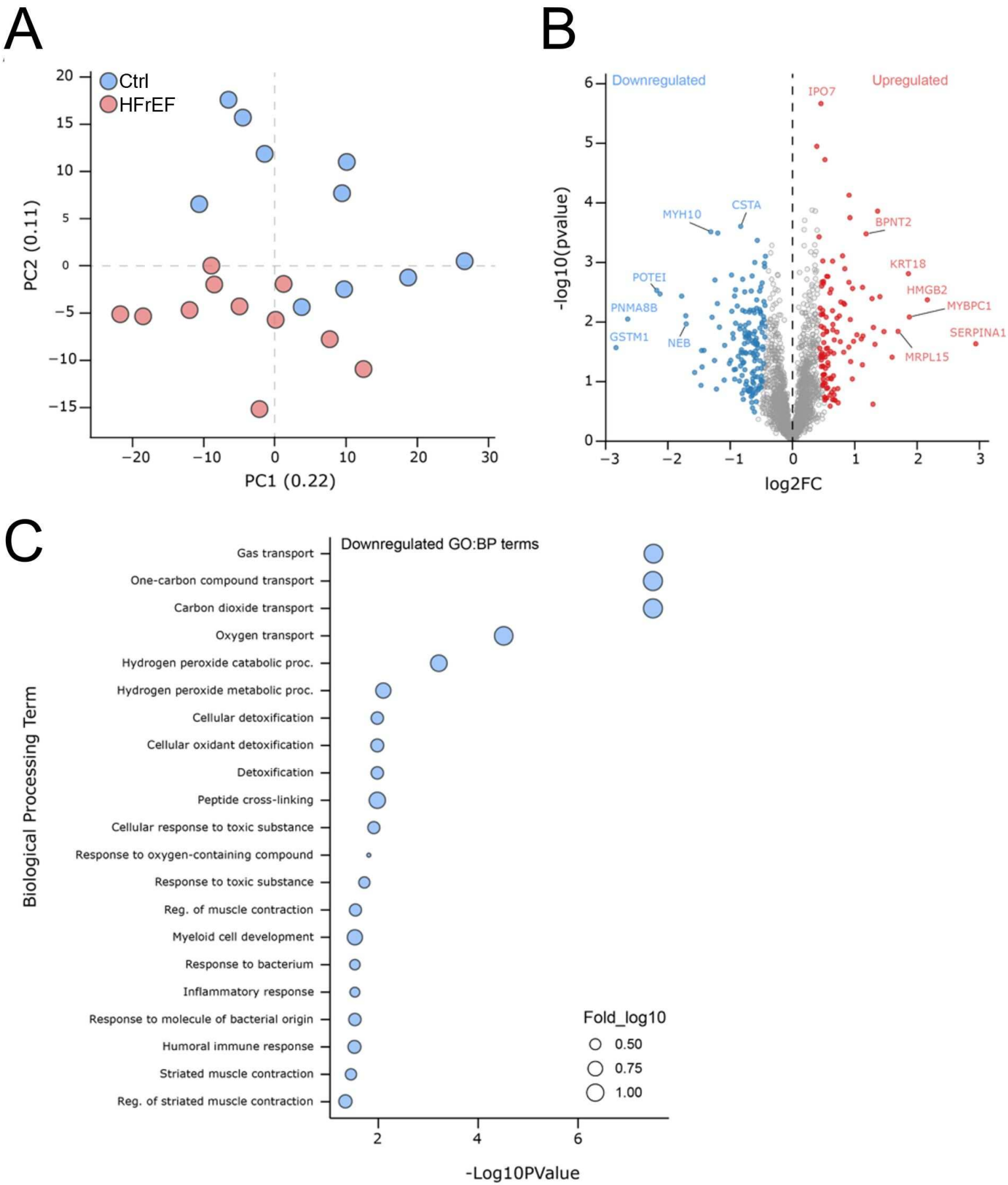
Normalized fluorescence intensity (a.u.)
Time (sec)
Ctrl
HFrEF
B
P1 (%)
0.0013
0.0019
Ctrl
HFrEF
Slow
Fast
C
P2 (%)
0.0013
0.0019
D
T1 (sec)
E
T2 (sec)
F
Relative myosin ATP consumption (a.u.)
0.0141
0.0814
G
Relative myosin ATP consumption (a.u.)
6MWT (m)
Slow
Fast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HFrEF
PC2 (0.11)
PC1 (0.22)
B
Downregulated
Upregulated
IPO7
MYH10
CSTA
BPNT2
KRT18
POTEI
HMGB2
PNMA8B
MYBPC1
GSTM1
NEB
SERPINA1
MRPL15
-log10(pvalue)
log2FC
C
Downregulated GO:BP terms
Biological Processing Term
Gas transport
One-carbon compound transport
Carbon dioxide transport
Oxygen transport
Hydrogen peroxide catabolic proc.
Hydrogen peroxide metabolic proc.
Cellular detoxification
Cellular oxidant detoxification
Detoxification
Peptide cross-linking
Cellular response to toxic substance
Response to oxygen-containing compound
Response to toxic substance
Reg. of muscle contraction
Myeloid cell development
Response to bacterium
Inflammatory response
Response to molecule of bacterial origin
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Striated muscle contraction
Reg. of striated muscle contraction
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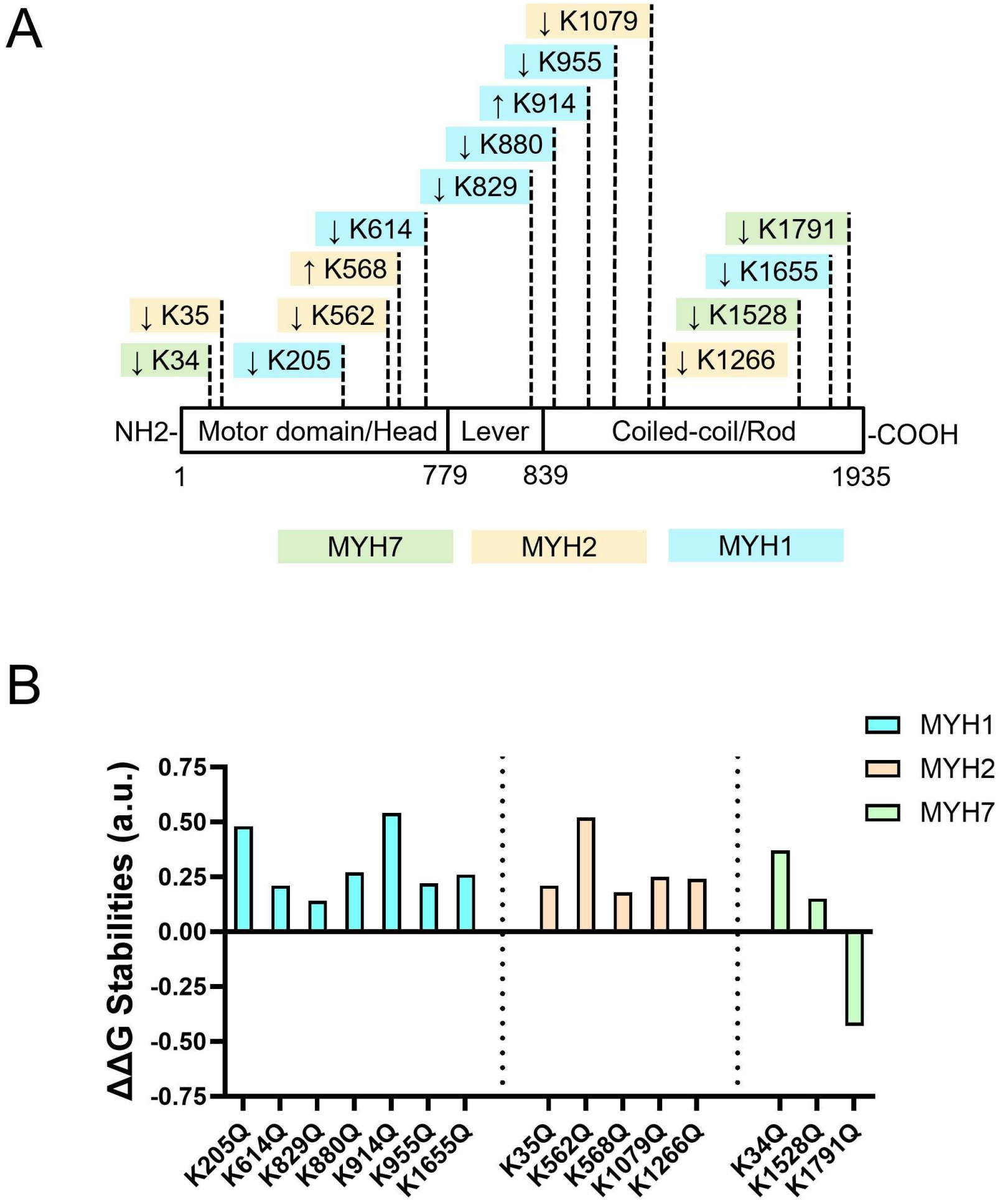
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↓ K880
↓ K829
↓ K614
↑ K568
↓ K562
↓ K35
↓ K34
↓ K205
↓ K1791
↓ K1655
↓ K1528
↓ K1266
NH2-
Motor domain/Head
Lever
Coiled-coil/Rod
-COOH
1
779
839
1935
MYH7
MYH2
MYH1
B
MYH1
MYH2
MYH7
ΔΔG Stabilities (a.u.)
0.75
0.50
0.25
0.00
-0.25
-0.50
-0.75
K205Q
K614Q
K829Q
K880Q
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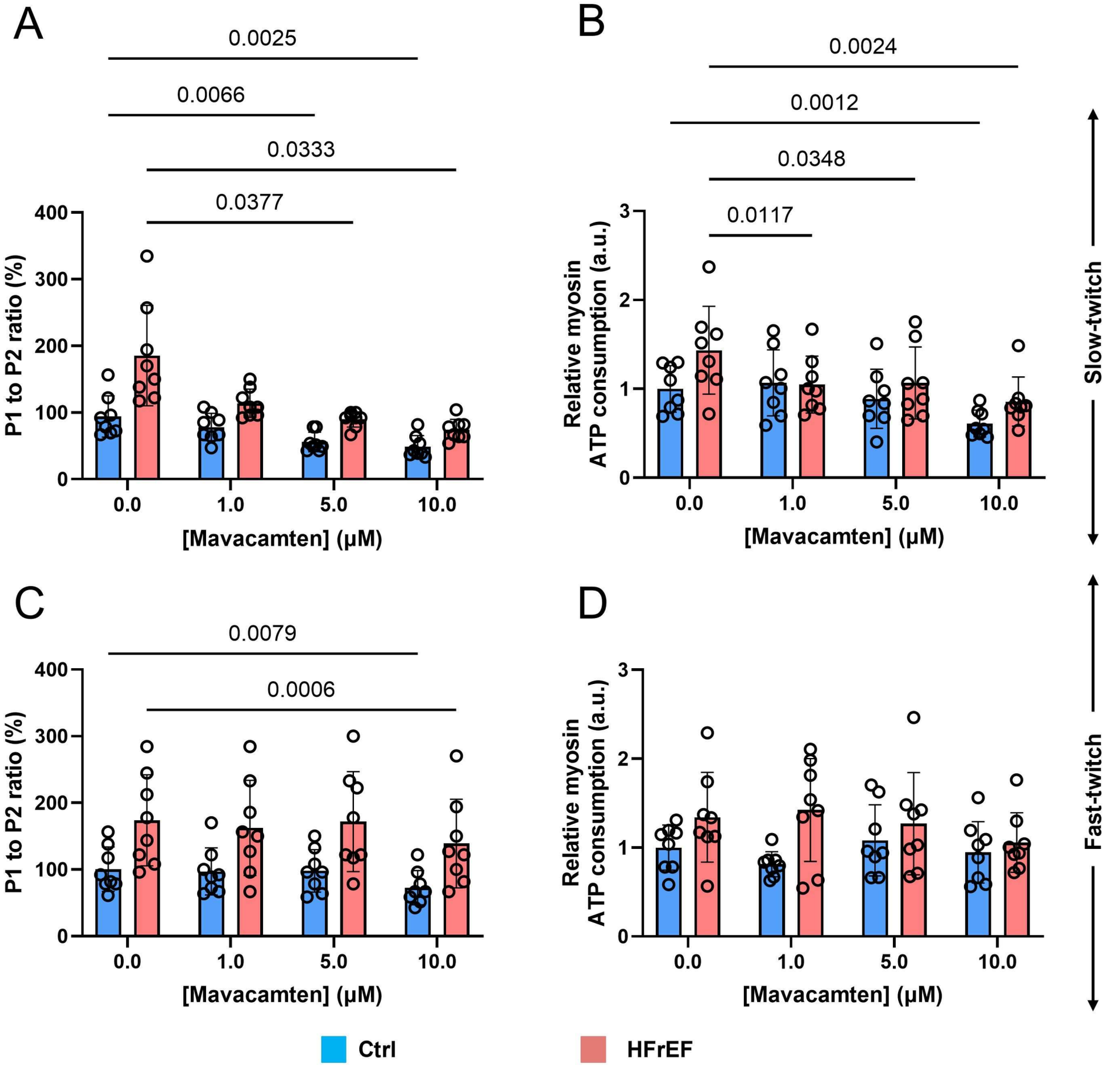
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0.0377
P1 to P2 ratio (%)
400
300
200
100
0
0.0
1.0
5.0
10.0
[Mavacamten] (μM)
B
0.0024
0.0012
0.0348
0.0117
Relative myosin ATP consumption (a.u.)
3
2
1
0
0.0
1.0
5.0
10.0
[Mavacamten] (μM)
Slow-twitch
C
0.0079
0.0006
P1 to P2 ratio (%)
400
300
200
100
0
0.0
1.0
5.0
10.0
[Mavacamten] (μM)
D
Relative myosin ATP consumption (a.u.)
3
2
1
0
0.0
1.0
5.0
10.0
[Mavacamten] (μM)
Fast-twitch
Ctrl
HFrEF