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Title: “ Improving the beneficial effects of HDAC8 inhibition by simultaneously activation of SIRT1 in a zebrafish model of Duchenne muscular dystrophy”

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Keywords: zebrafish, DMD, SIRT1, HDAC8, acetylome

ABSTRACT

Duchenne Muscular Dystrophy (DMD) is a severe genetic disorder characterized by progressive skeletal muscle degeneration, chronic inflammation, fibrosis, and mitochondrial dysfunction. Although gene- and cell-based therapies hold great promise, significant limitations still restrict their broad application, highlighting the need for complementary pharmacological approaches targeting secondary pathological mechanisms. Histone deacetylase (HDAC) inhibitors and Sirtuin 1 (SIRT1) activators represent promising therapeutic strategies, as HDAC8 is involved in cytoskeletal remodeling, whereas SIRT1 mainly regulates mitochondrial metabolism and energy homeostasis. In this study, we investigated the therapeutic efficacy of combining selective HDAC8 inhibition (PCI-34051) with SIRT1 activation (SRT2104) in a zebrafish model of DMD. Combined treatment

significantly improved muscle structure and function, reduced inflammatory cell recruitment, and enhanced mitochondrial activity compared with single-drug administration. Importantly, comparable therapeutic efficacy was achieved using reduced doses of both compounds. Proteomic and acetylomic analyses revealed distinct but complementary molecular signatures induced by the two treatments. HDAC8 inhibition predominantly modulated pathways associated with cytoskeletal organization, epigenetic regulation, ribosome biogenesis, and muscle structural remodeling, whereas SIRT1 activation mainly affected mitochondrial function, oxidative metabolism, amino acid catabolism, and cellular bioenergetics. Overall, these findings demonstrate that combined HDAC8 inhibition and SIRT1 activation effectively ameliorate key pathological features of DMD in zebrafish and provide mechanistic insights into the complementary pathways targeted by the two compounds. This work supports the development of combinatorial epigenetic strategies as potential therapeutic approaches for DMD.

Keywords

HDAC8; SIRT1; DMD; acetylome; mitochondria; zebrafish

LIST OF ACRONYMS

DAPC: dystrophin-associated protein complex

DMD: Duchenne Muscular Dystrophy

DMSO: dimethyl sulfoxide

HDACi: histone deacetylase inhibitors

MO: morpholino

OCR: Oxygen consumption rates

PCI: PCI-34051 (HDAC8 inhibitor)

SRT: SRT2104 (SIRT1 activator)

TMRM: Tetramethylrhodamine methyl ester

WISH: Whole-mount in situ hybridization

INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is an X-linked degenerative genetic disorder caused by mutations in the *DMD* gene encoding dystrophin protein. DMD has an incidence of 1 in 3,500-5,000 live male births and a prevalence of 7.1 in 100,000 males worldwide [1]. Muscle degeneration appears as early as 2–5 years of age, advancing gradually to cardiac and respiratory impairments, which ultimately lead to premature death. Mutations within the *DMD* gene disrupt the dystrophin-associated protein complex (DAPC), resulting in sarcolemmal fragility, increased myofiber susceptibility to damage, and impaired response to mechanical stress. These defects ultimately lead to myofiber loss, chronic inflammation, muscle weakness, and progressive skeletal muscle degeneration [2]. Although remarkable progress has been made with genetic approaches aimed at restoring dystrophin expression or function, targeting downstream pathological mechanisms remains important to improve the efficacy of therapeutic interventions, depending on tackling either the lacking gene or the downstream consequences [3]. Moreover, since current genetic approaches seem to have many drawbacks [4], there is a clear need to develop strategies targeting secondary pathological mechanisms that can benefit a broader population of DMD patients. Within this framework, HDAC inhibitors and SIRT1 activators have emerged as highly selective pharmacological approaches and are currently being investigated for their therapeutic potential [5], [6].

Histone deacetylases (HDACs) represent a large family of enzymes that are key regulators of nucleosome histone deacetylation but are also involved in the deacetylation of non-histone proteins [7], [8]. Givinostat (ITF2357) is an inhibitor of class I and class II HDACs that has shown beneficial effects in multiple preclinical models of DMD. Among them givinostat administration reduces inflammation and fibrosis, enhanced muscle regeneration, and improved muscle histopathology. More recently, clinical studies have indicated that givinostat can slow disease progression and preserve muscle function in patients with DMD, leading to its approval by the FDA and conditional marketing authorization in the European Union [9]. Using a zebrafish *dmd* model, we previously

demonstrated that selective inhibition of HDAC8 has comparable effects to those of givinostat, providing evidence that targeting a single HDAC isoform may also represent a successful strategy [10]. In this context, HDAC8 seems to be mainly involved in the deacetylation of cytoskeleton-associated targets, including cortactin in smooth muscle [11], α -actin [12] and α -tubulin [10]. In addition, HDAC8 inhibition has been reported to negatively affect signalling pathways such as TGF- β [13]. Importantly, aberrant TGF- β signaling is a hallmark of DMD pathogenesis, where it suppresses myogenesis while promoting fibrotic tissue deposition. [14].

Sirtuin 1 (SIRT1) is a NAD⁺-dependent class III histone deacetylase that modulates the activity of different nuclear and cytoplasmic proteins, and thereby regulates diverse cellular processes. In skeletal muscle, SIRT1 activation promotes mitochondrial biogenesis and fatty acid oxidation by regulating PGC-1 α and FOXO transcription factors [15]. Given the contribution of mitochondrial dysfunction and metabolic abnormalities to muscle weakness in DMD, therapeutic strategies aimed at restoring mitochondrial function have gained considerable attention in recent years. Consistently, SIRT1 overexpression induces a switch toward a slower, more oxidative muscle fiber type, thus preventing muscle atrophy and degeneration. Moreover, SIRT1 overexpression promotes a remarkable improvement in muscular parameters and functional performance in dystrophic mouse models [16]. All these findings highlight the crucial role of SIRT1 in the DMD pathophysiology. Different attempts have been made to increase SIRT1 expression or activity in *mdx* mice. To date, one of the most promising approaches seems to be the use of resveratrol (REV), an allosteric modulator reported to influence SIRT1 activity by affecting K_m values for both NAD⁺ and the acetylated peptide, thereby stabilizing substrate-enzyme interactions [17]. However, REV is known to act on a wide range of enzymes, and both its specificity and the extent to which its effects are mediated by direct SIRT1 activation are still debated [18]. Therefore, more selective SIRT1 activators are currently under development, among which SRT2104 represents one of the most advanced compounds in clinical studies. SRT2104 is a highly selective and potent SIRT1 activator, with a

reported K_m of 450 nM, which is 100-fold lower than that of REV (K_m = 46.2 μ M) [18]. We have previously assessed the effects of SRT2104 administration in the *mdx* mouse model, obtaining very promising results [19]. Long-term-treated mice showed improved muscle structure and functional performance, consistent with increased regeneration and reduced inflammation and fibrosis.

Here, we evaluate the efficacy of a novel combination therapy for DMD patients based on selective HDAC8 inhibition using the PCI-34051 compound, and SIRT1 activation through SRT2104 administration. Our results showed that *dmd* zebrafish larvae treated with PCI-34051 and SRT2104 exhibited reduced skeletal muscle degeneration and inflammation. Notably, the combination therapy enabled the use of lower doses of each compound, maximizing the effects of the single treatment and minimizing the risk of possible side effects. In addition, we analyzed the acetylation profile of HDAC8-inhibited and SIRT1-activated zebrafish larvae to identify the respective acetylation targets. In parallel, given demonstration that HDAC8 inhibition may affect proliferation and differentiation of zebrafish neurons and murine neural stem cells, we assessed potential neurotoxicity and found no detectable adverse effects of PCI-34051 and SRT2104 at lower doses.

MATERIAL AND METHODS

Zebrafish husbandry and manipulation

Zebrafish (*Danio rerio*) AB strain, Tg(*alpha-actin*) and Tg(*mpeg1:mCherry*) [20] were maintained and bred at the University of Milan (Aut. Prot. n. n. 0035597/23 —February 28, 2023, protocol n. 60/2023, approved by the Animal Welfare Body of the University of Milan, OPBA). according to international (EU Directive 2010/63/EU) and national guidelines (Italian decree No 26 of the 4th of March 2014). All animal experiments comply with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Embryos were collected by natural spawning, staged according to Kimmel et al. [21], and raised at 28°C in fish water (Instant Ocean, 0.1% Methylene Blue) in Petri dishes, according to established techniques. At 1 day post fertilization (dpf), 0.003% 1-phenyl-2-thiourea

(PTU; Sigma-Aldrich) was added to the fish water to prevent pigmentation. Embryos were washed, dechorionated, and anesthetized with 0.016% tricaine (Ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich) prior to observation, microinjection, and imaging. A limitation to the generalisability of the study is that it did not consider gender/sex issues.

***dmd* zebrafish model**

dmd morpholinos (*dmd*-MOs, GeneTools LLC, Oregon, USA) were used as described in [22] [23]:

dmd-MO1 5'-TTGAGTCCTTTAATCCTACAATTTT-3';

dmd-MO6 5'-GCCATGACATAAGATCCAAGCCAAC-3'.

MOs were co-injected at the concentration of 0.6 (*dmd*-MO1) and 1 (*dmd*-MO6) pmol/embryo, as described by [10], in 1X Danieau buffer (pH = 7.6). A standard control morpholino (ctrl-MO) was injected in parallel.

HDACi and SIRT1 drug treatment

PCI-34051 and SRT2104, were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration was 0.5% for the high-dose treatments and 0.25% for the low-dose treatments. In all experiments, embryos treated with the corresponding concentration of DMSO alone were used as vehicle controls. Drugs were added to the fish water and renewed daily. 1 dpf embryos were put in 24-wells plate, 15 embryos/well and kept at 28°C in the dark until they reached the 3 dpf developmental stage. PCI-34051 drug (PCI, Cayman Chemicals, Ann Arbor, MI, USA) was used for HDAC8 inhibition. The initial concentration of 37.5 µM PCI was selected based on our previous evidence in which this dose consistently rescued the dystrophic muscle phenotype [24]. Embryos were treated with PCI 37.5 [24] or 18.5 µM. and SRT-2104 (Cayman Chemical Company, Ann Arbor, MI, USA), to inhibit SIRT1, at the concentration of 10 or 5 µM.

Muscle lesion analysis through birefringency

Muscle lesions in zebrafish larvae were assessed by birefringence, by setting the anesthetized embryos on a polarizing filter and placing a second polarizing filter over the objective lens as previously described [25]. Larvae were oriented to maximize the brightness of the trunk muscle through the crossed filters. Images of larvae and sections were acquired using a microscope equipped with a digital camera with LAS Leica Imaging software (Leica, Wetzlar, Germany). To quantify the birefringence in the trunk muscle using the Fiji software the ROI around the trunk of the larva (using polygon section) was set, all the ROI were added in the ROI panel and the image was converted to 8 bits. We applied the ConvertSet scale (using the pixel of the acquired images), adjusted the threshold (setting the lower level according to that of the *dmd*-MO group) and measured the area and/or mean gray value [26]. Images were processed using Adobe Photoshop software.

Neutrophil and macrophage staining and count

Mature neutrophils were stained using the Sudan black colorimetric assay following previously established protocols [27]. Red macrophages were counted in the *Tg(mpeg1:mCherry)* larvae. Images of each embryo were acquired using a stereomicroscope equipped with a digital camera and LAS Leica imaging software (Leica, Wetzlar, Germany). The acquired images were then processed and analyzed using Fiji (ImageJ) software.

The same ROI around the caudal hematopoietic tissue (CHT) was set for each larva using the rectangle selection tool. Stained dots for Sudan black or red macrophages were manually counted in the CHT region and, when present, in the upper muscle part. The percentage of dots in the trunk (near the damaged muscle tissue) was estimated as: number of dots in the trunk/total number of dots within the ROI.

Whole mount *in situ* hybridization (WISH) and quantifications

Whole-mount *in situ* hybridization (WISH) experiments were conducted according to a standardized protocol optimized for each specific probe used [28], [29]. Each stained embryo was captured using

a stereomicroscope equipped with a digital camera and LAS Leica imaging software (Leica). The acquired images were processed and analyzed using Fiji (ImageJ) software. The signal intensity and area were quantified using established methods described previously [30]. The basic hybridization signal (brightness and spatial disposition) was qualitatively defined according to the DMSO-treated group and classified as normal. Hybridization signals that qualitatively differed from the control pattern were classified as altered. When informative, the number of larvae displaying normal or altered expression was converted into percentages and represented as histograms.

Assessment of Mitochondrial Membrane Potential Using TMRM

Tetramethylrhodamine methyl ester (TMRM), a cell-permeant fluorescent dye that selectively accumulates in polarized mitochondria, was used to monitor their function [31]. Mitochondrial membrane potential was assessed in zebrafish embryos at 2 dpf. One hour after treatment renewal, embryos were exposed to TMRM (300 nM) supplemented with CsH (2 μ M) and incubated in the dark for 24 h. At 3 dpf, larvae were washed, anesthetized, and mounted in 0.8–1% low-melting agarose. TMRM fluorescence was imaged by confocal microscopy (Leica Stellaris 8 DLS) using constant acquisition settings, and multiple z-stacks were collected for each embryo. TMRM fluorescence analysis was performed at single fiber level of trunk muscles by using Fiji software.

Determination of the gene expression level

Reverse transcription-PCR and real-time quantitative-PCR (RT-qPCR) assays were carried out to detect the mRNA expression levels of mitochondrial and inflammatory genes. Total RNA was extracted from human cells and zebrafish embryos using NucleoZOL reagent (Machery-Nagel, Düren, DE) according to the manufacturer's instructions. Concentration and purity of RNA were measured using the Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). To avoid possible genomic contamination, RNA was treated with RNase-free DNase I (Roche Diagnostics, Basel, SW). 1 μ g of DNase-treated RNA was reverse-transcribed by means of the

ImPromiITM Reverse Transcription System kit (Promega, Madison, WI, USA), using a mixture of random primers and oligo(dT), following manufacturer's protocol. qPCRs were performed in a total volume of 20 μ l containing 1X iQ SYBR Green Super Mix (Promega), using proper amount of synthesized cDNA. qPCRs were performed using the BioRad iQ5 Real Time Detection System kit (Biorad) following manufacturer's guidelines. Thermocycling conditions were the following: 95°C for 10 min, 95°C for 10 s, and 55°C for 30 s. All reactions were performed at least in triplicate for 40 cycles. Primers used for mRNA expression analysis are listed in Supplementary Table 1. The relative expression level of each gene was calculated according to the $2^{-\Delta\Delta Ct}$ method [32]. For normalization purposes, *rpl8* and β -actin were used as internal reference genes. The mean and SEM of at least three independent experiments were reported on graphs.

Assessment of mitochondrial bioenergetics using Seahorse XF Analyzer

Mitochondrial respiration and glycolytic activity were assessed using an Agilent Seahorse XF-24 extracellular flux analyzer. The sensor cartridge was hydrated overnight in Seahorse calibrant solution at 28°C according to the manufacturer's instructions. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in 3 dpf zebrafish larvae previously selected by birefringence at 2 dpf to identify *dmd*-MO. Following selection, larvae were incubated with DMSO, SRT (5 or 10 μ M), PCI (18.7 or 37.5 μ M), or the corresponding drug combinations. Individual larvae were placed into 24-well Seahorse XF24 islet capture microplates, and measurements were performed at 28.5°C. OCR and ECAR were recorded under basal conditions and following sequential injections of oligomycin (10 μ M), FCCP (1 μ M, injected twice), antimycin A (1 μ M), and rotenone (1 μ M), allowing the calculation of basal respiration, maximal respiration, spare respiratory capacity, and glycolytic activity. At least 15 larvae were analyzed for each experimental condition [28].

Sample preparation for acetylome

Pools of zebrafish larvae (N=250) treated with PCI (37.5 μ M), SRT (10 μ M) or DMSO as control, upon de-yolking, were analysed in biological replicates (three for DMSO, 4 for PCI, 4 for SRT treatment). Each sample was lysed using M-PER buffer (Thermo Fisher Scientific) with the addition of protease inhibitors (Halt™ Protease Inhibitor Cocktail (100X), Thermo Scientific, Rockford, IL) and of phosphatase inhibitors (PhosStop, Merck). The pellets were discarded, and the supernatants were subjected to BCA analysis to quantify the total protein content, using BSA as standard (Thermo Scientific, Rockford, IL). 1 mg of total proteins from each sample was digested using the FASP (Filter Aided Sample Preparation) protocol [33]. Briefly, proteins were diluted to 200 μ l with 0.1 M Tris/HCl pH 7.4. Cysteines were reduced with 50 μ l of 0.1 M DTE (1,4-dithioerythritol) in Tris/HCl pH 7.4 and incubated at 95 °C for 1 min; then samples were centrifuged on Amicon Ultra-3K at 14,000 x g for 10 min with a solution of urea 8 M. Cysteines were alkylated with 100 μ l 0.05 M IAA (iodoacetamide) in urea 8 M added on the filter and left at room temperature for 5 min; samples were centrifuged on Amicon Ultra-3K at 14,000 x g for 10 min with a solution of urea 8 M and then incubated overnight at 37 °C in the presence of sequencing grade trypsin (Sigma-Aldrich, St Louis, MO) 1:50 (w/w), upon dilution of urea up to 2 M with 50 mM ammonium bicarbonate buffer. Peptides were purified and desalted with OASIS PRIME HLB C18 cartridges (Waters, Milford, MA). The samples were eluted in 1 ml (0.5 ml + 0.5 ml) of ACN (acetonitrile) and MeOH (methanol) 90/10 and dried under nitrogen. The dried peptide pellet was resuspended in 1.5 ml of Immunoaffinity Purification Bind buffer (IAP) from the PTMScan HS Acetyl-Lysine Motif kit from Cell Signaling Technology (Cat.#46784, CST Inc.). AcK-containing peptides were enriched following the manufacturer's instructions.

Proteomic and acetylotomic analyses by LC-MS/MS

The eluted/acetylated peptides and the unmodified peptides were subsequently desalted using home-made Stage Tips C18 and injected in a capillary chromatographic system (Vanquish Neo, Thermo

Scientific, Bremen, Germany) for peptide separations on a 75 μm i.d. $\times$ 15 cm reverse phase silica capillary column, packed with 1.9 μm ReproSil-Pur 120 C18-AQ (Dr. Maisch GmbH, Germany).

A 100 min-gradient of eluents A (pure water with 0.1% v/v formic acid) and B (acetonitrile with 0.1% v/v formic acid) was used to achieve separation (from 5% to 29% of B in 88 min, 300 nL/min flow rate). MS analyses were performed using a Q-Exactive mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray ion source (Proxeon Biosystems). Each sample was analysed in technical triplicates for label-free quantitative (untargeted) proteomics and duplicates for the acetylome analysis. Full scan spectra were acquired with the lock-mass option, resolution set to 70,000 and mass range from m/z 300 to 2000. The ten most intense ions (charge exclusion: unassigned, 1, 6-8, >8) were selected to be fragmented (ddMS2). MS/MS spectra were acquired with resolution set to 17,500, NCE set to 25 with an isolation window of 2 m/z .

Proteome and acetylome data analysis

All MS/MS data were analysed using Mascot (version 2.6, Matrix Science) search engine to search for the zebrafish_proteome 20250120 (47,561 sequences; 34,755,906 residues). Searches were performed with the following settings: trypsin as proteolytic enzyme; 2 missed cleavages allowed; carbamidomethylation on cysteine as fixed modification; protein N-terminus-acetylation, methionine oxidation, acetylation on lysine residues as variable modifications; mass tolerance was set to 5 ppm and to 0.02 Da for precursor and fragment ions, respectively. All data were then analysed by MaxQuant software (v. 1.6.1.0) [34] for label-free protein quantification based on the precursor intensity, using the above search parameters, except for ± 20 ppm for fragment ions mass tolerance. FDR (False Discovery Rate) of 1% was selected for both peptide and protein identification, with a minimum of two peptides per protein with at least one unique. The complete dataset of identified and quantified proteins was subjected to: log transformation; filtering for protein/sites with at least 50% quantification rate in one or more conditions; imputation of missing values, both protein/site- and condition-specific; centering of data across their median, as an adaptation from PhosR script [35];

normalization for each AcK site with its corresponding protein; The statistical analysis and the subsequent hierarchical clustering were performed using an in-house developed R script. GO analyses were performed using ClusterProfileR and all detected proteins as background.

Semithin Histological Sections

For semithin histological sections, zebrafish larvae at 3 dpf were fixed overnight with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1M sodium cacodylate buffer pH 7.4, washed in the same buffer, postfixed in 2% osmium tetroxide in 0.1M sodium cacodylate buffer, rinsed ten times in water and left at 4°C overnight in a solution of 0.5% uranyl acetate. Samples were then washed and dehydrated in an ethanol series, infiltrated with epoxy resin and embedded in fresh resin.

Statistical analysis

Each experiment was performed at least three times with an average number of embryos for each category of 15-20. Statistical analyses were performed with GraphPad Prism software version 8.0.2 (La Jolla, CA, USA). The Gaussian data distribution of all datasets was assessed by Shapiro–Wilk or Kolmogorov–Smirnov normality test. Mann–Whitney test was used when comparing two groups. When comparing more than two groups, one-way ANOVA followed by Tukey post-hoc correction was used for normally distributed datasets whereas Kruskal–Wallis followed by Dunn’s multiple comparisons test was used for non-normally distributed datasets. Data were considered significant if $p < 0.05$.

RESULTS

SIRT1 activation positively regulates mitochondrial activity in zebrafish.

Since SRT2014 (hereafter SRT) treatment has never been assessed in zebrafish, we first performed a short-term drug administration in wild-type zebrafish embryos by dissolving the compound in the E3 growth medium. Embryos at 1 dpf were treated for 72 hours with SRT at 2.5, 5, and 10 μ M, or with dimethyl sulfoxide (DMSO) as a vehicle control. Drug administration was renewed daily (Fig. 1A). To verify the activity of SRT on SIRT1, we analysed mitochondrial function, as SIRT1 activation is expected to enhance the transcription of mitochondrial genes [36]. Consistently, we observed a dose-dependent increase in the expression of genes essential for mitochondrial protein import and biogenesis, such as *translocase of inner mitochondrial membrane 23 b* (*tim23b*) and *mitochondrially encoded ATP synthase membrane subunit 6* (*mt-atp6*). By contrast, expression of the orthologue *tim23a* was decreased in an SRT dose-dependent manner. One possible explanation is that this opposite regulation may reflect a compensatory response to the upregulation of *tim23b* (Fig. 1B). To further confirm the effects of SRT on mitochondrial activity, we performed a Tetramethylrhodamine methyl ester (TMRM) assay to measure mitochondrial membrane potential. As shown in Figure 1C-D, the red fluorescent signal of TMRM, indicative of healthy mitochondria with a high negative electrical potential within the mitochondrial matrix, was increased after SRT administration in a dose-dependent manner. Collectively, these data show that SRT treatment can improve both mitochondrial function and homeostasis in zebrafish larvae.

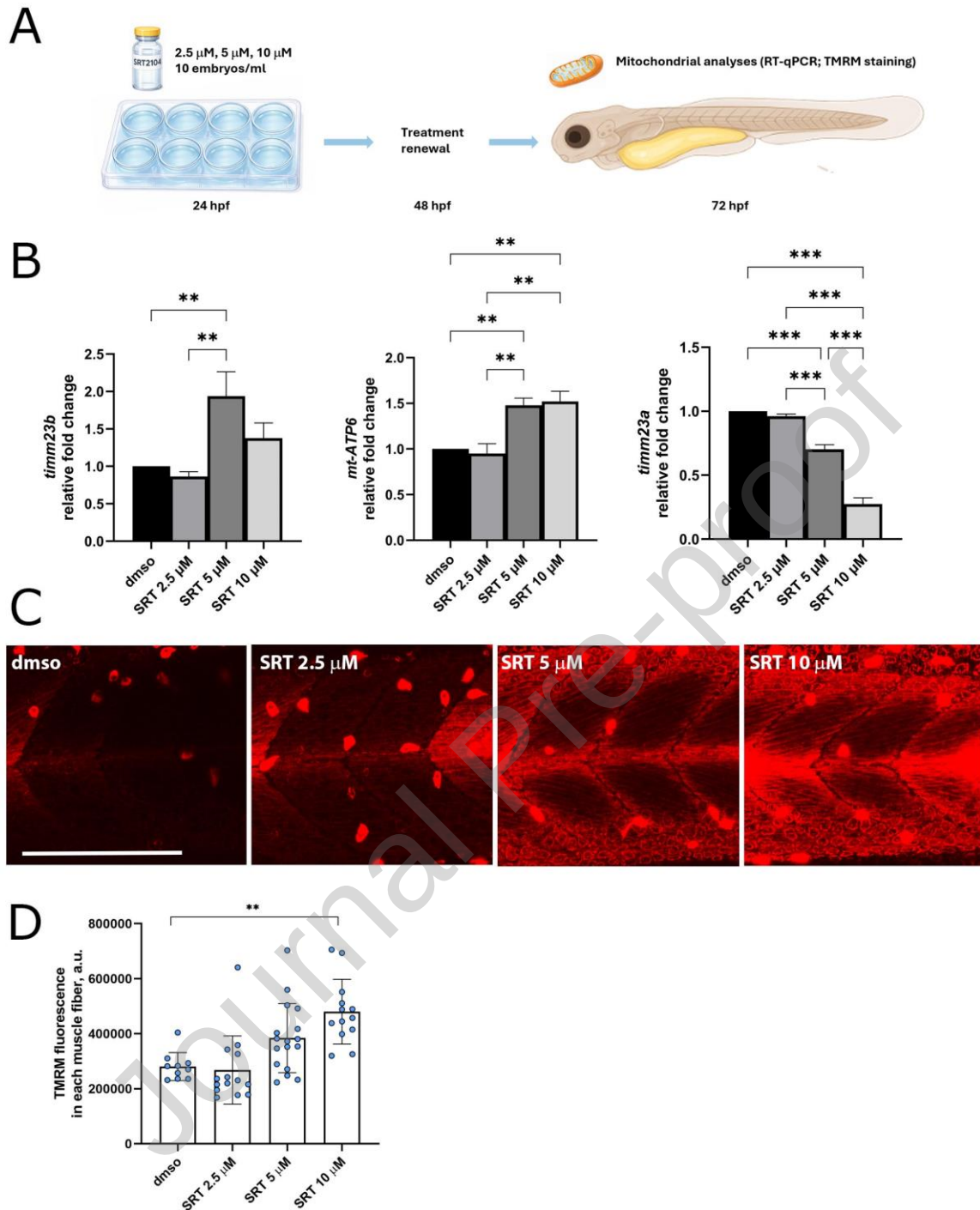


Figure 1. SRT treatment improves mitochondrial function in zebrafish larvae. (A) Schematic representation of the time-scale experimental setting for the SRT pharmacological treatments of the zebrafish model. Image created with Biorender. (B) Relative mRNA expression of *timm23b*, *mt-ATP6*, and *timm23a* in larvae treated with the vehicle (DMSO) or SRT at the indicated concentrations. Data are presented as mean fold change $\pm$ SD from three biological replicates. One Way ANOVA with Tukey post hoc analysis. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. (C-D) Representative images of TMRM fluorescence and its quantification of 3 dpf wild-type larvae from the different experimental groups: DMSO; SRT 2.5 μ M; SRT 5 μ M; SRT 10 μ M. TMRM in red shows the mitochondrial membrane potential in muscle. Scale bar, 100 μ m. The graph in D reports the quantification of TMRM signal in single trunk muscle fibers for each analyzed sample for

each condition. A total number of 54 trunk muscle fibers (DMSO, n = 10; SRT 2.5 μ M, n = 14; SRT 5.0 μ M, n = 17; SRT 10 μ M, n = 13) have been analyzed from at least 3 larvae per condition. Statistical analysis has been performed by using Kruskal–Wallis followed by Dunn’s multiple comparisons test. **p<0.01. Scale bar, 100 μ m.

Single or combined HDAC8 and SIRT1 modulation reduces skeletal muscle damage in *dmd* zebrafish

Birefringence is commonly used as an indicator of muscle integrity in zebrafish larvae, with damaged muscle exhibiting reduced birefringence compared with intact tissue [26]. *dmd*-MO-injected embryos (hereafter named *dmd*) were treated with DMSO, 10 μ M SRT or 37.5 μ M PCI, administered as described above for SRT, and muscle integrity was assessed by polarized-light birefringence at 3 dpf (Fig. 2A). Compared with controls, *dmd* larvae displayed a markedly altered muscle phenotype characterized by a significant reduction in birefringence signal, consistent with myofiber damage. Treatment with PCI or SRT partially rescued the muscle phenotype and increased birefringence. Notably, *dmd* larvae treated with the combination (PCI at 37.5 μ M + SRT at 10 μ M, hereafter combo) showed a major improvement in birefringence, with almost complete recovery of normal muscle phenotype (Fig. 2A, C).

To gain further insight into myotome structure, semi-thin histological cross-sections at the trunk level were analyzed in controls and *dmd* larvae treated with single or combined therapy. Sections from 3 dpf *dmd* larvae showed disrupted and disorganized muscle fibers, whereas myotome structure was partially restored following either single or combined treatments (Fig. 2B).

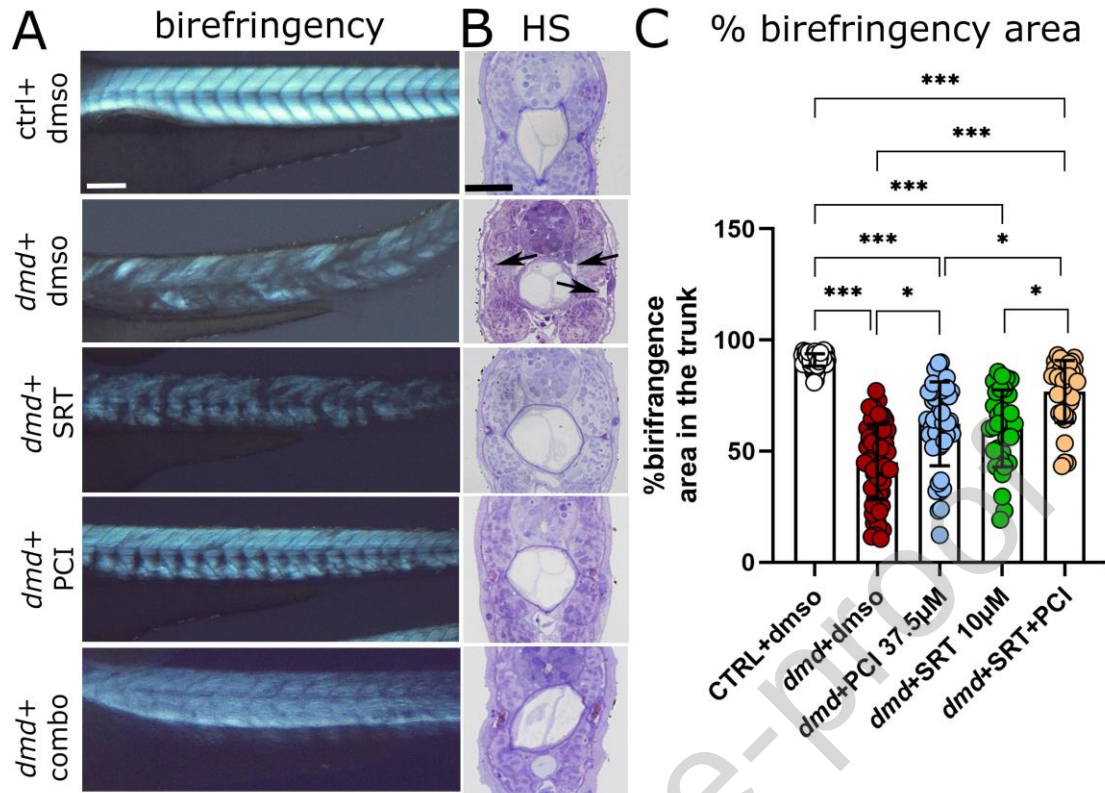


Figure 2. Rescue of the skeletal muscle phenotype of *dmd* larvae treated with PCI and SRT, alone or in combination. (A) Birefringence signal in the trunk-tail region of 3 dpf larvae treated with: DMSO as a vehicle, SRT 10µM, PCI 37.5µM of the different experimental groups. (B) Semithin histological cross-section of the trunk of 3 dpf larvae of different categories of larvae. Arrows indicate damaged skeletal myotome. (C) Graphs of the percentage of birefringence signal in the embryonic trunk shown in A across the different experimental groups. Scale bar indicates 100 µm. Data belongs to biological triplicates and are presented as mean ± SD. One Way ANOVA, Tukey post hoc. Each dot represents an embryo. ***p<0.001; **p<0.01; *p<0.05; not significant data are not shown.

HDAC8 inhibition modulates inflammation, macrophage activity and extracellular matrix remodelling.

The *dmd* zebrafish model displayed a significantly higher proportion of neutrophils compared with controls, consistent with elevated inflammation in damaged muscle tissue (Fig. 3A-D). SRT (10 µM) treatment slightly reduced the total number of neutrophils in the trunk/tail region, although not significantly (Fig. 3A, D). However, this reduction was not accompanied by a decrease in the proportion of neutrophils localized within the damaged muscle tissue. Conversely, treatment of *dmd* larvae with 37.5 µM PCI reduced both the total number of neutrophils in the trunk/tail region and the proportion of neutrophils recruited to damaged muscle. Combined SRT + PCI treatment further reduced the total number of neutrophils and their recruitment to damaged muscle compared with either treatment alone (Fig. 3A, D).

To investigate macrophage activation and extracellular matrix remodelling, we analysed the expression of the *matrix metalloproteinase-9 (mmp9)* by whole-mount in situ hybridization (WISH). Mmp9 is predominantly produced by activated macrophages and plays a key role in extracellular matrix remodelling and the development of fibrosis [37]. Consistent with this, *dmd* larvae displayed a marked increase in the number of *mmp9*-positive cells in the trunk/tail region compared with wild-type controls (Fig. 3B, E), indicating enhanced macrophage activation and matrix remodelling. PCI treatment significantly reduced the number of *mmp9*-positive cells, whereas SRT alone had no significant effect. The combined PCI/SRT treatment produced a reduction comparable to PCI alone, suggesting that HDAC8 inhibition is the main contributor to limiting macrophage activation and the associated matrix remodelling response (Fig. 3B, E).

To determine whether these changes were associated with altered macrophage recruitment to dystrophic muscle, macrophages were visualized in Tg(*mpeg1:mCherry*) larvae. As expected, *dmd* larvae exhibited increased red-macrophage accumulation (Fig. 3C, F). Notably, only PCI/SRT treatment, significantly reduced macrophage accumulation compared with untreated *dmd* larvae (Fig. 3C, F). This supports the conclusion that HDAC8 inhibition has a predominant effect in attenuating inflammation and macrophage-mediated extracellular matrix remodelling. However, SIRT1 activation can further contribute to limit fibrotic response in dystrophic muscle.

Finally, mitochondrial activity in trunk muscles was assessed by performing TMRM assay in the skeletal muscle reporter line Tg(*α-actin:GFP*) (in green) (Fig. 3G, H). Control embryos exhibited low basal TRMR fluorescence (in red), whereas *dmd* larvae showed minimal signal, consistent with mitochondrial dysfunction. SRT treatment increased TMRM fluorescence, in line with SIRT1-mediated mitochondrial activation. PCI treatment also increased TMRM fluorescence intensity, likely reflecting an improved recovery of muscle integrity. The combined treatment yielded the strongest TMRM signal. Together, these results demonstrate that HDAC8 inhibition and SIRT1 activation cooperatively improve mitochondrial function, muscle phenotype, and inflammatory balance in *dmd* larvae.

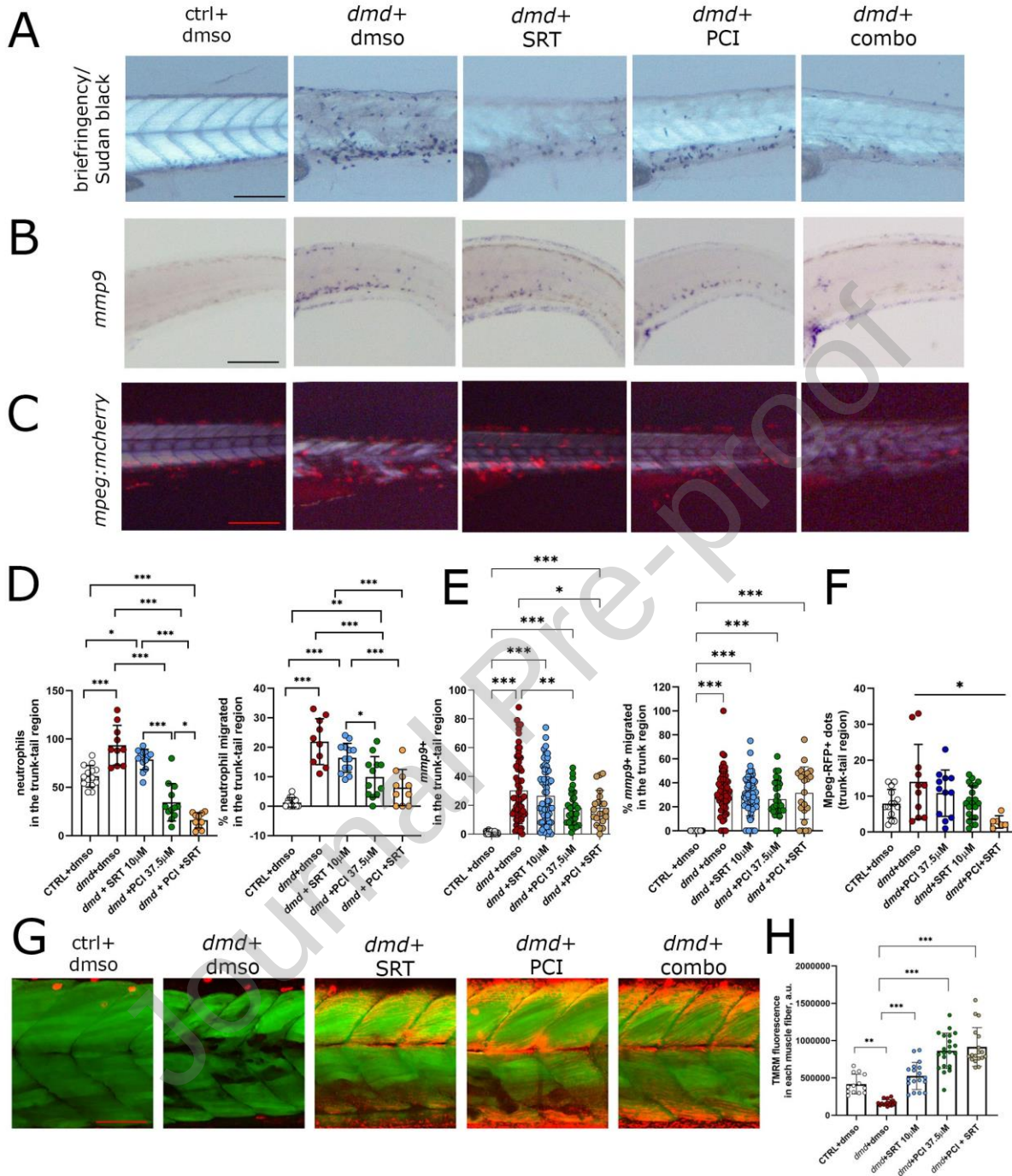


Figure 3. PCI and SRT administration impact on innate immune system response and mitochondrial activation.

(A) Tail region of 3 dpf analysed through birefringency and stained with Sudan black for detecting mature neutrophils (purple dots) in the different embryo categories. (B) Tail region of 3 dpf stained through WISH with the *mmp9* probe, for the detection of the metalloprotease secreted by macrophages. (C) Tail region of 3 dpf *mpeg1:mCherry* macrophage reporter transgenic larvae. (D) The graphs indicate the number of neutrophils in the trunk-tail region and the percentage of neutrophils migrated at the muscle lesion; each dot represents an embryo. (E) The graph indicates the number of

positive *mmp9*⁺ dots in the trunk-tail region and the number of *mmp9*⁺ cells migrated at the muscle lesion; each dot represents an embryo. (F) The graph indicates the number of *mpeg*⁺ macrophages in the trunk-tail region. (G) Representative images of merged green signal of Alpha-actin positive muscles and red signal of TMRM as an index of the mitochondrial membrane potential. (H) The graph reports the quantification of TMRM signal in single trunk muscle fibers for each analyzed sample for each condition. Statistical analysis has been performed by using non parametric ONE-WAY ANOVA. ***p*<0.01, ****p*<0.001. Scale bar, 100 μ m. A total number of 87 trunk muscle fibers (DMSO, *n* =13; *dmd* morphants + DMSO, *n* = 17; *dmd* morphants + SRT 10 μ M, *n* = 21; *dmd* morphants + PCI 37 μ M, *n* = 18; *dmd* morphants + SRT/PCI combination, *n* = 18) have been analyzed from at least 3 larvae per condition. Data are presented as mean $\pm$ SD. Statistical significance in D-F was assessed by One Way ANOVA, Tukey post hoc. ****p*<0.001,***p*<0.01:**p*<0.05. Statistical analysis in H has been performed by using Kruskal–Wallis followed by Dunn’s multiple comparisons test. ****p*<0.001, ***p*<0.01. Scale bar, 100 μ m.

Neurodevelopment is largely preserved following PCI and SRT treatment except at the highest PCI dose

To assess the potential neurodevelopmental effects of PCI and SRT, wild-type embryos treated or not with PCI and/or SRT were assessed for the expression of the neuronal markers *islet1a* and *olig2* at 28–30 hpf, 2 dpf, 2.5 dpf, 3 dpf (Supplementary Fig. 1,2,3).

In addition, *dmd*-MO larvae treated with the same drug regimens were analysed at 3 dpf (Fig. 4). Larvae were classified as displaying either a normal or altered expression pattern based on the distribution of the probe-labelled domains: *islet1a*, which labels the preoptic region, retina, subpallium and hypothalamus [37]; *olig2* which labels motor neurons, oligodendrocytes and their progenitors [36]. Although treated larvae displayed differences in the spatial distribution of *islet1a* and *olig2* expression compared with stage-matched controls, these changes were not indicative of a loss of neuronal areas. Instead, they reflected the normal temporal dynamics of marker expression during zebrafish neurodevelopment, as the patterns observed in treated larvae corresponded to those typically found at earlier developmental stages (Supplementary Fig. 1,2). Consistent with this interpretation, these apparent differences were no longer detectable at 3 dpf in all treatment groups

except those receiving the highest PCI dose (37.5 μ M), alone or in combination with SRT (Suppl. Fig. 3A-B). We next assessed neuromotor behaviour in response to different sensory stimuli by analysing swimming activity elicited by a “tap” stimulus (Suppl. Fig. 3C), or light–dark transition assay (Suppl. Fig. 3D) in wild-type larvae treated with different doses of PCI at 3 dpf using the Ramona Optics MCAM™ system [37][38]. In the tap stimulus assay, PCI-treated larvae showed a statistically significant reduction in distance travelled following the second and third taps. Similarly, in the light–dark transition assay, PCI-treated larvae displayed reduced activity during the dark phase, thereby attenuating the difference between light and dark periods. No differences were detectable using the 18.7 μ M PCI (Suppl. Fig. 3C-D).

Further, we assessed the expression of *islet1a* and *olig2* in *dmd*-MO injected larvae at 3 dpf. *Islet1a* expression remained comparable to untreated morphants under all treatment conditions, indicating preservation of the neuronal areas labelled by this marker (Fig. 4A-B). In contrast, altered *olig2* expression was observed only in larvae treated with 37.5 μ M PCI (Fig. 4C-D). Interestingly, the milder effect of PCI on *islet1a* expression in *dmd*-MO compared with wild-type larvae is consistent with our previous observation that HDAC8 is upregulated in dystrophic muscle and the response to HDAC8 inhibition may differ between healthy and dystrophic larvae [24].

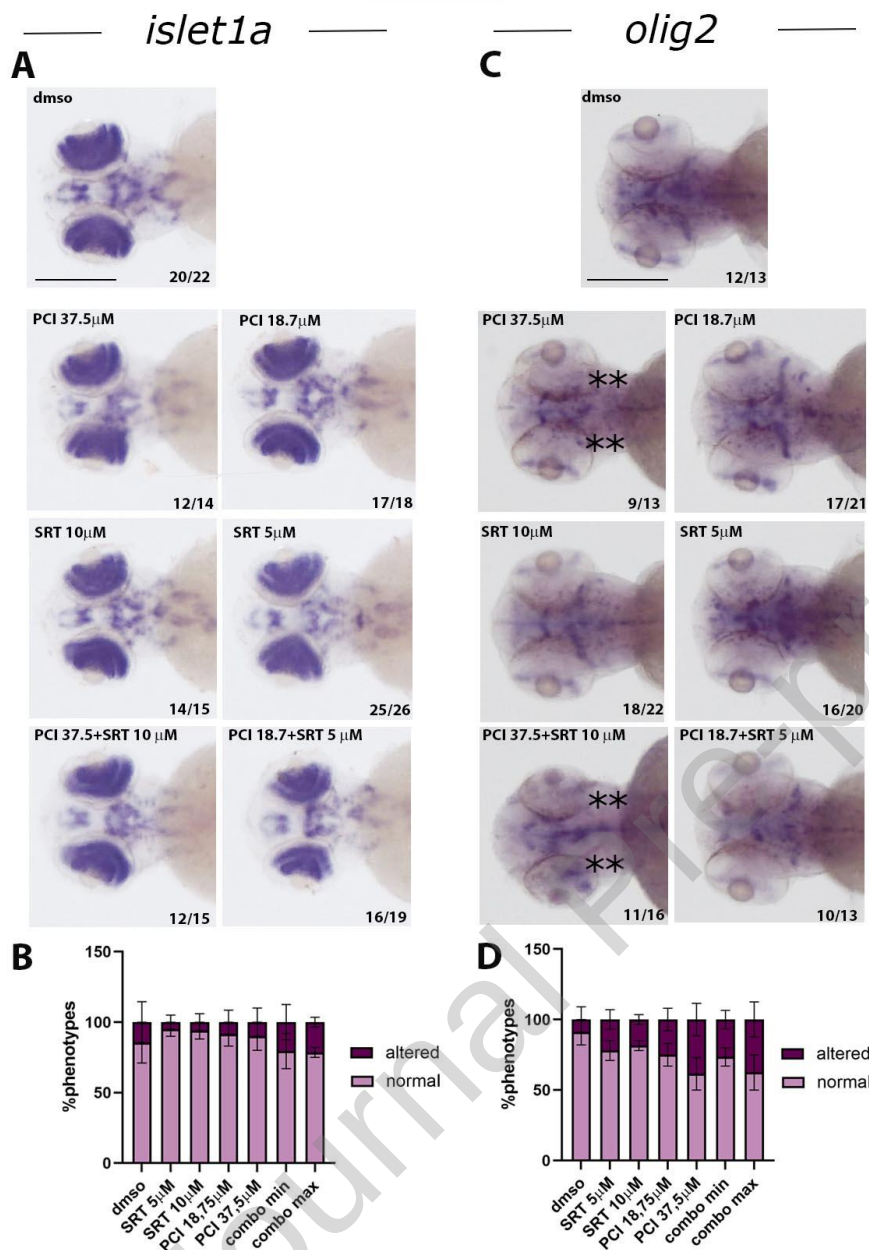


Figure 4. Evaluation of neuronal marker expression in 3 dpf *dmd*-MO zebrafish larvae following PCI and SRT treatments. (A,C) Representative WISH images showing the expression of *islet1a* (A) and *olig2* (C) in 3 dpf *dmd*-MO larvae treated with PCI, SRT, or their combination. Larvae were classified according to the distribution of the probe-labelled domains as displaying either a normal or altered expression pattern (asterisks). (B,D) Quantification of larvae displaying normal or altered *islet1a* (B) and *olig2* (D) expression patterns. Numbers in each panel indicate larvae showing the representative phenotype/total larvae analysed. Scale bars: 100 μ m.

Reduced PCI and SRT doses effectively rescue skeletal muscle damage and inflammation of *dmd* zebrafish

Given that PCI at 37.5 μ M showed potential neurotoxic effects, we next evaluated lower doses of PCI (18.7 μ M), and SRT (5 μ M), administered either individually or in combination. All treatment

conditions partially improved birefringence and restored muscle integrity in *dmd* larvae. Notably, PCI at 18.7 μ M achieved a degree of muscle recovery comparable to that observed with the higher dose (37.5 μ M) (Compare Fig. 5A-B with 2A-B), suggesting that dose reduction does not compromise therapeutic efficacy while it minimizes toxicity. Inflammation was also assessed by quantifying neutrophil recruitment using Sudan Black staining (Fig. 5C). While treatment with a reduced dose of PCI (18.7 μ M) or SRT (5 μ M) alone did not significantly decrease the total number of neutrophils or their migration into muscle tissue, the combined treatment preserved a robust anti-inflammatory effect, markedly reducing both parameters to levels close to those observed in control larvae (Fig. 5D).

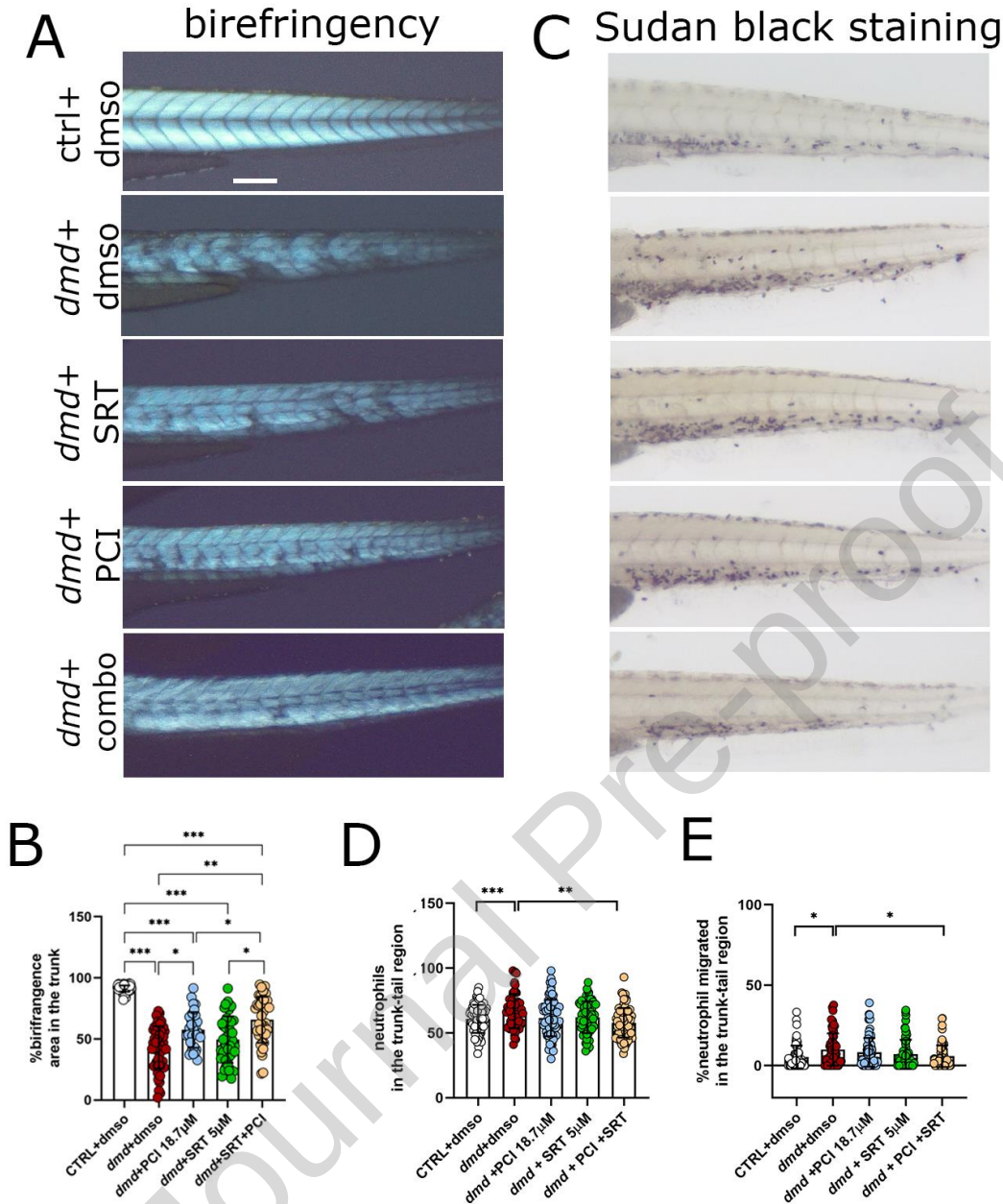


Figure 5. Rescue of the skeletal muscle phenotype and inflammation of *dmd* zebrafish treated with lower doses of PCI and SRT, alone and in combination. (A) The images show the birefringence signal, read-out of muscle integrity, in the trunk-tail region of 3 dpf larvae of the different experimental conditions. Scale bar 100 μ m. (B) Quantification graph of the birefringence area of the different categories of larvae. Each dot indicates the % of birefringence in an embryo. (C) Tail region of 3 dpf stained with Sudan black staining for detecting mature neutrophils (black dots) in the different embryo categories. (D) Graph showing the total neutrophil counts, and (E) the number of neutrophils migrated at the muscle. Each dot represents the total number or the percentage of neutrophils in an embryo. Data are presented as mean $\pm$ SD. Data belongs to biological triplicates and are presented as mean $\pm$ SD. One Way ANOVA, Tukey post hoc. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

To determine whether simultaneous HDAC8 inhibition and SIRT1 activation improve mitochondrial function in dystrophic muscle, we evaluated mitochondrial bioenergetics by Seahorse extracellular flux analysis in *dmd*-MO treated with PCI, SRT, or their combination. *dmd*-MO displayed a pronounced impairment of mitochondrial respiration, characterized by significantly reduced basal and maximal oxygen consumption rates (OCR) compared with control larvae (Fig. 6). Moreover, they

exhibited an almost complete loss of spare respiratory capacity, indicating a markedly reduced ability of mitochondria to respond to increased energetic demands. Treatment with either SRT or PCI alone partially restored mitochondrial respiration, although the extent of recovery depended on drug concentration. Lower concentrations of both drugs alone or in combination preferentially improved maximal OCR, whereas higher doses of PCI and the combined treatment produced a more evident increase in mitochondrial reserve capacity. The low-dose combination (SRT 5 μ M + PCI 18.7 μ M) significantly enhanced maximal respiration, while the high-dose combination (SRT 10 μ M + PCI 37.5 μ M) produced the strongest effect, restoring maximal OCR and markedly increasing spare respiratory capacity to values approaching those observed in control larvae. Analysis of glycolysis by extracellular acidification rate (ECAR) revealed comparatively modest alterations across experimental groups. Although *dmd*-MO showed reduced glycolytic activity compared with controls, pharmacological treatments induced only limited changes in ECAR profiles, indicating that the beneficial effects of SRT and PCI primarily result from improved mitochondrial oxidative metabolism rather than enhanced glycolysis.

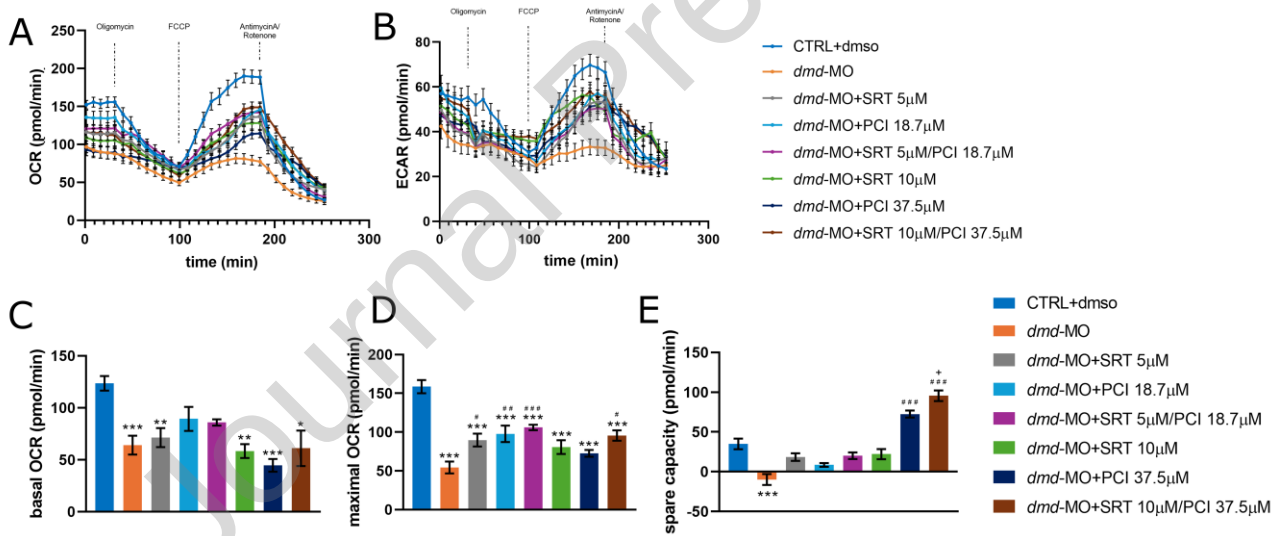


Figure 6. Mitochondrial respiratory function is improved by PCI and SRT in *dmd*-MO larvae (A). Seahorse XF kinetic profiles of oxygen consumption rate (OCR) and (B) extracellular acidification rate (ECAR) measured in control larvae (CTRL+dmsol) and *dmd*-MO treated with vehicle, SRT; 5 or 10 μ M, PCI; 18.7 or 37.5 μ M, or their combinations. Mitochondrial respiration was assessed under basal conditions and following sequential injections of oligomycin (10 μ M), FCCP (1 and 2 μ M), and rotenone/antimycin A (1 μ M). Each condition is represented by symbols and colors reported in the legend on the right of graphs. Standard error bars are reported for each assessed condition. At least 15 samples for each condition have been analyzed. (C) Quantification of basal OCR, (D) maximal OCR, and (E) spare respiratory capacity for each condition is reported in the graphs. Data are presented as mean OCR $\pm$ SEM. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test (maximal OCR) and non-parametric Kruskal Wallis test (Basal OCR and spare respiratory capacity OCR). * p < 0.05, ** p < 0.01, *** p < 0.001 (versus CTRL+dmsol); # p < 0.05, ## p < 0.01, ### p < 0.001 (versus *dmd*-MO); + p < 0.05 (SRT 5 μ M + PCI 18.7 μ M versus SRT 10 μ M + PCI 37.5 μ M).

Proteome and acetylome analyses reveal distinct molecular signatures induced by HDAC8 inhibition and SIRT1 activation

Quantitative proteome and acetylome analyses were performed on zebrafish larvae treated with PCI (37,5 μ M) or SRT (10 μ M), using DMSO as a vehicle control. Comparable proteome depth was achieved across all samples, with approximately 2,300–2,900 proteins identified per condition and low variability among biological replicates, indicating high dataset quality (Supplementary Figure 4). A consistent number of acetylated lysine (AcK) sites was detected across samples, further confirming the robustness of the acetylome analysis. Principal component analysis (PCA) following batch correction showed clear segregation of samples according to treatment, in both proteomic and acetylotomic datasets. PCI- and SRT-treated samples clustered separately from DMSO controls and from each other, indicating that HDAC8 inhibition and SIRT1 activation induce distinct molecular remodeling at both the protein abundance and acetylation levels (Supplementary Fig. 4).

Gene Ontology (GO) enrichment analysis of upregulated proteins following HDAC8 inhibition (PCI vs DMSO) revealed significant enrichment of pathways associated with rRNA metabolic process, rRNA processing, and small ribosomal subunit maturation, indicating activation of ribosome biogenesis and translational machinery. In parallel, enrichment of terms related to cellular detoxification, response to toxic substances, and response to chemical stimulus suggests enhanced cytoprotective and stress-adaptive mechanisms. These signatures are consistent with improved cellular resilience and regenerative capacity observed in PCI-treated dystrophic larvae. Notably, processes linked to oxygen and redox metabolism, including hemoglobin metabolic process, were also upregulated, supporting a broader metabolic remodeling effect (Fig. 7A).

Comparative acetylome analysis between PCI- and DMSO-treated larvae identified significant changes in the acetylation status of proteins involved in chromatin regulation, metabolism, and muscle structure. Increased acetylation was detected on histone H3, together with altered acetylation of PTMAB-related proteins associated with epigenetic regulation, consistent with the transcription-related Gene Ontology enrichment observed following HDAC8 inhibition. Differential acetylation was also observed in Enoyl-CoA-associated enzymes involved in β -oxidation, indicating modulation of proteins linked to fatty acid metabolism. In addition, acetylation changes were detected in Kelch-domain-containing proteins and Parvalbumin, both associated with muscle structural and calcium-related functions, although the functional consequences of these modifications remain to be determined (Fig. 7B). Together, these data indicate that HDAC8 inhibition induces selective acetylation remodeling across nuclear, metabolic, and muscle-associated proteins.

In contrast, SIRT1 activation (SRT vs DMSO) predominantly upregulated pathways associated with alpha-amino acid and proteinogenic amino acid catabolic processes, highlighting a metabolic shift

toward enhanced substrate utilization and oxidative metabolism. Enrichment of terms related to cell-cell junction organization, endomembrane system organization, and microtubule-based transport indicates structural and trafficking remodeling, potentially contributing to improved cellular organization and mitochondrial dynamics. Additionally, modulation of spliceosomal snRNP assembly suggests active transcriptional and post-transcriptional reprogramming (Fig. 7C).

Acetylome analysis comparing SRT and DMSO-treated samples identified differential acetylation of proteins linked to immune cell migration, muscle metabolism, and mitochondrial function. *Asap1*, already exhibiting differential acetylation following SRT treatment [38] showed altered acetylation, consistent with its established role in regulating neutrophil and macrophage migration [38]. In addition, *Phb2a*, a mitochondrial scaffold protein with documented post-translational modification sites associated with pathological conditions, displayed altered acetylation status. These data indicate that SIRT1 activation reshapes the acetylation profile of proteins involved in inflammatory regulation, bioenergetics, and mitochondrial homeostasis (Fig. 7D).

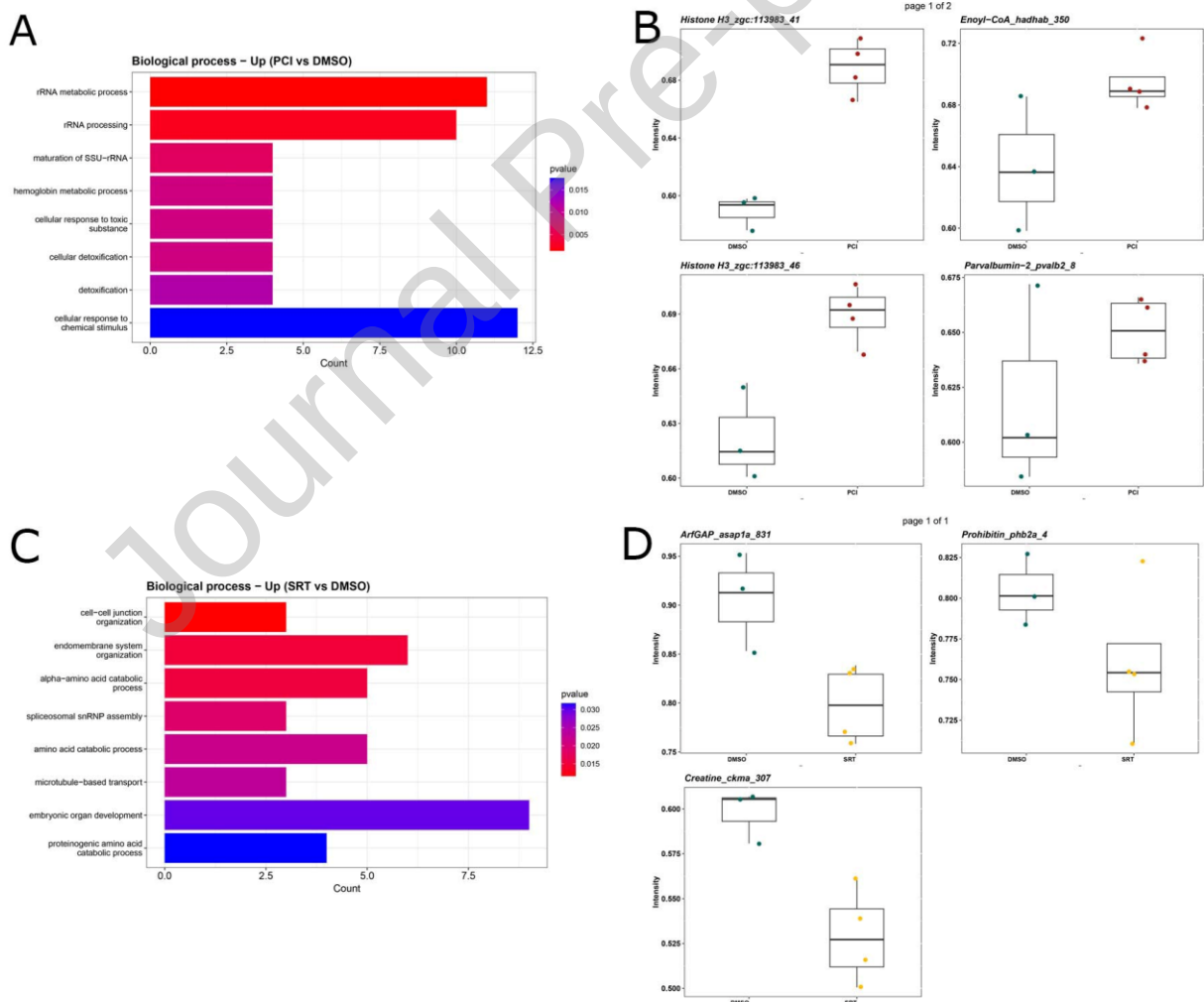


Figure 7. Functional enrichment and protein acetylation differences induced by PCI and SRT treatments. (A) Gene Ontology Biological Process enrichment of proteins upregulated in PCI vs DMSO. Bar length indicates protein count; color represents adjusted p -value. (B) Boxplots of selected acetylated sites increased in PCI-treated samples compared to

DMSO controls. Points represent biological replicates; boxes show median and interquartile range. (C) Gene Ontology Biological Process enrichment of proteins upregulated in SRT vs DMSO. Bar length indicates protein count; color represents adjusted *p*-value. (D) Boxplots of selected acetylated sites differentially abundant in SRT-treated samples compared to DMSO controls. Points represent biological replicates; boxes show median and interquartile range.

Together, these results demonstrate that HDAC8 inhibition and SIRT1 activation elicit distinct and complementary proteomic and acetylome signatures, providing a molecular rationale for their combined pharmacological efficacy in dystrophic muscle.

DISCUSSION

In this study, we demonstrate that combined modulation of HDAC8 and SIRT1, achieved through the selective HDAC8 inhibitor PCI-34051 (PCI) and the SIRT1 activator SRT2104 (SRT), shows a significant therapeutic effect in the *dmd* zebrafish model of Duchenne muscular dystrophy (DMD). Combined treatment improved muscle structure, reduced inflammatory response, and enhanced mitochondrial function, providing a strong pharmacological rationale for targeting complementary epigenetic regulators as a strategy that may complement current gene-based approaches for DMD. Pharmacological activation of SIRT1 has not previously been investigated in zebrafish. Here, we show that SRT induces a dose-dependent improvement in both mitochondrial function and homeostasis, evidenced by increased mitochondrial membrane potential and oxygen consumption. These data are consistent with the results obtained in *mdx* mice, in which SRT, activating Sirt1, improved mitochondrial function, promoting fatty acid oxidation [6]. Altogether these findings align with the established role of the AMPK/SIRT1/PGC-1 α signaling axis as a central regulator of mitochondrial biogenesis and oxidative metabolism in skeletal muscle [39]. Indeed, this pathway operates through a positive feedback loop wherein AMPK activation elevates NAD⁺ levels, thereby activating SIRT1, which in turn deacetylates and activates PGC-1 α to drive mitochondrial biogenesis and function, enhance oxidative metabolism. Disruption of this cascade contributes to the mitochondrial dysfunction observed in dystrophic muscle, and its pharmacological restoration represents a compelling therapeutic strategy.

Given that energy deficiency and mitochondrial dysfunction are central hallmarks of DMD pathology, our data further validate the *dmd* zebrafish as a translationally relevant model to study mitochondrial-targeted therapies and support SRT as an effective SIRT1-based intervention across vertebrate systems [40], [41] further supporting the potential of targeting disease-associated pathways to

ameliorate muscle pathology [39], [40]. In addition, the recent demonstration that HDAC11 deficiency improves the dystrophic phenotype in mdx mice [42] highlights the therapeutic relevance of selective HDAC modulation.

Selective HDAC8 inhibition has previously been shown to ameliorate dystrophic muscle phenotype in zebrafish by enhancing cytoskeletal stability, likely through modulation of non-histone substrates such as α -actin and α -tubulin [10]. Consistent with these reports, PCI treatment increased birefringence and partially restored myofiber organization. Notably, our findings extend the functional role of HDAC8 by providing the first *in vivo* evidence that its selective inhibition also enhances mitochondrial activity. Mitochondrial dysfunction is increasingly recognized as an early pathogenic event in DMD, contributing to defective ATP production, calcium dysregulation and impaired regenerative capacity. In agreement with previous reports, *dmd* morphants exhibited a marked reduction in basal and maximal mitochondrial respiration together with an almost complete loss of spare respiratory capacity, indicating a profound impairment of mitochondrial bioenergetic flexibility [31].

Our data demonstrate that pharmacological modulation of the SIRT1/HDAC8 axis substantially improves mitochondrial function, with combination therapy producing the most robust metabolic rescue. Interestingly, lower drug concentrations preferentially restored basal and maximal respiration, whereas the higher-dose combination induced the greatest recovery of spare respiratory capacity. Because spare respiratory capacity reflects the ability of mitochondria to meet increased energetic demands during physiological stress, its restoration suggests that combined epigenetic modulation enhances the metabolic resilience of dystrophic muscle cells.

The relatively small changes observed in ECAR further indicate that the therapeutic benefit is primarily mediated through restoration of oxidative phosphorylation rather than a compensatory increase in glycolytic metabolism. These findings are consistent with our previous evidence showing that HDAC8 inhibition and SIRT1 activation converge on mitochondrial homeostasis and support the concept of simultaneous targeting of complementary epigenetic pathways. Indeed, our proteomic analysis shows that PCI treatment predominantly affected acetylation of proteins linked to epigenetic regulation, cytoskeletal organization, and muscle structural components. Increased acetylation of histone H3 and other chromatin-associated proteins is consistent with HDAC8 canonical nuclear role and aligns with the enrichment of rRNA processing and transcription-related Gene Ontology terms observed in PCI-treated samples. Beyond nuclear targets, PCI altered acetylation of structural and contractile-associated proteins, including Kelch-domain proteins and Parvalbumin, suggesting remodeling of cytoskeletal and calcium-handling networks. Interestingly, we previously demonstrated that selective HDAC8 inhibition using PCI increases α -tubulin acetylation in dystrophic

muscle, contributing to improved cytoskeletal organization and muscle integrity [10]. Since α -tubulin acetylation is associated with microtubule stabilization and enhanced structural resilience [43], its modulation represents a key mechanism through which HDAC8 inhibition supports sarcolemmal stability in DMD. Increased α -tubulin acetylation may therefore contribute to improved mechanotransduction and reduced myofiber fragility, complementing the regenerative and anti-inflammatory effects observed in dystrophic models.

In contrast, SRT2104 reshaped the acetylation landscape primarily at the level of metabolic and mitochondrial-associated proteins, consistent with SIRT1's established role in metabolic regulation [44]. Differential acetylation of enzymes involved in β -oxidation and ATP buffering, such as Ckma, supports enhanced bioenergetic adaptation. Moreover, SRT modulated acetylation of mitochondrial regulators such as Phb2, suggesting an effect on mitochondrial architecture and respiratory function. This is coherent with the observed enhancement of mitochondrial membrane potential and oxidative metabolism. Importantly, SRT treatment also influenced acetylation of Asap1, a protein implicated in cytoskeletal dynamics and immune cell migration [38]. While PCI-driven acetylation changes were more structurally oriented within muscle fibers, SRT-dependent remodeling appears to extend to inflammatory cell regulation, potentially linking metabolic reprogramming with modulation of immune responses.

Chronic inflammation is a major secondary contributor to DMD progression. In this study, *dmd* zebrafish displayed elevated neutrophil numbers in the tail region, providing, to our knowledge, the first direct characterization of muscle-associated inflammation in this model. Treatment with SRT reduced total neutrophil numbers without affecting their localization to damaged muscle, suggesting a systemic anti-inflammatory effect. Conversely, PCI reduced both total neutrophils and their recruitment to injured muscle tissue, indicating a more direct role in regulating inflammatory cell migration. While PCI treatment markedly reduced neutrophil recruitment, its effects on macrophages appeared to involve both functional modulation and, when combined with SRT, reduced recruitment to dystrophic muscle. PCI significantly decreased the number of *mmp9*-positive cells, a marker of activated macrophages involved in extracellular matrix remodelling and fibrosis, suggesting that HDAC8 inhibition attenuates macrophage activation rather than simply reducing macrophage abundance. Matrix metalloproteinase-9 is a major mediator of pathological extracellular matrix turnover and fibrotic progression in dystrophic muscle, and its reduction is therefore consistent with a less pro-fibrotic inflammatory environment. Interestingly, although SRT alone did not significantly affect macrophage accumulation, the combined PCI/SRT treatment significantly reduced the number of *mpeg1:mCherry*-positive macrophages associated with muscle lesions, indicating that dual targeting of HDAC8 and SIRT1 limits macrophage recruitment to damaged tissue. Together, these

findings suggest that HDAC8 inhibition primarily restrains macrophage inflammatory activity and matrix-remodelling capacity, whereas its combination with SIRT1 activation further dampens macrophage accumulation at sites of muscle injury.

Based on these observations, we evaluated combined PCI and SRT treatment, demonstrating a cooperative improvement in muscle integrity and inflammation. Notably, comparable efficacy was achieved using reduced doses of both drugs, underscoring the translational relevance of this approach by potentially minimizing off-target effects associated with long-term epigenetic modulation.

An additional aspect addressed in this study was the potential toxicity associated with pharmacological modulation of HDAC8 and SIRT1. Previous studies have suggested that HDAC8 activity may play a role in neural development and differentiation, raising the possibility that its inhibition could affect the nervous system. For this reason, we specifically investigated neuronal marker expression and behavioural responses following drug administration. Our results revealed that only the highest PCI concentration induced detectable alterations in neural markers and locomotor behaviour, whereas the lower dose, either alone or in combination with SRT, was well tolerated while maintaining therapeutic efficacy. These findings allowed us to define a therapeutic window that balances efficacy and safety in the zebrafish model. A limitation of the present study is that the early larval MO-based model does not recapitulate the long-term clinical setting of DMD, in which pharmacological treatments are intended to preserve muscle function, delay disease progression and ultimately prolong patient survival. The transient nature of morpholino-mediated knockdown and its progressive dilution during development prevent us from determining whether the observed therapeutic effects are sustained over time or translate into improved survival. The primary objective of our work is to rapidly and cost-effectively assess the feasibility and therapeutic potential of a dual pharmacological therapy that has never been tested in DMD.

While the short duration of treatment in this study precludes conclusions on long-term efficacy, the high selectivity of HDAC8 and SIRT1 targeting suggests that chronic administration may be better tolerated than less specific epigenetic drugs. Future studies should focus on validating key sarcoplasmic and mitochondria-associated acetylation targets and assessing long-term outcomes, paving the way for optimized epigenetic combination therapies for DMD. A limitation of the present study is that PCI-34051 has not advanced to clinical development, largely due to suboptimal pharmacokinetic properties. Nevertheless, the beneficial effects observed following PCI-34051 treatment provide proof-of-concept evidence that selective HDAC8 inhibition may represent a therapeutically relevant mechanism in DMD. On the contrary, SRT2104 has been evaluated in several

Phase I and Phase II clinical studies and shown to be generally well tolerated. Consistently, our results further encourage the development and evaluation of next-generation SIRT1 modulators in DMD.

Conclusion

In conclusion, our study demonstrates that combined HDAC8 inhibition and SIRT1 activation effectively ameliorate key pathological features of Duchenne muscular dystrophy in zebrafish, including skeletal muscle damage, inflammation, and mitochondrial dysfunction. Importantly, the combined treatment enabled the use of lower drug doses while maintaining therapeutic efficacy, thereby reducing the risk of adverse effects associated with higher concentrations of HDAC8 inhibition.

Beyond the phenotypic improvements, proteomic and acetylomic analyses revealed that HDAC8 inhibition and SIRT1 activation regulate distinct yet complementary molecular pathways. While HDAC8 inhibition predominantly affected cytoskeletal organization, muscle-associated proteins, and epigenetic regulators, SIRT1 activation mainly targeted mitochondrial function, cellular metabolism, and bioenergetic processes. These findings provide a mechanistic basis for the enhanced efficacy observed following combined treatment.

Overall, our results highlight the potential of combinatorial epigenetic approaches for Duchenne muscular dystrophy and further support the zebrafish model as a rapid, cost-effective, and translationally relevant platform for therapeutic screening, dose optimization, and mechanistic studies. Future investigations will be required to validate the key molecular targets identified and to assess the long-term therapeutic benefits of this strategy in additional preclinical models.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the author(s) used ChatGPT to revise the language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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Author Contributions

Conceptualization, A.P.1 (Anna Pistocchi), C.D.P., A.P.2 (Alex Pezzotta); methodology S.C., A.B., A.P.2, O. G., L. L., L.B., E.C., S.B., and M.S.; acetylome methodology C.B. and A.A.; software, S.C., A.P.2, A.B., M.S., C.B. and A.A.; validation, S.C., A.P.2, and A.B.; investigation, A.P. 1, S.C., A.P.2, and A.B.; resources, A.P.2, and F.D.B.; data curation, A.P.1, A.P.2 and C.D.P.; writing—original draft preparation, A.P.1; S.C., A.B. and A.P.2; writing—review and editing, A.P.1; C.D.P., A.M. All authors have read and agreed to the published version of the manuscript. We thank Chiara De Simoni for the precious help in performing the analyses.

Institutional Review Board Statement

Zebrafish (*D. rerio*) were maintained at the University of Milan, Via Fratelli Cervi 93 —20054 Segrate, Italy (Protocol Authorization n. 0035597/23 —28 February 2023). AB strain zebrafish were maintained according to international (EU Directive 2010/63/EU) and national guidelines (Italian decree No 26 of the 4 March 2014). Embryos were staged and used until 5 days postfertilization (dpf), a time window in which zebrafish is not considered an animal model according to national guidelines (Italian decree No 26 of the 4 March 2014).

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Declaration of interest

NA

