White Rose Research Online

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk
Universities of Leeds, Sheffield and York

Deposited via The University of Leeds.

White Rose Research Online URL for this paper:
https://eprints.whiterose.ac.uk/id/eprint/245901/

Version: Accepted Version

Article:
Vahle, B., Weidner, S., Tomalka, A. et al. (2026) Targeting Mitochondrial Dysfunction With Elamipretide (SS-31) Improves Skeletal Muscle Performance in a HFpEF Rat Model. Circulation Heart Failure, 19 (8). e014397. ISSN: 1941-3289

https://doi.org/10.1161/circheartfailure.126.014397

This is an author produced version of an article published in Circulation: Heart Failure, made available via the University of Leeds Research Outputs Policy under the terms of the Creative Commons Attribution License (CC-BY), which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

Reuse
This article is distributed under the terms of the Creative Commons Attribution (CC BY) licence. This licence allows you to distribute, remix, tweak, and build upon the work, even commercially, as long as you credit the authors for the original work. More information and the full terms of the licence here: https://creativecommons.org/licenses/

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

UNIVERSITY OF LEEDS
University of Sheffield
UNIVERSITY of York

Targeting Mitochondrial Dysfunction with Elamipretide (SS-31) Improves Skeletal Muscle Performance in a HFpEF Rat Model

Beatrice Vahle, MSc, [1], Sven Weidner, PhD, [2], André Tomalka, PhD, [2], Antje Schauer, PhD, [1], Antje Augstein, PhD, [1], Anita Männel; BSc, [1], Peggy Barthel, BSc, [1], Janet Friedrich, BSc, [1], Grietje Beck, MD, [3], Siegfried Labeit, MD, [3], T Scott Bowen, PhD, [4], Tobias Siebert, PhD, [2,5], Axel Linke, MD, [1,†] and Volker Adams, PhD, [1, †]

Affiliations:

[1]Heart Center Dresden, Laboratory of Molecular and Experimental Cardiology, TU Dresden; 01307 Dresden, Germany

[2]Motion and Exercise Science, University of Stuttgart; 70569 Stuttgart, Germany

[3]Integrative Pathophysiology, Dept. for Anesthesiology & Intensive Operative Care, Medical Faculty Mannheim, University of Heidelberg; 68169 Mannheim, Germany

[4]School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds; Leeds, United Kingdom

[5]Stuttgart Center for Simulation Science, University of Stuttgart; 70569 Stuttgart, Germany.

†Shared last authorship

Short title: SS-31 Improves Skeletal Muscle Function in HFpEF

Address for correspondence:

Beatrice Vahle

Department of Internal Medicine and Cardiology

Laboratory of Experimental and Molecular Cardiology

Heart Center, University Clinic

University of Technology Dresden,

Fiedlerstraße 42, 01307 Dresden, Germany

beatrice.vahle@tu-dresden.de; Phone: +49 351 458 6627

Word count: 7241

Abstract:

Background

Exercise intolerance, promoted by skeletal muscle (SKM)- and mitochondrial dysfunction, has been identified as a therapeutic target in heart failure with preserved ejection fraction (HFpEF). In the context of mitochondrial dysfunction, altered cardiolipin integrity has been reported in the myocardium of HFpEF, suggesting Elamipretide, a cardiolipin stabilizing agent, as potential therapeutic approach. The present study investigated cardiolipin dysregulation in the SKM of HFpEF rats and analyzed the effect of Elamipretide treatment.

Methods

Female ZSF1 lean (n=10, control (con)) and obese rats (n=24, HFpEF) were included. At 20 weeks of age, HFpEF rats were randomized into two groups receiving NaCl (n=12) or Elamipretide (n=12) for 12 weeks. SKM tissue was collected for whole-muscle force, single-fiber mechanics, mitochondrial respiration, histology and molecular analyses.

Results

HFpEF rats exhibited reduced cardiolipin levels (-6.8%, $p = 0.007$) and maturation (shown via tafazzin expression), contractile dysfunction, titin hyperphosphorylation, fiber atrophy and increased oxidative stress markers. Elamipretide improved whole muscle (Sol: +8.2%, $p = 0.041$, EDL: +10.9%, $p = 0.016$) and single-fiber (Sol: +173.2%, $p < 0.001$, EDL: 66.0%, p = ns) contractile function and titin phosphorylation (Sol: -35.4%, $p < 0.001$, EDL: -40.2%, $p < 0.001$), while preventing atrophy development (Sol: +49%, $p = 0.001$, EDL: +54.8%, $p < 0.001$) Improved mitochondrial function, presumably by cardiolipin-mediated improvements in oxidative phosphorylation. could be associated with muscle force and cardiolipin integrity.

Conclusions

Our data highlight cardiolipin stabilization as a key modulator of mitochondrial and contractile function in HFpEF, emerging Elamipretide as a promising therapeutic approach for SKM dysfunction.

Key Words: ZSF1; HFpEF; Elamipretide; SS-31; mitochondria; skeletal muscle; titin

What is new:

- Cardiolipin was significantly reduced in the skeletal muscle of established heart failure with preserved ejection fraction (HFpEF).
- Contrary to the myocardium, Elamipretide improves skeletal muscle function in a rat model of HFpEF.
- HFpEF is associated with skeletal muscle mitochondrial dysfunction, which was attenuated by Ela treatment despite no change in mitochondrial content.
- HFpEF-induced hyperphosphorylation of titin at S11878 in the PEVK region was prevented by Elamipretide.
- Elamipretide reduced oxidative stress, which exhibits an inverse association with muscle force.

What are the clinical implications

- Elamipretide may represent a novel therapeutic option targeting skeletal muscle dysfunction in HFpEF.
- Combination therapy with agents targeting myocardial and metabolic pathways (e.g., GLP-1 receptor agonists and SGLT2 inhibitors) may be required for comprehensive HFpEF management.

Non-standard abbreviations

ANT	
CL	Cardiolipin
con	control
CSA	cross-sectional area
EDL	extensor digitorum longus
Ela/SS-31	Elamipretide
Fis1	mitochondrial fission protein 1
GDF8	myostatin
HF	heart failure
HFpEF	heart failure with preserved ejection fraction
MCU	mitochondrial calcium uniporter
MAFbx	Muscle Atrophy F-box gene
MHC	Myosin Heavy Chain
MLC	Myosin Light Chain
MuRF1	Muscle Ring-Finger Protein-1
NOX2	NADPH oxidase 2
ROS	reactive oxygen species
SDH	succinate-dehydrogenase
s_e	
SKM	skeletal muscle
Sol	soleus

SOD2	superoxide dismutase 2
TAZ	tafazzin
VAGE	vertical agarose gel electrophoresis
WB	western blot
ZSF1	zucker fatty spontaneously hypertensive heart failure F1 hybrid
rat	

1 INTRODUCTION

Heart failure with preserved ejection fraction (HFpEF) is a heterogeneous syndrome characterized by a wide range of clinical phenotypes, complicating the understanding of the underlying pathophysiology and finding effective treatments. One symptom that is common amongst all HFpEF patients is exercise intolerance, which contributes to reduced quality of life and increased morbidity.[1–3] Impairment of contractile protein expression and of ATP production by mitochondrial oxidative phosphorylation, both in skeletal muscle (SKM) and in the myocardium, are key contributors to exercise intolerance. These changes impact locomotion, ventilation, as well as cardiac contraction and relaxation.[4,5] In the context of mitochondrial dysfunction, earlier studies reported reduced cardiolipin (CL) and tafazzin (TAZ) levels in the myocardium of heart failure rats and patients.[6,7] While CL plays an important role in mitochondrial bioenergetics and cristae formation, TAZ is critically involved in CL maturation. Consistent with this mechanism, alterations in cristae morphology have been reported several times, in both animal models and patients with HFpEF.[7,8] However, it remains unclear if CL homeostasis is influenced in SKM by HFpEF, despite CL abnormalities being reported in other conditions such as diabetes, obesity, and sarcopenia.[9–12]

Current studies are investigating the effect of Elamipretide (Ela, SS-31) as a potential therapeutic approach for Barth syndrome, a disorder caused by mutations in the TAZ gene.[13]

Ela's mechanism of action involves its localization to the inner mitochondrial membrane, where it associates with CL, thereby stabilizing the membrane, enhancing ATP production, and reducing reactive oxygen species (ROS) formation. Consistent with this mechanism, studies have demonstrated beneficial effects of Ela treatment on ATP production in the heart, kidneys, neurons, and SKM.[14]

Therefore we investigated the effect of Ela on CL content and its maturation via TAZ and its therapeutic potential to rescue SKM pathology in HFpEF promoting mitochondrial function. Specifically, we evaluated mitochondrial properties and changes in contractile function in the SKM at both the whole-muscle and single-fiber levels in HFpEF, as well as changes in structural, and atrophy-related proteins. Our work identified Ela as a useful therapeutic intervention to attenuate mitochondrial dysfunction and overall muscle pathology in our preclinical HFpEF model, warranting further translational follow-up experiments.

2 MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

2.1 Study design

Female ZSF1 lean (n=10) and obese rats (n=24) were obtained from Charles River. At 20 weeks of age, the obese rats were randomized into 2 groups: a placebo group (n=12) receiving an osmotic micropump (Model 2006, Alzet, USA) containing NaCl and a treatment group (n=12) receiving Ela (3mg/kg/day). At 32 weeks of age, the rats were sacrificed to assess muscle function in the soleus (Sol) and extensor digitorum longus (EDL), as well as mitochondrial respiration (Sol). Fiber bundles (Sol and EDL) were prepared for single-fiber measurements. The remaining portions (Sol and EDL) were snap-frozen in liquid nitrogen for molecular analyses or embedded in paraffin for immunohistology.

A detailed study design and a characterization of the rats can be found in a previously published paper of our group[15] and in the supplemental material (Table S1). All experiments were approved by the local animal research council, TU Dresden and the Landesbehörde Sachsen (TVV 50/2023 and 26/2022) and are in accordance with the National Animal Welfare Act and the Laboratory Animal Welfare Ordinance.

2.2 Skeletal Muscle Function

Ex vivo muscle function (whole-muscle and single-fiber) was analyzed in the isolated EDL and Sol muscles as previously described.[16–19] For detailed information see supplementary materials.

2.3 Cardiolipin Assay

CL content was assessed using a Cardiolipin Assay Kit (ab241036, Abcam, UK) according to the manufacturer's recommendation.

2.4 Protein Carbonylation

Protein carbonylation was assessed with the OxyBlot Protein Detection Kit (S1750, Merck, USA) according to the manufacturer's recommendation. Post-translational modification was expressed as the ratio of modified to unmodified protein.

2.5 Mitochondrial Respiration

Mitochondrial respiration was assessed in saponin-permeabilized Sol muscle fibers using high-resolution oxygraphy, as recently described.[20,16] For detailed information see supplementary materials.

2.6 Enzyme Activity

Succinate-dehydrogenase (SDH) activities were assessed spectrophotometrically as previously described.[21]

2.7 Western Blot (WB)

WB analyses were performed as described in detail in the supplementary materials. Myosin Light Chain (MLC)-2 and α-actin expression were analyzed as previously described.[2,3]

2.8 Vertical agarose gel electrophoresis (VAGE)

Titin and myosin heavy chain (MHC) content as well as phosphorylation status were analyzed using VAGE as recently described.[2,3] For detailed information see supplementary materials.

2.9 Histology

Histological analysis was performed as recently described.[22] For detailed information see supplementary materials.

2.10 Statistical Analysis

Normal distribution was tested with the Kolmogorov-Smirnov-Test. Data were analyzed by appropriately using a one-way or two-way ANOVA followed by Tukey's post-hoc test for normally distributed data. For non-normally distributed variables, the Kruskal–Wallis test followed by Dunn's post hoc correction was applied. Results are presented as median ± 95% confidence interval. Correlation was assessed using simple linear regression. A p-value < 0.05 was considered statistically significant. For p-values between 0.05 and 0.1, Cohen's d effect size (d) was calculated. A d-value > 0.8 indicates a large effect between the groups. All analyses were performed using GraphPad Prism version 10.5.0 (GraphPad Software, USA).

3 RESULTS

3.1 Ela has positive effects on muscle physiology in HFpEF

Specific force (force per cross-sectional area [N/cm^2]) in the whole Sol muscle was significantly reduced in HFpEF rats compared to controls, starting at 15 Hz and progressively declining at higher stimulation frequencies. Starting at 80 Hz, Ela treatment significantly increased specific force compared to the HFpEF rats (Figure 1A,B), although values remained lower than in controls.

To identify whether these impairments originated at the myofilament level, we assessed single-fiber maximum specific force, which was significantly reduced in HFpEF rats compared

198 to both, controls and Ela-treated rats (Figure 1C). Notably, no significant difference was
199 observed between the controls and Ela-treated rats, indicating that Ela restored muscle force
200 and specifically rescued myofilament function (Figure 1C). This is also reflected in the force
201 response during muscle fiber stretching (Figure S1A,B). At the end of the stretch, HFpEF rats
202 generate significantly less specific force (se) than the controls. This applies to both passive and
203 active stretches. Ela eliminates these force differences (Figure 1D,E).

204 According to s_e, HFpEF rats exhibited significantly reduced stiffness compared with
205 controls and Ela-treated rats during both passive and active stretches. Ela restored stiffness in
206 both conditions (Figure 1F,G).

207 In the Sol, fiber CSA was significantly reduced only in the slow but not fast fibers of
208 HFpEF rats when compared to controls (Figure 1H). Fiber distribution differed significantly in
209 HFpEF rats compared to controls and Ela-treated rats, affecting both slow and fast fibers,
210 accompanied by a shift toward fast fibers in HFpEF rats (Figure 1I). Strikingly, alterations in
211 both, Sol slow fiber CSA and proportion, were reversed following Ela treatment (Figure 1H,I).

212 We next explored whether effects were similar in the EDL. Compared to controls, there
213 was a trend for specific force in the EDL ($p = 0.07$, $d = 0.63$) to be lower in HFpEF rats.
214 However, following Ela treatment, force was significantly increased compared to HFpEF rats
215 at 50, 80, and 150 Hz (Figure 2A,B).

216 At the single-fiber level, maximum specific force was significantly reduced in HFpEF
217 rats compared to controls independent of Ela treatment (Figure 2C). This is also reflected in
218 the force response during muscle fiber stretching (Figure S1C,D). The Ela-treated muscle fibers
219 generated less specific force at the end of the stretch (s_e) than the controls. This applies to both
220 passive ($p = 0.06$, $d = 1.33$) and active stretches (Figure 2D,E). Ela increased specific active
221 force compared to HFpEF, while passive force was only slightly increased ($p = 0.09$, $d = 1.13$)

but forces remained below the control group (Figure 2D,E). According to s_e, HFpEF rats exhibited significantly reduced stiffness compared to controls during both passive and active stretches. During active stretching, Ela-treated fibers showed a significant recovery of passive, but not active, stiffness to control levels (Figure 2F,G).

Structurally, EDL CSA was significantly reduced in HFpEF rats compared to controls in fast but not slow fibers (Figure 2H). Fiber distribution differed significantly in HFpEF rats compared to controls and Ela-treated rats, affecting both slow and fast fibers, accompanied by a shift toward fast fibers in HFpEF rats (Figure 2I). Notably, alterations in both - EDL CSA and fiber distribution - were reversed by Ela treatment (Figure 2H,I).

3.2 Ela does not fully restore depressed CL and TAZ expression but ROS

CL content was significantly reduced in both the Sol and the EDL of HFpEF animals, with a slight, but not significant impact by Ela treatment (Figure 3A,B). To assess indirect evidence of CL maturation, TAZ expression was evaluated. TAZ levels were significantly decreased in both - the Sol and EDL - of HFpEF rats and remained significantly reduced in the EDL, but not in the Sol ($p = 0.06$, $d = 0.98$), of Ela-treated rats compared to controls (Figure 3C,D).

We next assessed markers of ROS, as cardiolipin abnormalities have been associated with increased mitochondrial ROS production. In the EDL and Sol, NADPH oxidase 2 (NOX2) expression did not significantly differ between groups (Figure 3E,F). In the EDL, superoxide dismutase 2 (SOD2) expression was numerically reduced ($p = 0.06$, $d = 1.24$) in HFpEF rats suggesting a trend towards impaired ROS detoxification and was slightly increased by Ela treatment. In the Sol, SOD2 expression did not differ significantly between groups (Figure 3G,H). Actin carbonylation, an indirect marker of ROS-induced oxidative modifications, was significantly increased in the EDL of HFpEF-rats compared to controls but did not differ

246 significantly following Ela treatment, whereas no differences were observed in the Sol between
247 groups (Figure 3I,J).

248 *3.3 Ela improves SKM mitochondrial respiration in HFpEF*

249 To assess whether Ela impacts SKM mitochondrial respiration, we measured oxygen
250 consumption in permeabilized Sol fibers, evaluating the function of mitochondrial complexes
251 I, II and IV. Furthermore, in independent experiments, we also measured SDH activity as an
252 index of complex II activity. Complex I-stimulated oxygen consumption was significantly
253 reduced in HFpEF rats and numerically, but not significantly, increased following Ela
254 treatment (Figure 4A). Complex II-stimulated respiration was unchanged across all groups
255 (Figure 4B). However, SDH enzyme activity was significantly decreased in HFpEF rats and
256 was restored by Ela treatment (Figure 4C). Complex IV-stimulated oxygen consumption was
257 slightly, but not significantly, decreased in HFpEF rats, compared to controls ($p = 0.06$, $d =$
258 1.38) and numerically increased after Ela treatment (Figure 4D).

259 Sol protein expression of mitochondrial complexes IIV was significantly increased in
260 both HFpEF and Ela-treated rats, compared to controls (Figure 4E-H). Complex V did not
261 differ significantly between groups (Figure 4I). To assess mitochondrial content in SKM, porin
262 expression (Figure 4J) was analyzed, but no differences were observed.

263 To provide details about mitochondrial dynamics, protein expressions were determined
264 from mitochondrial calcium uniporter protein (MCU), mitochondrial fission protein 1 (Fis1)
265 and mitofusin 2 (MFN2). MCU expression was significantly upregulated in HFpEF and Ela-
266 treated rats compared to controls (Figure 4K).

267 In the Sol, Fis1 expression was unchanged across groups, whereas in the EDL a slight,
268 but not significant increase in the Ela-treated rats was observed when compared to HFpEF (p
269 $= 0.08$, $d = 1.00$), as well as control rats ($p = 0.09$, $d = 0.57$) (Figure S2A,B). Analyzing MFN2
270 revealed a significant upregulation of protein expression in the Sol of both, HFpEF and Ela-

treated rats, compared to controls (Figure S2C). In contrast, MFN2 expression in the EDL was significantly downregulated in HFpEF rats compared to controls, with no significant effect of Ela treatment (Figure S2D).

3.4 Correlations between whole-muscle force, mitochondrial function and (maturated) CL

In the EDL, maximal specific force correlated negatively with carbonylated actin ($r = -0.47$, $p = 0.038$). In the Sol, maximal specific force correlated positively with TAZ expression ($r = 0.65$, $p < 0.001$) and mitochondrial function, as measured by Complex I ($r = 0.45$, $p = 0.04$), Complex II ($r = 0.42$, $p = 0.03$), and Complex IV ($r = 0.49$, $p = 0.01$). When analyzing only control and HFpEF animals, SDH activity correlated positively with both CL content ($r = 0.68$, $p = 0.003$) and TAZ expression ($r = 0.75$, $p < 0.001$) (Figure 5A-D).

3.5 Titin hyperphosphorylation can be reversed with Ela

Titin alterations were analyzed to assess molecular changes in muscle fiber stiffness. In the Sol, total titin expression was significantly reduced in Ela-treated rats compared to both control and HFpEF rats. In contrast, titin expression in the EDL did not differ significantly across groups (Figure 6A,B). Analysis of titin revealed significantly increased phosphorylation levels in both, the Sol and EDL of HFpEF rats, compared to controls. This hyperphosphorylation was significantly reduced in Ela-treated rats (Figure 6C,D). A similar pattern was also observed for site-specific Serin(S)-11878 phosphorylation in both muscles, with significantly higher levels in HFpEF rats compared to controls, which was reversed by Ela treatment (Figure 6E,F). Overall, these data indicate that Ela restores titin regulation towards normal levels in HFpEF.

3.6 Ela partially reverses atrophy- and structural protein dysregulation in HFpEF

We analyzed a variety of structural sarcomeric proteins in the Sol and EDL. MHC and α-actin expression did not differ significantly in either the Sol or EDL between the three groups (Figure 7A-D). MLC -1 and -2 expression was unchanged in the Sol (Figure 7E,F), whereas in

the EDL MLC-1 remained unchanged (Figure 7G) while MLC-2 was significantly lower in both HFpEF and Ela-treated rats compared to controls (Figure 6H). In the Sol, α-actinin and telethonin expression did not differ significantly between groups (Figure 7I,J). In contrast, in the EDL, α-actinin and telethonin were significantly downregulated in HFpEF rats compared to controls, but for α-actinin this was prevented following Ela treatment (Figure 7K,L).

To assess molecular markers of muscle atrophy, we analyzed the expression of three different E3 ligases (MuRF1 (Muscle Ring-Finger Protein-1), Trim72, MAFbx (Muscle Atrophy F-box gene)) and one member of the transforming growth factor-beta family (myostatin (GDF8)). In the Sol, MuRF1 expression was significantly higher in HFpEF and Ela-treated rats compared to controls (Figure 8A), whereas in the EDL expression was numerically increased in HFpEF rats compared to controls ($p = 0.07$, $d = 1.17$) and significantly higher compared to Ela-treated rats (Figure 8B). Trim72, MAFbx (Figure S2E,G) and GDF8 (Figure 8C) were not different expressed in the Sol across groups. In the EDL, Trim72 was not altered (Figure S2F), while MAFbx showed no significant differences between groups (Figure S2H). GDF8 was significantly upregulated in the EDL of HFpEF rats compared to controls but this was prevented by Ela treatment (Figure 8D).

4 DISCUSSION

In line with the clinical phenotype, our HFpEF model, showed mitochondrial impairments, a shift from slow-to-fast muscle fibers, atrophy, and overall contractile weakness in the SKM. With respect to mitochondrial dysfunction, recent studies have reported CL dysregulation in the myocardium of HF patients and experimental animal models.[6,7] Ela binds to CL on the inner mitochondrial membrane, leading to beneficial effects on mitochondrial function, cristae formation, and ROS production.[14] In the present study, we investigated the effects of Ela treatment in HFpEF rats, with a focus on identifying whether targeting SKM

mitochondrial dysfunction could benefit muscle mass and function, which can be summarized as follows:

- CL content and TAZ-induced maturation were abnormal in HFpEF, and these changes were partly attenuated by Ela treatment alongside attenuated markers of oxidative stress
- HFpEF-induced mitochondrial dysfunction was ameliorated by Ela treatment
- Ela treatment prevented contractile dysfunction and maladaptive muscle remodeling
- Ela treatment mitigated detrimental changes in myofilament function and compliance in a muscle–specific manner

Taken together, these results indicate that in addition to the myocardium, CL dysregulation in HFpEF is also present in SKM. Furthermore, treatment of rats with HFpEF, using the clinically approved mitochondrial-specific peptide Ela, was able to rescue deficits in muscle contractile function and atrophy via restoring mitochondrial function, highlighting the potential for future translation.

4.1 Ela treatment improves mitochondrial function

Numerous studies have demonstrated impaired mitochondrial respiration in ZSF1 HFpEF rats and in HFpEF patients.[5,20] Additionally, dysregulation of CL levels and CL maturation via TAZ has been reported, but only in the myocardium.[7] In the present study, we extend these findings to SKM, showing reduced TAZ expression and CL levels in both slow- and fast-twitch muscles. In terms of myocardial alterations, another paper from our group demonstrated that Ela treatment improved mitochondrial respiration, but did not show further structural or functional benefits.[15]

Nevertheless, in the present study, Ela treatment improved SKM mitochondrial function, as reflected by enhanced oxygen consumption and enzymatic activity. Analysis of mitochondrial complex expression suggests a compensatory regulation in HFpEF rats, leading to increased complex levels without changes in overall mitochondrial content.[20] Furthermore, MCU expression was elevated in HFpEF, suggesting increased mitochondrial calcium content. Given that CL is critical for calcium channel stability[23], these findings imply impaired channel function, which appears to be partially restored by Ela treatment.

Our mitochondrial analyzes were limited to the Sol, a predominantly slow-twitch muscle that relies heavily on mitochondrial energy supply and thus exhibits high oxidative capacity. In contrast, the EDL, a fast-twitch muscle, depends primarily on anaerobic glycolysis.[24] Interestingly, analysis of mitochondrial fission and fusion proteins revealed the expected dysregulation in the EDL, whereas the Sol appeared to exhibit compensatory regulation. This suggests that the EDL relies minimally on mitochondrial energy, favoring glycolytic pathways, while the Sol actively maintains its mitochondrial energy supply as the primary energy source.

With respect to CL content, Ela treatment did not fully restore CL levels, consistent with previous reports showing improved mitochondrial function without changes in CL content [25,26]. Given that Ela binds directly to CL and stabilizes the lipid, it is also unsurprising that TAZ expression was not restored by Ela treatment.[14]

Mitochondrial dysfunction is closely linked to increased ROS production, which promotes inflammation and contributes to disease progression. Accordingly, improvements in mitochondrial function would be expected to reduce ROS production.[14,5] To address this, we assessed NOX2, a major source of ROS and contributor to diastolic dysfunction, SOD2, an antioxidative enzyme responsible for neutralizing ROS, and carbonylated actin, a marker of cellular oxidants.[27,28] Surprisingly, NOX2 expression was not altered in both muscles.

However, most studies report altered expression in the myocardium rather than in SKM of HF.[28] Furthermore, NOX2 represents only one of many potential ROS sources.[29] In our study, reduced antioxidative defense, reflected by increased protein oxidation, was evident in HFpEF rats but not in Ela-treated rats, suggesting a reduction in ROS burden with Ela treatment.[29] Interestingly, these changes were confined to the EDL, which is unexpected, given that ROS production is typically associated with aerobic metabolism of oxygen, whereas the EDL primarily relies on anaerobic glycolysis, in contrast to the Sol.[29] Nevertheless, previous work has shown that type II fibers exhibit lower antioxidative capacity, which may explain this finding.[30]

4.2 Physiological muscle dysfunction can be prevented by Ela treatment

HFpEF is well recognized to be associated with reduced muscle force and increased fatigability, partly attributed to a slow-to-fast fiber shift and muscle atrophy.[2,31,32]

In our study, we not only confirmed these alterations in the HFpEF rat model but also demonstrated that Ela treatment exerted beneficial effects on these parameters, which may contribute to improved exercise capacity. These changes were observed in both the Sol (slow-twitch) and the EDL (fast-twitch), indicating an overall enhancement of skeletal muscle function. With respect to muscle force, significant improvement by Ela treatment, likely attributable to the partial restoration of mitochondrial function, was evident. However, emerging evidence indicates that Ela may also enhance ADP flux, thereby accelerating ATP delivery to myosin ATPase and rapidly augmenting contractile performance, without requiring structural mitochondrial remodeling.[33] Furthermore, Ela has been reported to decrease S-glutathionylation of key contractile and mitochondrial proteins, including myosin, troponin and the adenosine nucleotide translocator (ANT).[34] Such redox modifications may alter Ca^{2+} sensitivity and force generation independently of ATP supply, providing an additional mechanism explaining the observed functional improvements.

Muscle atrophy was evident from the reduced CSA and further validated on the molecular level by altered expression of atrophy-related markers (MuRF1, GDF8, MAFbx and Trim72).[35,36] Interestingly, atrophy development appeared to be fiber-type dependent, predominantly affecting the dominant fiber type in each muscle. Consistent with previous reports, we observed a slow-to-fast fiber shift in both muscles, which is commonly linked to impaired exercise performance.[2,22] This fiber type shift could also be reversed by Ela treatment, which may contribute to the improved muscle force observed in the treated rats.

4.3 Sarcomere dysfunction and decreased fiber stiffness can be prevented by Ela treatment

Sarcomere function and stability are critical for exercise capacity and muscle performance[1], and particularly titin plays a key role in regulating muscle stiffness and contraction.[37] Force generation results from a complex interplay of the myofilaments actin, myosin, and titin[38,39], which is not yet fully understood.[38] In HFpEF rats, we found a ≈60% reduction in isometric maximum force at the single-fiber level in both the Sol and EDL. This finding suggests impaired myofilament integrity and fiber structure in HFpEF, which may compromise intrafiber force transmission and overall contractile performance.

HFpEF was also associated with reduced force responses to passive stretch (Figs. 1D and 2D, and Fig. S1A, C). In permeabilized fibers, this parameter primarily reflects the mechanical behavior of non-cross-bridge structures, including titin, nebulin, and associated cytoskeletal and extracellular matrix components. This is because cross-bridge cycling is absent under these conditions due to a lack of Ca^{2+} dependent activation and actomyosin ATP turnover.[40] Consequently, the observed response is interpreted as an index of passive stiffness and viscoelastic resistance to stretch. Reduced active and passive force therefore reflects altered sarcomeric mechanics, likely involving titin-based properties, post-translational modifications, and/or cytoskeletal disruption. Notably, increased titin phosphorylation at S11878 in HFpEF contrasted with reduced passive stiffness[3], indicating that broader sarcomeric or cytoskeletal

alterations may override phosphorylation-dependent stiffening effects. Further microstructural analyzes at the fiber and sarcomere level are required to better define the impact of HFpEF on myofilament organization. Interestingly, titin hyperphosphorylation was reversed by Ela treatment, likely contributing to improved muscle function (force and stiffness), especially in the Sol of treated rats. In contrast, structural proteins, including MLC-2, α-actinin, and telethonin, were impaired in the EDL. These deficits were not rescued by Ela treatment. Importantly, normalization of passive stiffness at the single-fiber level should not be interpreted as direct evidence of improved whole-muscle function. Overall, HFpEF induces coordinated impairment of active and passive sarcomeric mechanics, with Ela providing partial, fiber-type–specific restoration of function.

4.4 Limitations

Several limitations of the study should be acknowledged, including:

1) The study was conducted in female ZSF1 rats, a well-validated metabolic model of HFpEF. While this model mimics many key aspects of the human disease, it does not fully capture the complete clinical phenotype. Consequently, extrapolation of these findings to other HFpEF subtypes, such as purely hypertension-driven HFpEF (e.g., Dahl salt–sensitive rats), is limited and requires further investigation. Notably, previous studies have highlighted that the ZSF1 model closely reflects the SKM alterations observed in HFpEF patients[2,3] and community-based studies indicate that the majority of patients are elderly women[3], supporting the relevance of our experimental approach. Nevertheless, it may not be possible to transfer the positive results to the total HFpEF population, including male patients.

2) We were unable to obtain a direct measurement of ROS production in SKM. Nevertheless, upstream regulators and downstream consequences were assessed, providing valuable insights into ROS-related mechanisms. It should be noted, however, that numerous

other pathways potentially contributing to ROS production were not investigated in the present study.

3) No histological analyses, such as electron microscopy were performed. These analyses would provide insights into mitochondrial morphology, particularly cristae structure, under conditions with and without Ela treatment.

4.5 Clinical implications

Overall, our findings highlight Ela as a promising therapeutic strategy in HFpEF by attenuating CL reductions and mitochondrial dysfunction in both SKM and the heart, likely through CL-mediated improvements in oxidative phosphorylation. Since Ela did not show further beneficial effects on myocardial structure or function[15], it may primarily be considered a therapeutic option targeting skeletal muscle dysfunction. Nevertheless, combining Ela with additional therapies, primarily targeting myocardial dysfunction/metabolic disorders (e.g. GLP-1[41,42] and SGLT2i[7,43]), may be the next innovative step to achieve comprehensive treatment of HFpEF.

Acknowledgments: We thank the Light Microscopy Facility, a Core Facility of the CMCB Technology Platform at TU Dresden, for excellent support.

Funding: No funding

CRediT authorship contribution statement: Beatrice Vahle: Data Curation, Formal analysis, Investigation, Visualization, Writing - original draft. **Sven Weidner:** Formal analysis, Investigation, Writing – review & editing. **André Tomalka:** Data curation, Visualization, Writing - original draft. **Antje Schauer:** Conceptualization, Project administration. **Antje Augstein:** Conceptualization, Project administration. **Anita Männel:** Investigation. **Peggy Barthel:** Investigation. **Janet Friedrich:** Investigation. **Grietje Beck:** Writing – review & editing. **Siegfried Labeit:** Writing – review & editing. **T Scott Bowen:** Writing – review & editing**. Tobias Siebert:** Formal analysis, Resources, Supervision, Writing - original draft. **Axel Linke:** Resources, Supervision. **Volker Adams:** Data Curation, Formal analysis, Investigation, Writing - original draft.

Ethics Approval: The study was conducted according to the guidelines of the Declaration of Helsinki and the guidelines of the local animal ethics committee, the National Animal

Welfare Act and the Laboratory Animal Welfare Ordinance. All experiments and procedures were approved by the local animal research council, TU Dresden and the Landesbehörde Sachsen (TVV 50/2023 and 26/22).

Competing interests: The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Axel Linke reports grants from Novartis, personal fees from Medtronic, Abbott, Edwards Lifesciences, Boston Scientific, Astra Zeneca, Novartis, Pfizer, Abiomed, Bayer, Boehringer and others from Picardia, Transverse Medical, Claret Medical, outside the submitted work. The funders had no role in the design of the study, in the collection, analyses or interpretation of data, in the writing of the manuscript, or in the decision to publish the results. All other authors declare no competing interests.

5 References

1. Vahle B, Heilmann L, Schauer A, Augstein A, Jarabo M-EP, Barthel P, Mangner N, Labeit S, Bowen TS, Linke A, et al. Modulation of Titin and Contraction-Regulating Proteins in a Rat Model of Heart Failure with Preserved Ejection Fraction: Limb vs. Diaphragmatic Muscle. *Int J Mol Sci*. 2024;25. doi: 10.3390/ijms25126618.
2. Vahle B, Klädtke R, Schauer A, Bowen TS, Wisløff U, Linke A, Adams V. Skeletal Muscle Alterations in Different Phenotypes of Heart Failure with Preserved Ejection Fraction. *Int J Mol Sci*. 2025;26:6196. doi: 10.3390/ijms26136196.
3. Vahle B, Schauer A, Augstein A, Jarabo M-EP, Friedrich J, Barthel P, Männel A, Mangner N, Labeit S, Bowen TS, et al. MyoMed205 Counteracts Titin Hyperphosphorylation and the Expression of Contraction-Regulating Proteins in a Rat Model of HFpEF. *J Cachexia Sarcopenia Muscle*. 2025;16:e13843. doi: 10.1002/jcsm.13843.
4. Anderson M, Parrot CF, Haykowsky MJ, Brubaker P, Ye F, Upadhya B. Skeletal muscle abnormalities in heart failure with preserved ejection fraction. *Heart Fail Rev*. 2023;28:157–168. doi: 10.1007/s10741-022-10219-9.
5. Kumar AA, Kelly DP, Chirinos JA. Mitochondrial Dysfunction in Heart Failure With Preserved Ejection Fraction. *Circulation*. 2019;139:1435–1450. doi: 10.1161/CIRCULATIONAHA.118.036259.
6. Sparagna GC, Chicco AJ, Murphy RC, Bristow MR, Johnson CA, Rees ML, Maxey ML, McCune SA, Moore RL. Loss of cardiac tetralinoleoyl cardiolipin in human and experimental heart failure. *Journal of Lipid Research*. 2007;48:1559–1570. doi: 10.1194/jlr.M600551-JLR200.
7. Schauer A, Adams V, Kämmerer S, Langner E, Augstein A, Barthel P, Männel A, Fabig G, Alves PKN, Günscht M, et al. Empagliflozin Improves Diastolic Function in HFpEF by Restabilizing the Mitochondrial Respiratory Chain. *Circ Heart Fail*. 2024;17:e011107. doi: 10.1161/CIRCHEARTFAILURE.123.011107.
8. Chaanine AH, Joyce LD, Stulak JM, Maltais S, Joyce DL, Dearani JA, Klaus K, Nair KS, Hajjar RJ, Redfield MM. Mitochondrial Morphology, Dynamics, and Function in Human Pressure Overload or Ischemic Heart Disease With Preserved or Reduced Ejection Fraction. *Circulation: Heart Failure*. 2019;12:e005131. doi: 10.1161/CIRCHEARTFAILURE.118.005131.
9. Ritov VB, Menshikova EV, Azuma K, Wood R, Toledo FGS, Goodpaster BH, Ruderman NB, Kelley DE. Deficiency of electron transport chain in human skeletal

519 muscle mitochondria in type 2 diabetes mellitus and obesity. *Am J Physiol Endocrinol*
520 *Metab*. 2010;298:E49-58. doi: 10.1152/ajpendo.00317.2009.
521 10. Yoo Y, Yeon M, Yoon M-S, Seo Y-K. Role of cardiolipin in skeletal muscle function
522 and its therapeutic implications. *Cell Commun Signal*. 2025;23:36. doi: 10.1186/s12964-
523 025-02032-2.
524 11. Lumini JA, Magalhães J, Oliveira PJ, Ascensão A. Beneficial effects of exercise on
525 muscle mitochondrial function in diabetes mellitus. *sports med*. 2008;38:735–750. doi:
526 10.2165/00007256-200838090-00003.
527 12. Ferrara PJ, Lang MJ, Johnson JM, Watanabe S, McLaughlin KL, Maschek JA, Verkerke
528 ARP, Siripoksup P, Chaix A, Cox JE, et al. Weight loss increases skeletal muscle
529 mitochondrial energy efficiency in obese mice. *life. metab.* 2023;2. doi:
530 10.1093/lifemeta/load014.
531 13. Sabbah HN. Barth syndrome cardiomyopathy: targeting the mitochondria with
532 elamipretide. *Heart Fail Rev*. 2021;26:237–253. doi: 10.1007/s10741-020-10031-3.
533 14. Tung C, Varzideh F, Farroni E, Mone P, Kansakar U, Jankauskas SS, Santulli G.
534 Elamipretide: A Review of Its Structure, Mechanism of Action, and Therapeutic
535 Potential. *Int J Mol Sci*. 2025;26. doi: 10.3390/ijms26030944.
536 15. Schauer A, Jahn D, Vahle B, Barthel P, Männel A, Fabig G, Linke A, Adams V,
537 Augstein A. Mitochondrial Targeting by Elamipretide Improves Myocardial
538 Bioenergetics Without Translating into Functional Benefits in HFpEF. *Int J Mol Sci*.
539 2026;27. doi: 10.3390/ijms27021060.
540 16. Alves PKN, Schauer A, Augstein A, Prieto Jarabo M-E, Männel A, Barthel P, Vahle B,
541 Moriscot AS, Linke A, Adams V. Leucine Supplementation Prevents the Development
542 of Skeletal Muscle Dysfunction in a Rat Model of HFpEF. *Cells*. 2024;13:502. doi:
543 10.3390/cells13060502.
544 17. Tomalka A, Weidner S, Hahn D, Seiberl W, Siebert T. Power Amplification Increases
545 With Contraction Velocity During Stretch-Shortening Cycles of Skinned Muscle Fibers.
546 *Front Physiol*. 2021;12:644981. doi: 10.3389/fphys.2021.644981.
547 18. Tomalka A, Rode C, Schumacher J, Siebert T. The active force-length relationship is
548 invisible during extensive eccentric contractions in skinned skeletal muscle fibres. *Proc*
549 *Biol Sci*. 2017;284. doi: 10.1098/rspb.2016.2497.
550 19. Weidner S, Tomalka A, Rode C, Siebert T. How velocity impacts eccentric force
551 generation of fully activated skinned skeletal muscle fibers in long stretches. *Journal of*
552 *Applied Physiology*. 2022;133:223–233. doi: 10.1152/japplphysiol.00735.2021.
553 20. Winzer EB, Schauer A, Langner E, Augstein A, Goto K, Männel A, Barthel P, Jannasch
554 A, Labeit S, Mangner N, et al. Empagliflozin Preserves Skeletal Muscle Function in a
555 HFpEF Rat Model. *Int J Mol Sci*. 2022;23. doi: 10.3390/ijms231910989.
556 21. Adams V, Bowen TS, Werner S, Barthel P, Amberger C, Konzer A, Graumann J, Sehr
557 P, Lewis J, Provaznik J, et al. Small-molecule-mediated chemical knock-down of
558 MuRF1/MuRF2 and attenuation of diaphragm dysfunction in chronic heart failure. *J*
559 *Cachexia Sarcopenia Muscle*. 2019;10:1102–1115. doi: 10.1002/jcsm.12448.
560 22. Espino-Gonzalez E, Tickle PG, Benson AP, Kissane RWP, Askew GN, Egginton S,
561 Bowen TS. Abnormal skeletal muscle blood flow, contractile mechanics and fibre
562 morphology in a rat model of obese-HFpEF. *J Physiol*. 2021;599:981–1001. doi:
563 10.1113/JP280899.
564 23. Fuentes JM, Morcillo P. The Role of Cardiolipin in Mitochondrial Function and
565 Neurodegenerative Diseases. *Cells*. 2024;13:609. doi: 10.3390/cells13070609.
566 24. Crupi AN, Nunnelee JS, Taylor DJ, Thomas A, Vit J-P, Riera CE, Gottlieb RA,
567 Goodridge HS. Oxidative muscles have better mitochondrial homeostasis than glycolytic

muscles throughout life and maintain mitochondrial function during aging. *Aging (Albany NY)*. 2018;10:3327–3352. doi: 10.18632/aging.101643.
25. Chatfield KC, Sparagna GC, Chau S, Phillips EK, Ambardekar AV, Aftab M, Mitchell MB, Sucharov CC, Miyamoto SD, Stauffer BL. Elamipretide Improves Mitochondrial Function in the Failing Human Heart. *JACC Basic Transl Sci*. 2019;4:147–157. doi: 10.1016/j.jacbts.2018.12.005.
26. Allen ME, Pennington ER, Perry JB, Dadoo S, Makrecka-Kuka M, Dambrova M, Moukdar F, Patel HD, Han X, Kidd GK, et al. The cardiolipin-binding peptide elamipretide mitigates fragmentation of cristae networks following cardiac ischemia reperfusion in rats. *Commun Biol*. 2020;3:389. doi: 10.1038/s42003-020-1101-3.
27. Ciacka K, Tymiński M, Gniazdowska A, Krasuska U. Carbonylation of proteins-an element of plant ageing. *Planta*. 2020;252:12. doi: 10.1007/s00425-020-03414-1.
28. Teuber JP, Essandoh K, Hummel SL, Madamanchi NR, Brody MJ. NADPH Oxidases in Diastolic Dysfunction and Heart Failure with Preserved Ejection Fraction. *Antioxidants*. 2022;11:1822. doi: 10.3390/antiox11091822.
29. Zhou Y, Li H, Xia N. The Interplay Between Adipose Tissue and Vasculature: Role of Oxidative Stress in Obesity. *Front. Cardiovasc. Med.* 2021;8:650214. doi: 10.3389/fcvm.2021.650214.
30. Watanabe D, Lamboley CR, Lamb GD. Effects of S-glutathionylation on the passive force-length relationship in skeletal muscle fibres of rats and humans. *Journal of Muscle Research and Cell Motility*. 2020;41:239–250. doi: 10.1007/s10974-019-09563-5.
31. Saw EL, Ramachandran S, Valero-Muñoz M, Sam F. Skeletal muscle (dys)function in heart failure with preserved ejection fraction. *Curr Opin Cardiol*. 2021;36:219–226. doi: 10.1097/HCO.0000000000000824.
32. Adams V, Schauer A, Augstein A, Kirchhoff V, Draskowski R, Jannasch A, Goto K, Lyall G, Männel A, Barthel P, et al. Targeting MuRF1 by small molecules in a HFpEF rat model improves myocardial diastolic function and skeletal muscle contractility. *J Cachexia Sarcopenia Muscle*. 2022;13:1565–1581. doi: 10.1002/jcsm.12968.
33. Dhanekula AS, Harrison BR, Pharaoh G, Mattson-Hughes A, Tarantini S, Stuppard R, DeRoo SC, Burke CR, Hwang B, Pal JD, et al. Mitochondrial Dysfunction Drives Age-Related Degeneration of the Thoracic Aorta. *bioRxiv : the preprint server for biology*. 2025. doi: 10.1101/2025.06.13.659620.
34. Campbell MD, Martín-Pérez M, Egertson JD, Gaffrey MJ, Wang L, Bammler T, Rabinovitch PS, MacCoss M, Qian W-J, Villen J, et al. Elamipretide effects on the skeletal muscle phosphoproteome in aged female mice. *GeroScience*. 2022;44:2913–2924. doi: 10.1007/s11357-022-00679-0.
35. Bodine SC, Baehr LM. Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogin-1. *Am J Physiol Endocrinol Metab*. 2014;307:E469-84. doi: 10.1152/ajpendo.00204.2014.
36. Jung S-Y, Ko Y-G. TRIM72, a novel negative feedback regulator of myogenesis, is transcriptionally activated by the synergism of MyoD (or myogenin) and MEF2. *Biochemical and Biophysical Research Communications*. 2010;396:238–245. doi: 10.1016/j.bbrc.2010.04.072.
37. Freundt JK, Linke WA. Titin as a force-generating muscle protein under regulatory control. *J Appl Physiol (1985)*. 2019;126:1474–1482. doi: 10.1152/japplphysiol.00865.2018.
38. Hessel AL, Ma W, Mazara N, Rice PE, Nissen D, Gong H, Kuehn M, Irving T, Linke WA. Titin force in muscle cells alters lattice order, thick and thin filament protein formation. *Proc Natl Acad Sci U S A*. 2022;119:e2209441119. doi: 10.1073/pnas.2209441119.

39. Tomalka A, Weidner S, Hahn D, Seiberl W, Siebert T. Cross-Bridges and Sarcomeric Non-cross-bridge Structures Contribute to Increased Work in Stretch-Shortening Cycles. *Front Physiol*. 2020;11:921. doi: 10.3389/fphys.2020.00921.
40. Fitts RH. The cross-bridge cycle and skeletal muscle fatigue. *J Appl Physiol (1985)*. 2008;104:551–558. doi: 10.1152/japplphysiol.01200.2007.
41. Ibrahim R, Han W, Wang W, Kau E, Said N, Forst B, Pham HN, Abdelnabi M, Salih M, Ali NB, et al. Glucagon-Like Peptide-1 Receptor Agonists Versus Bariatric Surgery in Patients With Obesity and Heart Failure With Preserved Ejection Fraction. *Journal of the American Heart Association*. 2025;14:e044577. doi: 10.1161/JAHA.125.044577.
42. Packer M, Zile MR, Kramer CM, Baum SJ, Litwin SE, Menon V, Ge J, Weerakkody GJ, Ou Y, Bunck MC, et al. Tirzepatide for Heart Failure with Preserved Ejection Fraction and Obesity. *N Engl J Med*. 2025;392:427–437. doi: 10.1056/NEJMoa2410027.
43. Anker SD, Butler J, Filippatos G, Ferreira JP, Bocchi E, Böhm M, Brunner-La Rocca H-P, Choi D-J, Chopra V, Chuquiure-Valenzuela E, et al. Empagliflozin in Heart Failure with a Preserved Ejection Fraction. *N Engl J Med*. 2021;385:1451–1461. doi: 10.1056/NEJMoa2107038.

6 Figure Legends

Fig. 1. Physiological muscle parameters of the Soleus. Specific (spec.) whole-muscle force depending on the stimulation frequency (**A**) and corresponding maximal (max.) specific muscle force (**B**). For single fibers maximal specific force (**C**), specific force at the end of passive (**D**) and active (**E**) stretch as well as passive (**F**) and active (**G**) stiffness were evaluated. Cross-sectional area, separated into slow- and fast-twitch fibers (**H**), and fiber-type distribution (**I**) were analyzed in the Soleus from ZSF1-control (con), untreated ZSF1-HFpEF rats (HFpEF), and Elamipretide (Ela)-treated ZSF1-HFpEF rats (HFpEF+Ela) at 32 weeks of age. Results are expressed as mean ± SEM (n = 5–12 per group). Representative histological images are shown in (**J**), with slow fibers positively stained. ** $p < 0.005$, *** $p < 0.001$ indicate HFpEF rats versus controls; $ $p < 0.05$, $$ $p < 0.005$, $$$ $p < 0.001$ indicate HFpEF rats versus HFpEF+Ela. For the single fiber measurements data were obtained from 12 fibers (three rats) for con, 15 fibers (four rats) for HFpEF, and 8 fibers (two rats) for Ela. Statistics: two-way ANOVA (A, H, I), one-way ANOVA (B, C, D, E, F, G).

Fig. 2. Physiological muscle parameters of the extensor digitorum longus (EDL). Specific (spec.) whole-muscle force depending on the stimulation frequency (**A**) and corresponding maximal (max.) specific muscle force (**B**). For single fibers maximal specific force (**C**), specific force at the end of passive (**D**) and active (**E**) stretch as well as passive (**F**) and active (**G**) stiffness were evaluated. Cross-sectional area, separated into slow- and fast-twitch fibers (**H**), and fiber-type distribution (**I**) were analyzed in the EDL from ZSF1-control (con), untreated ZSF1-HFpEF rats (HFpEF), and Elamipretide (Ela)-treated ZSF1-HFpEF rats (HFpEF+Ela) at 32 weeks of age. Results are expressed as mean ± SEM (n = 5–12 per group). Representative histological images are shown in (**J**), with slow fibers positively stained. # $p < 0.05$ indicate control rats versus HFpEF+Ela, $ $p < 0.05$ indicate HFpEF rats versus HFpEF+Ela. For the single fiber measurements data were obtained from 13 fibers (three rats) for con, 8 fibers (two rats) for HFpEF, and 10 fibers (two rats) for Ela. Statistics: two-way ANOVA (A, H, I), one-way ANOVA (B, C, D, E, F, G).

Fig. 3. Cardiolipin (CL) and ROS markers. CL content (**A,B**), tafazzin (TAZ) (**C,D**), NADPH oxidase 2 (NOX2) (**E,F**) and superoxide dismutase 2 (SOD2) (**G,H**) expression, as well as actin carbonylation (**I+J**) was quantified by CL assay or Western Blot analysis of skeletal muscle homogenates of the soleus (Sol) (left side) and extensor digitorum longus (EDL) (right side), obtained from ZSF1-control (con) and untreated ZSF1-HFpEF rats (HFpEF) as well as treated ZSF1-HFpEF rats (HFpEF+Ela (Elamipretide)) at 32 weeks of age. The results are expressed as mean (**A,B**) / x-fold versus control (**C-J**) ± SEM (n = 7–12 per group). Representative blots are shown below (c = con, HF = HFpEF, Ela = HFpEF+Ela). Statistics: one-way ANOVA (A, B, D, E, F, G) and Kruskal-Wallis (C).

Fig. 4. Mitochondrial respiration and protein expression from the Soleus. Oxygen consumption of soleus (Sol) muscle tissue from complex I (**A**), II (**B**) and IV (**D**), as well as succinat dehydrogenase (SDH) (complex II) activity (**C**). Complex I (**E**), II (**F**), III (**G**), IV (**H**) and V (**I**), as well as porin (**J**) and mitochondrial calcium uniporter (MCU) (**K**) expression, quantified by Western Blot analysis of skeletal muscle homogenates of the Sol, obtained from ZSF1-control (con) and untreated ZSF1-HFpEF rats (HFpEF) as well as treated ZSF1-HFpEF rats (HFpEF+Ela (Elamipretide)) at 32 weeks of age. The results are expressed as x-fold versus control ± SEM (n = 6–12 per

683 group). Representative blots are shown below (c = con, HF = HFpEF, Ela =
684 HFpEF+Ela). Statistics: one-way ANOVA (A - K).

685

686 **Fig. 5. Correlation analyses.** Correlation between maximal (max.) specific (spec.) force and
687 carbonylated actin (**A**), as well as taffazin (TAZ) expression (**B**) and between succinat
688 dehydrogenase (SDH) activity and cardiolipin (CL) content (**C**), as well as TAZ
689 expression (**D**) of skeletal muscle homogenates from the extensor digitorum longus
690 (EDL) (**A**) and soleus (Sol) (**B-D**), obtained from ZSF1-control (con) and untreated
691 ZSF1-HFpEF rats (HFpEF) (**C-D**) as well as treated ZSF1-HFpEF rats (HFpEF+Ela
692 (Elamipretide)) (**A-B**) at 32 weeks of age. The results are expressed as x-fold versus
693 control ± SEM (n = 5-12 per group).

694

695 **Fig. 6. Titin modulation in the soleus (Sol) and extensor digitorum longus (EDL).** Titin
696 expression (**A,B**), phosphorylation (**C,D**), and site-specific Serin-(S)11878 titin
697 phosphorylation (**E,F**), were quantified by vertical agarose gel electrophoresis analysis
698 of skeletal muscle homogenates of the Sol (left side) and EDL (right side) obtained
699 from ZSF1-control (con), ZSF1-HFpEF rats (HFpEF), and Elamipretide (Ela)-treated
700 ZSF1-HFpEF rats (HFpEF+Ela) at 32 weeks of age. The results are expressed as x-fold
701 versus control ± SEM (n = –12 per group). Representative gels and blots are shown
702 below (c = con, HF = HFpEF, Ela = HFpEF+Ela). Statistics: one-way ANOVA (A, B,
703 D, E, F) and Kruskal-Wallis (C).

704

705 **Fig. 7. Sarcomere protein modulation in the soleus (Sol) and extensor digitorum longus**
706 **(EDL).** Myosin heavy chain (MHC) (**A,C**), α-actin (**B,D**), myosin light chain (MLC)-
707 2 (**E,G**) expression, and MLC-2 phosphorylation (**F,H**), α-actinin (**G,I**) and telethonin
708 (**J,L**) expression were quantified by Western Blot analysis of skeletal muscle
709 homogenates from the Sol (left panels) and EDL (right panels), obtained from ZSF1-
710 control (con), ZSF1-HFpEF rats (HFpEF), and Elamipretide (Ela)-treated ZSF1-
711 HFpEF rats (HFpEF+Ela) at 32 weeks of age. The results are expressed as x-fold versus
712 control ± SEM (n = 7-12 per group). Representative blots are shown below (c = con,
713 HF = HFpEF, Ela = HFpEF+Ela). Statistics: one-way ANOVA (A – L).

714

715 **Fig. 8. Atrophy-related protein expression.** MuRF1 (**A,B**), and myostatin (GDF8) (**C,D**)
716 expression were quantified by western blot analysis of skeletal muscle homogenates
717 from the soleus (Sol) (left side) and extensor digitorum longus (EDL) (right side),
718 obtained from ZSF1-control (con), ZSF1-HFpEF rats (HFpEF), and Elamipretide (Ela)-
719 treated ZSF1-HFpEF rats (HFpEF+Ela) at 32 weeks of age. The results are expressed
720 as x-fold versus control ± SEM (n = 5-11 per group). Representative blots are shown
721 below (c = con, HF = HFpEF, Ela = HFpEF+Ela). Statistics: one-way ANOVA (A -
722 D).
723