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HDAC8 regulates protein kinase D phosphorylation in skeletal myoblasts in response to stress signaling

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ABSTRACT

Skeletal muscle differentiation involves activation of quiescent satellite cells to proliferate, differentiate and fuse to form new myofibers; this requires coordination of myogenic transcription factors. Myogenic transcription is tightly regulated by various intracellular signaling pathways, which include members of the protein kinase D (PKD) family. PKD is a family of serine-threonine kinases that regulate gene expression, protein secretion, cell proliferation, differentiation and inflammation. PKD is a unique PKC family member that shares distant sequence homology to calcium-regulated kinases and plays an important role in muscle physiology. In this report, we show that class I histone deacetylase (HDAC) inhibition, and in particular HDAC8 inhibition, attenuated PKD phosphorylation in skeletal C2C12 myoblasts in response to phorbol ester, angiotensin II and dexamethasone signaling independent of changes in total PKD protein expression. As class I HDACs and PKD signaling are requisite for myocyte differentiation, these data suggest that HDAC8 functions as a potential feedback regulator of PKD phosphorylation to control myogenic gene expression.

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1. Introduction

Skeletal muscle represents 35–45% of total body mass, marking it is as the most abundant and an important tissue within individuals [1]. Skeletal muscle is essential for thermogenesis, metabolism and its primary function in movement and postural support [1,2]. Therefore, maintenance of muscle tissue is vital and must undergo adaptive potential and regeneration of myofibers to maintain quality of life and reduce mortality among individuals [1–3]. Muscle regeneration depends on myogenic differentiation of satellite cells to proliferate, differentiate and fuse to form multinucleated myotubes and ultimately new myofibers [3,4]. Muscle differentiation is a highly conserved process and regulation occurs at the molecular level through expression of multiple myogenic transcription factors, and intracellular signaling cascades that include protein kinase C isoforms (PKC) and protein kinase D (PKD) [2,3,5,6].

PKD belongs to a large family of serine/threonine kinases that includes ten isoenzymes that transduce countless signals implicated in the regulation of membrane trafficking, cell survival, migration and myogenic differentiation and proliferation [7]. The N-terminus of PKD contains a regulatory region with a tandem repeat of zinc finger-like cysteine-rich motifs that display high affinity for diacylglycerol (DAG) and phorbol esters such as phorbol 12-myristate 13-acetate (PMA) to promote its phosphorylation, redistribution to the nucleus and therefore its catalytic activity [3,7–9]. Indeed, PKD phosphorylation at serine residues 744/748 (S744/S748) within the activation loop has been shown essential for PKD activity and translocation, and is requisite for myogenic differentiation and proliferation [10].

Histone deacetylase (HDAC) inhibition has been identified as a potential therapeutic in skeletal muscle disease and atrophy [11,12]. HDACs function in catalyzing the removal of acetyl groups from lysine residues on histone proteins [13]. As a result, lysine residues retain their positive charge and histone proteins are more tightly bound to DNA leading to inhibition of gene transcription. There are 18 known mammalian HDACs that are grouped into four distinct classes: class I (HDACs 1, 2, 3, and 8); class II (HDACs 4, 5, 6, 7, 9 and 10); class III (Sirts 1–7), and class IV (HDAC 11). Animal and cellular

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models have documented class I HDACs as major regulators in muscle atrophy, wasting and disease [14–16]. In addition, recent reports suggest that class I HDACs can also regulate intracellular signaling cascades such as the mitogen-activated protein kinases (MAPKs) [17,18].

Interestingly, previous studies have demonstrated that PKD phosphorylates class IIa HDACs, which drives HDAC translocation to the cytosol leading to gene transcription; this is important for angiogenesis, pathological cardiac hypertrophy and immune cell function [6,19–22]. Here, we report for the first time that class I HDAC inhibition, in particular HDAC8 inhibition, attenuated stress-induced PKD phosphorylation in myoblasts. In addition, we report that the published HDAC1 and 2-selective inhibitor Romidepsin also inhibited HDAC8 in C2C12 myoblasts, suggesting that its selectivity is reduced in cell culture. Combined, these reports suggest that class I HDACs can regulate PKD phosphorylation, which may ultimately drive PKD-mediated class IIa HDAC translocation from the nucleus and lead to increased myogenic gene transcription.

2. Methods

2.1. Cell culture

Experiments were conducted in mouse C2C12 myoblasts and myotubes that were propagated in DMEM supplemented with 10% FBS. Cells were cultured to 80% confluence and passaged every 2–3 days at 37 °C in 5% CO2. C2C12 myoblasts can be differentiated into mature myotubes by replacing 10% FBS with 2% DES (horse serum) when cells reach 80% confluence. Differentiated myotubes were treated 4-days post differentiation as indicated in the results. Myoblasts and myotubes were pre-treated with HDAC inhibitors for 24 h prior to agonist(s) treatment. Myoblasts were pre-treated with siRNAs for 72 h prior to PMA treatment as indicated in figures and figure legends. All siRNAs were purchased from Sigma: non-targeting siRNA control (Cat#SIC001), siRNA HDAC1 (Cat# SASI_Mm01_0015_9497), siRNA HDAC1 (Cat#SASI_Mm01_00159502), siRNA HDAC2 (Cat#SASI_Mm01_0010_0070), siRNA HDAC2 (Cat#SASI_Mm01_00100_699), siRNA HDAC3 (Cat#SASI_Mm01_0016_2230) and siRNA HDAC8 (Cat#SASI_Mm01_0010_7278). Treatment times for phorbol-12-myristate-13-acetate (PMA), dexamethasone, or angiotensin II are indicated in figures and figure legends.

2.2. Materials

We purchased HDAC inhibitors from: 1) Trichostatin A (TSA; Sigma Cat#025M4024V), 2) Apicidin (Api; Enzo Cat#BML-GR340-0005), 3) diphenylacetohydroxamic acid (DPAH; Sigma; Cat#D6071), 4) Tubastatin A (TubA; Selleckchem; Cat#S8049) 5), Romidepsin (Selleckchem: Cat#S3020), and 6) RGFP966 (Selleckchem; Cat#S7229). PMA (Sigma; Cat# P1585), dexamethasone (Sigma; Cat# D4902), angiotensin II (Sigma; Cat#A9525). Recombinant HDAC1 (rHDAC1; Reaction Bio Corp; Cat#KDA-21-365), recombinant HDAC2 (rHDAC2; Reaction Bio Corp; Cat#KDA-21-277), recombinant HDAC3 (rHDAC3: Reaction BioCorp Cat# KDA-22-278) and 4) recombinant HDAC8 (rHDAC8; Reaction Bio Corp: Cat# KDA-21-285).

2.3. Immunoblotting

Cultured cell monolayers were washed one time with ice cold PBS and scraped into ice-cold lysis buffer containing PBS (pH 7.4), 0.5% Triton X-100, 300 mM NaCl and protease/phosphatase inhibitor cocktail (Thermo Fisher; Cat#VF300184). Samples of the cell lysate were sonicated prior to clarification by centrifugation at 16,000 g × 10 min and protein concentrations were determined using a BCA Protein Assay Kit (Pierce; Cat#VC-297330-296335). Proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to nitrocellulose membranes (Genesee Scientific, Cat#84–875). Transferred membranes were blocked with 5% milk and probed with indicated primary antibodies for phospho-PKD (S744/S748; Cell Signaling, Cat#2054), phospho-PKD (S916; Cell Signaling, Cat#2051), total PKD (Biosource, Cat#8715), acetyl-histone H3 (Cell Signaling, Cat#9441), acetyl-tubulin (Santa Cruz; Cat#sc-23950), HDAC1 (Cell Signaling, Cat#5356), HDAC2 (Cell Signaling, Cat#5113), HDAC3 (Cell Signaling, Cat#3949) and HDAC8 (Cell Signaling, Cat#66042). Horseradish Peroxidase (HRP)-conjugated secondary antibodies (Southern Biotech) were used at a concentration of 1:2000 in 5% milk and 1X TBST. SuperSignal West Pico chemiluminescence system (Genesee Scientific, Cat#210508–24) and a ChemiDoc XRS + imager (BioRad) were used to detect protein. Densitometry was performed using Image J software and statistical analyses conducted via GraphPad Prism software.

2.4. HDAC activity assay

Recombinant HDACs 1, 2, 3 and 8 was prepared in 1X PBS (200–800ng/ul). Vehicle (DMSO) or HDAC inhibitors, Apicidin (1 μM) and Romidepsin (1μ) was added to recombinant HDACs and incubated at 37 °C for 2 h. The class 1 specific HDAC substrate was added (5 μL of 1 mM stock solution), and plates incubated at 37 °C for 2 h. Developer/stop solution was added (50 μL per well of PBS with 1.5% Triton X-100, 3 μM TSA, and 0.75 mg/mL trypsin) and plates incubated at 37 °C for 20 min. Subsequent to deacetylation, trypsin was used to release AMC, resulting in increased fluorescence. AMC fluorescence was measured via BioTek Synergy 2 plate reader, with excitation and emission filters of 360 nm and 460 nm, respectively.

2.5. Statistics

Statistical analysis was completed by ANOVA followed by Turkey's post-hoc unless otherwise stated in the results. Statistical significance is defined as $p < 0.05$. GraphPad Prism software was used for statistical analysis.

3. Results

3.1. Class I HDAC inhibition attenuated PKD phosphorylation in C2C12 myoblasts in response to phorbol-12-myristate-13-acetate (PMA)

To establish a model for PKD activation, we first stimulated C2C12 myoblasts and myotubes with PMA (50 nM); total PKD and phosphorylated PKD expression at serine (S) residues S744/S748 and S916 was then examined via immunoblot over time. Phosphorylation of S744/S748 as well as S916 is essential for the activation of PKD [23]. We observed several interesting findings from this experiment. First, expression of total PKD as well as phosphorylated PKD at S744/S748 was markedly greater in myoblasts compared to myotubes (Fig. 1A). Second, PMA rapidly (5 min) and transiently increased phosphorylated PKD at S744/S748 in myoblasts and to a lesser extent in myotubes (Fig. 1A). Third, PMA rapidly and transiently increased PKD phosphorylation at S916 in both myoblasts and myotubes (Fig. 1A).

PKD phosphorylation plays an important role in muscle biology that includes hypertrophy/atrophy, proliferation and function [24]. Moreover, there has been evidence to link PKD to HDAC regulation

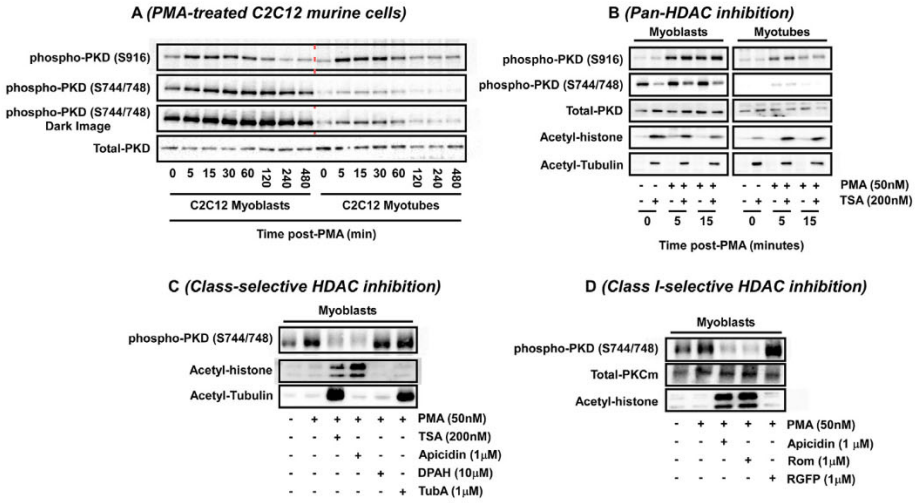

Fig. 1. Class I HDAC inhibition attenuated PKD phosphorylation (S744/S748) in C2C12 myoblasts. A) C2C12 myoblasts and myotubes (d4) were stimulated with phorbol-12-myristate-13-acetate (PMA; 50 nM) and cells lysed over time. Immunoblotting was performed to examine phosphorylated PKD (phospho-PKD; S916; S744/S748) and total PKD protein expression. B). Myoblasts and day 4 differentiated myotubes were pretreated with TSA (200 nM) for 24 h prior to stimulation with PMA (50 nM). Cells were lysed and immunoblotted to examine phosphorylated (S916 and S744/S748) and total PKD as well as acetylated histone (acetyl-histone) and acetylated tubulin (acetyl-tubulin). C) Myoblasts were pre-treated with TSA (200 nM), apicidin (1 μM), DPAH (10μM), Tubastatin A (TubA: 1 μM) for 24 h prior to 30 min PMA (50 nM) stimulation. Cells were lysed and immunoblotted for phosphorylated PKD (S744/S748), acetyl-histone and acetyl-tubulin. D) Myoblasts were pre-treated with romidepsin (Rom; 1 μm) or RGFP966 (RGFP; 1 μM) for 24 h prior to 30 min PMA (50 nM) stimulation. Cells were lysed and immunoblotted for phosphorylated and total PKD (S744/S748), acetyl-histone.

[6,25,26]. As HDAC inhibitors have recently gained interest as potential therapeutics in pre-clinical models of heart failure and skeletal muscle diseases [27], we next examined the role for HDAC inhibitors on PKD phosphorylation. Similar to Fig. 1A, we observed increased total PKD and phosphorylated PKD at S744/S748 in myoblasts compared to myotubes (Fig. 1B). We report that PMA increased PKD phosphorylation at S744/S748 in myoblasts and to a lesser extent in myotubes. Moreover, we showed that PKD phosphorylation of S744/S748 was attenuated with treatment of the pan-HDAC inhibitor trichostatin A (TSA) in myoblasts; this affect was not observed in myotubes (Fig. 1B). However, TSA failed to attenuate PKD phosphorylation of S916 in myoblasts or myotubes (Fig. 1B). Therefore, we next examined which class of HDACs regulate PKD phosphorylation at S744/S748 in myoblasts.

There are eighteen known mammalian HDACs that are ubiquitously expressed and are categorized into four classes: class I HDACs (1, 2, 3, and 8), class II (4, 5, 6, 7, 9, and 10), class III, which are sirtuins (Sirt 1–7), and the lone class IV HDAC (HDAC11) [28,29]. It should be noted that TSA does not target the sirtuins for inhibition [30]. To confirm which HDAC is responsible for attenuation of PKD phosphorylation, C2C12 myoblasts were pre-treated with TSA or a Class 1 HDAC inhibitor: Apicidin (1 μM), Class IIa HDAC inhibitor: DPAH (10μm) or Class IIb inhibitor: Tubastatin A (1 μM) for 24 h prior to PMA stimulation. PMA-induced PKD phosphorylation (S744/S748) was attenuated with TSA, similar to above (Fig. 1B), and apicidin, but not DPAH or tubastatin A (TubA) (Fig. 1C). Acetylated histone H3 (acetyl-histone) and acetylated tubulin (acetyl-tubulin) were used as positive controls to show efficacy of our HDAC inhibitors. These findings suggest that class I HDAC inhibition attenuates PKD phosphorylation at S744/S748. As a next step, myoblasts were then exposed to isoenzyme specific inhibitors for HDAC 1, 2, and 3 to determine which HDAC is responsible for attenuating PKD phosphorylation. Here we report that romidepsin treatment (HDAC 1 and 2 inhibition) attenuated PMA-induced PKD phosphorylation, but not RGFP966 (RGFP; HDAC3 inhibitor) (Fig. 1D). These data infer that HDAC1 and HDAC2 regulate PKD phosphorylation at S744/S748.

3.2. Class I HDAC inhibition attenuated PKD phosphorylation in response to multiple agonists in skeletal myoblasts

Data above demonstrated that class I HDAC inhibition could attenuate PMA-induced PKD phosphorylation, yet does not indicate if HDAC inhibition would attenuate PKD phosphorylation in response to myoblast stress in general. To address this question, we examined PKD phosphorylation in response to angiotensin II (AngII; 10 μM) and dexamethasone (Dex; 1 μM); two compounds known to drive skeletal muscle atrophy and impair skeletal muscle repair after injury [31,32]. Here, we report Dex and AngII rapidly (15 min) and transiently increased PKD S744/S748 phosphorylation as indicated via immunoblot and line graph (Fig. 2A); Dex- and AngII-induced PKD phosphorylation was confirmed at 15 min in which S744/S748 phosphorylation was significantly increased (Fig. 2B). Importantly, Dex- and AngII-induced PKD S744/S748 phosphorylation was significantly attenuated by the HDAC1/2 inhibitor romidepsin (Fig. 2C) demonstrating that HDAC inhibitors can decrease PKD S744/S748 phosphorylation in myoblasts in response to stress.

3.3. HDAC8 regulates PKD phosphorylation

Current findings report that romidepsin is an HDAC1 and HDAC2-selective inhibitor [33]. Based on this, we next used small interfering RNAs (siRNAs) to silence HDAC1 and HDAC2. We again report that PMA induced PKD S744/S748 phosphorylation in our siRNA control transfected myoblasts (Fig. 3A). Interestingly, neither knockdown of HDAC1 (siH1) nor HDAC2 (siH2) was sufficient to attenuate PMA-induced PKD phosphorylation (Fig. 3A). As a next step, we examined PKD phosphorylation with knockdown of HDACs 1 and 2 in combination. Again, PMA increased PKD phosphorylation in our siRNA control transfected cells. In addition, siRNA knockdown of HDACs 1 and 2 (siH1/2) with two different targeting sequences was sufficient to knockdown HDACs 1 and 2 but not sufficient to inhibit PMA-induced PKD phosphorylation (Fig. 3B).

While romidepsin has been reported to only target HDACs 1 and 2 [33], we next examined this small molecule inhibitor against

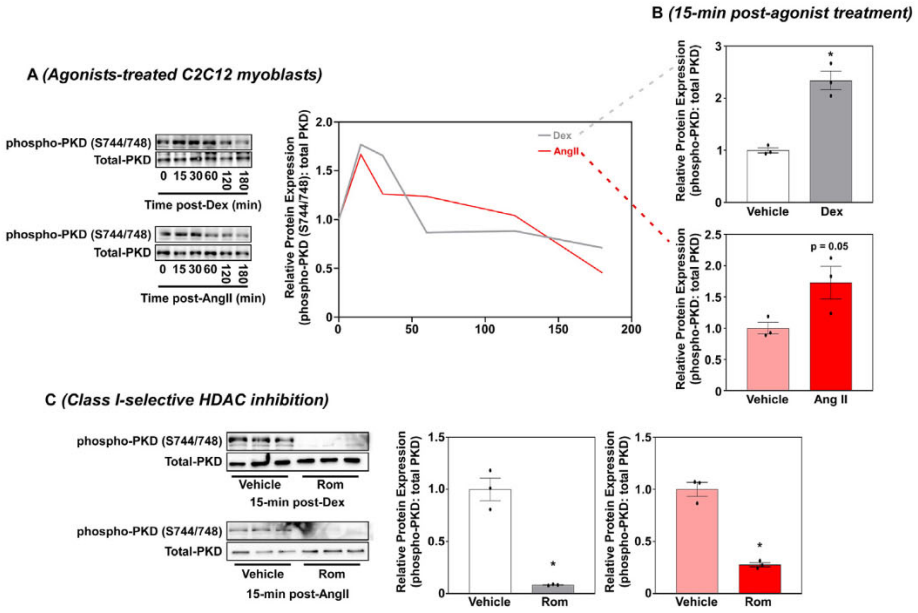

Fig. 2. Class I HDAC inhibition attenuated stress-induced signaling in C2C12 myoblasts. A) C2C12 myoblasts were stimulated with Dexamethasone (Dex; 1 μM) of angiotensin II (AngII; 10 μM) and cells lysed over time. Immunoblotting was performed to examine phospho-PKD (S744/S748) and total PKD protein expression and image J used to quantify relative changes in PKD phosphorylation overtime. GraphPad Prism software was used for graph generation. B) Myoblasts were treated with vehicle, Dex (1 μM) or AngII (10 μM) for 15 min (n = 3/treatment) prior to immunoblotting and quantification of phospho-PKD (S744/S748) normalized to total PKD. GraphPad software was used to generate graphs with student's t-test with Welch's correction used to asses significance ($p < 0.05$). C) Myoblasts were pretreated with Rom (1 μM) for 24 h prior to stimulation with Dex (1 μM) or AngII (10μM). 15 min post-treatment cells were lysed and immunoblotted for phospho-PKD (S744/S748) and total PKD protein expression and quantified with Image J software. Graphs were generated and statistical analysis performed via GraphPad software. Student's t-test with Welch's correction was used to asses significance ($p < 0.05$).

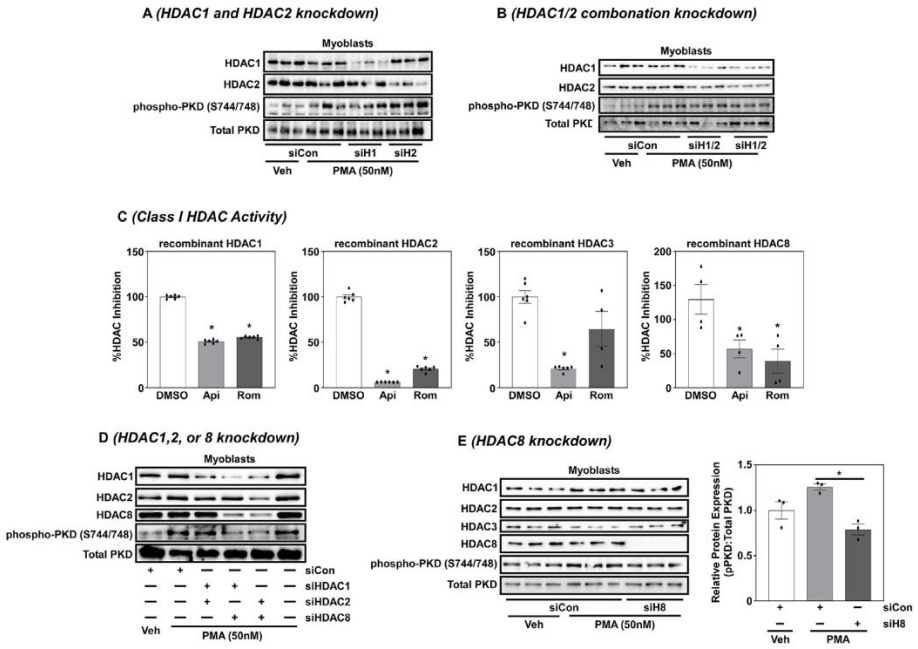

Fig. 3. HDAC8 regulates PKD phosphorylation in C2C12 myoblasts in response to PMA signaling. A) C2C12 myoblasts were transfected with a non-targeting control silencing RNA (siControl) or siRNA targeting HDAC1 (siH1) and HDAC2 (siH2) for 72 h and then stimulated with PMA (50 nM) for 30 min. Cells were lysed and immunoblotted for HDAC1, HDAC2, phosphorylated PKD (phopho-PKD; S744/S748) and total PKD. B) C2C12 myoblasts were transfected with an siControl or two different siRNAs targeting HDACs1 and 2 in combination (sH1/2) for 72 h prior to PMA (50 nM) stimulation for 30min. Cells were lysed and immunoblotted for HDAC1, HDAC2, phospho-PKD (S744/S748) and total PKD. C) Recombinant HDAC1, HDAC2, HDAC3 and HDAC8 were incubated with a class I-selective HDAC substrate prior to fluorescent analysis via the BioTeck Synergy 2 plate reader. D) Myoblasts were transfected with siControl or silencing RNAs in combination of HDACs 1, 2 or 8 for 72 h. Cells were then stimulated with PMA (50 nM) for 30 min, lysed and immunoblotted for HDAC1, HDAC2, HDAC8, phospho-PKD (S744/S748) and total PKD. E) Myoblasts were transfected with siControl or silencing RNA for HDAC8 for 72 h. Cells were then stimulated with PMA (50 nM) for 30min, lysed and immunoblotted for HDAC1, HDAC2, HDAC3, HDAC8, phospho-PKD (S744/S748) and total PKD. Images were quantified using Image J software and graphs were generated and statistical analysis performed via GraphPad software. One-way ANOVA with Tukey's post-hoc analysis was used to determine significance ($p < 0.05$).

recombinant HDACs 1, 2, 3, and 8 activity; all of these are class I HDACs. We report for the first time that romidepsin significantly inhibited HDACs 1, 2 and 8 activity but not HDAC3 (Fig. 3C). Apicidin on the other hand could inhibit all four class I HDACs (Fig. 3C). Based on these new findings, we next knocked down HDACs 1, 2, and 8 in combination. We report that PMA-induced PKD phosphorylation was only attenuated when HDAC8 was knocked down as observed in the HDAC1/8 (siH1/8) and HDAC2/8 (siH2/8) knockdown lanes (Fig. 3D). Moreover, we show that HDAC1/2 knockdown again failed to attenuate PKD phosphorylation (Fig. 3D). To confirm that HDAC8 regulates PKD phosphorylation, we next used siRNA to target HDAC8 individually. We observed that PMA induced PKD phosphorylation was significantly attenuated with HDAC8 knockdown (Fig. 3E).

4. Discussion

This report is the first to demonstrate that HDAC8 regulates PKD phosphorylation in C2C12 myoblasts independent of changes in total PKD expression. Moreover, we show that romidepsin does not selectively target HDACs 1 and 2, but also inhibits HDAC8 *in vitro* and in cell culture.

Interestingly, PKD phosphorylation of class IIa HDACs results in HDAC cytosolic translocation, which alleviates HDAC-mediated suppression of myogenic transcription factors (e.g. MEF2) resulting in muscle remodeling. Various stimuli that include work load, hormonal signaling and stress signaling regulates skeletal muscle regeneration, remodeling and differentiation; this is tightly controlled by myogenic transcription factors including myocyte enhancer factor-2 (MEF2) [34]. MEF2 activity is tightly regulated through association with class IIa HDACs to repress gene expression [34]. Myotube differentiation, for example, requires phosphorylation of class IIa HDACs by PKD, which is requisite for HDAC nuclear export and activation of MEF2 target genes [34]. Here, we report that class I HDAC inhibition attenuated PKD phosphorylation at S744/S748, sites necessary for PKD activity. Additionally, class I HDAC inhibition has been shown to attenuate myogenesis and myotube differentiation through inhibition of muscle specific genes such as myocyte heavy chain (MHC) in myoblasts [14]. Thus, these data suggest a potential mechanism by which class I HDACs control PKD phosphorylation to tightly regulate myogenic gene transcription via Class IIa HDAC nuclear localization. Studies are currently underway to address this question. It would also be important to determine if class I HDAC-mediated regulation of PKD phosphorylation is left unchecked in response to skeletal muscle diseases.

PKD is a serine-threonine kinase that functions in cell signaling, gene expression, protein secretion, inflammation and myogenic differentiation and proliferation [24]. Early reports demonstrated that PKD inhibition blocked myotube differentiation [35]. Consistently, we observed that PKD phosphorylation decreased with differentiation, suggesting that early activation of PKD is critical for myotube differentiation, while downregulation of PKD in mature myotubes is necessary for myotube maintenance. However, emerging evidence indicates that PKD is involved in pathological conditions linked to a variety of human diseases that include cancer, cardiovascular disease and inflammatory diseases [24]. Previous studies have demonstrated that PKD inhibition blunts pathological cardiac hypertrophy and that cardiac specific PKD knockout mice were resistant to cardiac hypertrophy and fibrosis in response to pressure overload [36]. PKD inhibition was also shown to enhance cardiac function and alleviated diabetic-induced cardiac remodeling and dysfunction [37].

To date, most studies have focused on HDACs 1 and 2 in skeletal muscle diseases such as atrophy/wasting, and myopathy [11,38,39]. These studies show that HDAC1 induced muscle fiber atrophy during disuse and pharmacological HDAC1 and 2 inhibition using MS-275 and trichostatin A (TSA) attenuated muscle fiber atrophy and contractile dysfunction [11,38]. However, we observed that HDAC8 controlled PKD phosphorylation at S744/S748. Interestingly, pharmacological or genetic HDAC8 inhibition was shown to attenuate cardiac hypertrophy and transverse aortic constriction (TAC)-induced heart failure [40,41]. More recently, HDAC8 was shown to be increased in patients with Duchene muscular dystrophy (DMD) and dystrophic zebra fish; not surprisingly, HDAC8 specific inhibition rescued skeletal muscle defects and improved cytoskeleton organization [42]. Together, this suggests that HDAC8 inhibition is potentially protective in models of skeletal muscle atrophy/wasting or myopathies by attenuating stress-induced PKD phosphorylation. While more work is needed regarding the physiological importance for HDAC8-mediated regulation of PKD phosphorylation in skeletal muscle, these data are the first to show the importance for this understudied class I HDAC in the regulation of skeletal muscle signal transduction, and in particular PKD signaling.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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