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3 **1 HDAC8 regulates canonical Wnt pathway to promote differentiation in skeletal muscles**

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1 **Running Title:** HDAC8 role in skeletal muscle differentiation

2

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9

10 **Keywords:** HDAC8, skeletal muscle, rhabdomyosarcoma, Wnt, zebrafish

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1  
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3 1 **Abstract**  
4

5 2 Histone deacetylase 8 (HDAC8) is a class 1 histone deacetylase and a member of the cohesin  
6 3 complex. *HDAC8* is expressed in smooth muscles but its expression in skeletal muscle has not been  
7 4 described. We show for the first time that *HDAC8* is expressed in human and zebrafish skeletal  
8 5 muscles. Using RD/12 and RD/18 rhabdomyosarcoma cells with low and high differentiation  
9 6 potency respectively, we highlight a specific correlation with *HDAC8* expression and an advanced  
10 7 stage of muscle differentiation. We inhibit HDAC8 activity through the specific PCI-34051 inhibitor  
11 8 in murine C2C12 myoblasts and zebrafish embryos and we observed skeletal muscles  
12 9 differentiation impairment. We also found a positive regulation of the canonical Wnt signalling by  
13 10 HDAC8 that might explain muscle differentiation defects. These findings suggest a novel  
14 11 mechanism through which HDAC8 expression in a specific time window of skeletal muscle  
15 12 development positively regulates canonical Wnt pathway that is necessary for muscle  
16 13 differentiation.  
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3 **1 Introduction**  
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6 2 Skeletal muscle is necessary to accomplish fundamental functions such as the maintenance of the  
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8 3 body structure, motility and metabolism by storing and consuming energy. Skeletal muscle  
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10 4 development is a multistep process in which myogenic cells are committed to proliferating  
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12 5 myogenic precursors that then differentiate into myoblasts and myocytes that fuse to form a  
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14 6 multinucleated myotube. Several signals are essential for the regulation of skeletal muscle  
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16 7 differentiation involving transcription factors, signalling molecules, transduction pathways and  
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18 8 epigenetic modifications. Among these, the histone deacetylases (HDACs) are frequently part of the  
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20 9 regulatory elements of muscle genes (Sincennes, Brun, & Rudnicki, n.d.). The HDAC family  
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22 10 comprises at least 18 different enzymes classified in four classes in mammals, and has been  
23  
24 11 originally identified for histone deacetylation activity and nucleosome stability. Recent evidence  
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26 12 pinpoints their role in deacetylation also of non-histone targets such as p53 and alpha-tubulin (de  
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28 13 Leval et al., 2006) as well as in gene transcription (Grunstein, 1997; Megee, Morgan, Mittman, &  
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30 14 Smith, 1990). Skeletal muscle is necessary to accomplish fundamental functions such as the  
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32 15 maintenance of the body structure, motility and metabolism by storing and consuming energy.  
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34 16 Skeletal muscle development is a multistep process in which myogenic cells are committed to  
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36 17 proliferating myogenic precursors that then differentiate into myoblasts and myocytes that fuse to  
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38 18 form a multinucleated myotube. Several signals are essential for the regulation of skeletal muscle  
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40 19 differentiation involving transcription factors, signalling molecules, transduction pathways and  
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42 20 epigenetic modifications. Among these, the histone deacetylases (HDACs) are frequently part of the  
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44 21 regulatory elements of muscle genes (Sincennes, Brun, & Rudnicki, 2016). The HDAC family  
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46 22 comprises at least 18 different enzymes classified in four classes in mammals, and has been  
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48 23 originally identified for histone deacetylation activity and nucleosome stability. Recent evidence  
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50 24 pinpoints their role in deacetylation also of non-histone targets such as p53 and alpha-tubulin (de  
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52 25 Leval et al., 2006) as well as in gene transcription (Grunstein, 1997; Megee et al., 1990).  
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1 HDAC8 is the last cloned and characterized member of class I HDACs (Buggy et al., 2000; Van  
2 den Wyngaert et al., 2000), it diverges from other class I enzymes as the C-terminal protein-binding  
3 domain is not present, probably indicating a functional specialization during evolution (Gregoretta,  
4 Lee, & Goodson, 2004)(Somoza et al., 2004). HDAC8 is ubiquitously expressed and can localize to  
5 either the nucleus or the cytoplasm interacting with non-histone proteins such as the cohesin protein  
6 SMC3, estrogen receptor a (ERRa), p53, inv(16) fusion protein (Deardorff et al., 2012; Durst,  
7 Lutterbach, Kummalu, Friedman, & Hiebert, 2003; Wilson, Tremblay, Deblois, Sylvain-Drolet, &  
8 Giguère, 2010; Wu et al., 2013). Moreover, in normal human tissues *HDAC8* is expressed by  
9 smooth muscle including vascular and visceral smooth muscle cells, myoepithelial cells, and  
10 myofibroblasts (Durst et al., 2003; Wu et al., 2013) where interacts with cortical actin-binding  
11 protein cortactin and Smooth Muscle Actin (SMA) and regulates smooth muscle contraction  
12 (Buggy et al., 2000; J. Li et al., 2014; Olson et al., 2014).

13 In this study, we describe for the first time a specific *HDAC8* expression in human and zebrafish  
14 (*Danio rerio*) skeletal muscle and murine and human myogenic cells. In particular, we have  
15 analyzed the time course of *HDAC8* expression during skeletal muscle differentiation in murine  
16 C2C12 myoblasts and zebrafish. We noticed that *HDAC8* is mainly expressed when differentiation  
17 is already started; moreover, in rhabdomyosarcoma derived cell lines RD/12 and RD/18 with low  
18 and high differentiation potency respectively, the increment of *HDAC8* expression during the  
19 differentiation is prominent in RD/18 than in RD/12 cell line. We also demonstrate that HDAC8  
20 promotes muscle differentiation *in vitro* and *in vivo* as the pharmacological block of its deacetylase  
21 activity inhibits myogenesis in the C2C12 cellular model and in zebrafish. This function is  
22 accomplished through the canonical Wnt pathway that is down-regulated when HDAC8 activity is  
23 inhibited. Our results link for the first time the HDAC8 activity to broad aspects of skeletal muscle  
24 development and open new possibility in the use of HDAC8 specific inhibitors (*i.e.* PCI-34051)  
25 (Balasubramanian et al., 2008)) for therapeutic intervention on skeletal muscle diseases.

26

## 1 **Material and Methods**

### 3 *Animals*

4 Zebrafish (*Danio rerio*) embryos were raised and maintained under standard conditions and national  
5 guidelines (Italian decree 4th March 2014, n.26). All experimental procedures were approved by  
6 IACUC (Institutional Animal Care and Use Committee). Zebrafish AB strains obtained from the  
7 Wilson lab, University College London, London, United Kingdom were maintained at 28°C on a 14  
8 h light/10 h dark cycle. Embryos were collected by natural spawning, staged according to Kimmel  
9 and colleagues (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995) and raised at 28°C in fish  
10 water (Instant Ocean, 0,1% Methylene Blue in Petri dishes), according to established techniques.  
11 We express the embryonic ages in hours post fertilization (hpf) and days post fertilization (dpf).  
12 After 24 hpf, to prevent pigmentation 0,003% 1-phenyl-2-thiourea (Sigma-Aldrich, Saint Louis,  
13 Missouri, USA) was added to the fish water. Embryos were washed, dechorionated and  
14 anaesthetized, with 0.016% tricaine (Ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich),  
15 before observations and picture acquisitions. Embryos were fixed overnight in 4%  
16 paraformaldehyde (Sigma-Aldrich) in PBS at 4 °C, then dehydrated stepwise to methanol and  
17 stored at -20 °C.

### 19 *C2C12 and rhabdomyosarcoma cells*

20 C2C12 cells were maintained in growth medium Dulbecco's modified Eagle medium (DMEM)  
21 supplemented with 10% fetal bovine serum (FBS, Euroclone, Pero, Italy), 100 IU/mL penicillin and  
22 100 µg/mL streptomycin in a humidified incubator at 37 °C with 5% CO<sub>2</sub>. After reaching 80–90%  
23 confluence, cells were washed in phosphate-buffered saline (PBS) and differentiated in DMEM  
24 medium with Horse Serum 2% (HS, Thermo Fisher Scientific, Waltham, MS, USA). The medium  
25 was changed every 48 hours and cultured up to 9 days of differentiation.

1 RD/12 and RD/18 cell lines were two different clone originally isolated from the human embryonal  
2 rhabdomyosarcoma cell lines RD by Lollini and colleagues (Lollini et al., 1991). Cells were  
3 cultured in DMEM supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin and either  
4 10% fetal bovine serum or 2% horse serum. The culture medium was renewed every 48-72 hours up  
5 to 11 days of culture in differentiation medium.

### 6 7 ***RT-PCR and quantitative real time PCR (qPCR)***

8 Total RNAs were isolated from C2C12, RD/12, RD/18 cells and zebrafish embryos at different  
9 developmental stages using Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to  
10 the producer's instructions. After treatment with DNase I RNase-free (Roche, Basel, Switzerland)  
11 to avoid possible genomic contamination, 1µg of RNA was reverse-transcribed using the “ImProm-  
12 II™ Reverse Transcription System” (Promega, Madison, WI, USA) and a mixture of oligo(dT) and  
13 random primers according to manufacturer's instructions. qPCRs on C2C12 and  
14 rhabdomyosarcoma RNAs were carried out in a total volume of 20 µl containing 1X SsoAdv  
15 Universal SYBR Green Super Mix (Bio-Rad, Hercules, CA, USA), using proper amount of the RT  
16 reaction. qPCRs were performed using the CFX-96™ (Bio-Rad). Relative expression of *HDAC8*  
17 was normalized with different reference genes, in particular TATA-box binding protein (TBP) and  
18 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for C2C12 cell line while Actin  
19 and beta-2-microglobulin (B2M) for rhabdomyosarcoma cell lines.

20 qPCRs in zebrafish were carried out in a total volume of 20 µl containing 1X iQ SYBR Green  
21 Super Mix (Promega), using proper amount of the RT reaction. PCRs were performed using the  
22 BioRad iCycler iQ Real Time Detection System (BioRad). For normalization purposes, *rpl8*  
23 expression levels were tested in parallel with the gene of interest. Primer list in Supplementary  
24 Table 1.

### 25 26 ***In situ hybridization, histological analysis and immunohistochemistry***



1  
2  
3 1 Whole mount *in situ* hybridization (WISH) experiments, were carried out as described by Thisse  
4  
5 2 and colleagues (Thisse & Thisse, 2008). Antisense riboprobes were previously *in vitro* labelled with  
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7 3 modified nucleotides (*i.e.* digoxigenin, fluorescein, Roche). *hdac8* probe was cloned in our  
8  
9 4 laboratory. Primer list in Supplementary Table 1. WISH experiments were done at least in 3 batches  
10  
11 5 of embryos of (minimum 30 embryos for each category). Immunohistochemistry analysis was  
12  
13 6 carried out on 6 um-thick cryosections from human skeletal muscle biopsy. The muscle biopsy was  
14  
15 7 performed after informed consent, snap-frozen in isopentane/liquid nitrogen, and maintained in  
16  
17 8 liquid nitrogen. Cryosections were permeabilized in cold methanol (MetOH) 50% for 1 minute and  
18  
19 9 MetOH 100% for 1 minute. Cryosections were hydrated with PBS and then blocked for 30 min at  
20  
21 10 room temperature in Normal Goat Serum (NGS) 1X and incubated with primary and secondary  
22  
23 11 antibodies. Primary antibodies were anti-HDAC8 (1:100) (polyclonal clone (H-145): sc-11405,  
24  
25 12 Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-Lamin B (1:100) (monoclonal  
26  
27 13 clone, Novocastra/YLEM, New Castle-upon-Tyne, UK). Secondary antibody were Alexa 488-  
28  
29 14 conjugated goat anti-mouse IgG or Alexa 546-conjugated goat anti-rabbit IgG, (Invitrogen Life  
30  
31 15 Technologies, Carlsbad, CA, USA) both diluted 1:2000. As control, sections were incubated either  
32  
33 16 with isotype specific IgG or the primary antibody was omitted. Sections were examined either  
34  
35 17 under a Zeiss fluorescence microscope. Immunohistochemistry in zebrafish was carried out as  
36  
37 18 described in Pistocchi and colleagues (Pistocchi, Gaudenzi, et al., 2013). Primary antibody was  
38  
39 19 mouse anti-sarcomeric (MF20, DSHB, diluition 1:4). Secondary antibody was EnVision+ System-  
40  
41 20 HRP Labelled Polymer anti-mouse (Dako, Glostrup, Denmark). Images of embryos and sections  
42  
43 21 were acquired using a microscope equipped with a digital camera with LAS Leica imaging software  
44  
45 22 (Leica, Wetzlar, Germany). Images were processed using the Adobe Photoshop software and when  
46  
47 23 necessary, different focal images planes of the same image have been took separately and later  
48  
49 24 merged in a single image.  
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## 26 ***Injections***

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3 1 Injections were carried out on 1- to 2-cell stage embryos; the dye tracer rhodamine dextran was also  
4  
5 2 co-injected. To repress *hdac8* mRNA translations, one morpholino was synthesized (Gene Tools  
6  
7 3 LLC, Philomath OR, USA) targeting *hdac8*-ATG. and used at the concentration of 1 pmole/embryo  
8  
9 4 in 1x Danieau buffer (pH 7,6). A standard control morpholino oligonucleotide (ctrl-MO) was  
10  
11 5 injected in parallel (Nasevicius & Ekker, 2000). ATG-*hdac8*-MO: 5'-  
12  
13 6 CATTACTGTCGCTTTTTTCACTCAT-3'.  
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### 16 7

### 18 8 ***PCI-34051 treatment***

19  
20 9 For C2C12 cells, HDAC8 inhibitor PCI-34051 (PCI) (Cayman Chemical; Ann Arbor, MI, USA)  
21  
22 10 was administrated at 25  $\mu$ M together with differentiating-medium; negative controls were treated  
23  
24 11 with the solvent Dimethyl-sulfoxide (DMSO). The PCI was changed every 24 hours until myogenic  
25  
26 12 differentiation. Zebrafish embryos after the shield developmental stage (6 hpf), were treated with  
27  
28 13 150  $\mu$ M PCI added to the fish water at 28°C kept in dark. As a control DMSO was used at the same  
29  
30 14 concentration. The PCI was changed every 24 hours and the embryos are let grown until the desired  
31  
32 15 developmental stage. For dose-dependent assays in zebrafish, the PCI was administrated at 50, 100,  
33  
34 16 150, 250  $\mu$ M  
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### 38 17

### 40 18 ***Western Blotting***

41  
42 19 Whole cell extracts from at least 30 zebrafish embryos were classically prepared in RIPA buffer (50  
43  
44 20 mM Tris-HCl pH 7.4, 1% NP-40, 150 mM NaCl, 0.25% sodium deoxycholate, 1mM EDTA, 1mM  
45  
46 21 PMSF, protease inhibitors Roche) (2  $\mu$ l/embryo or 1 $\mu$ l/ tail). Yolk was previously removed from  
47  
48 22 embryos to avoid yolk protein contamination. The protein concentration was determined using a  
49  
50 23 Micro BCA protein assay kit according to the manufacturer's instructions (Euroclone). 30-40  $\mu$ g of  
51  
52 24 each sample were loaded onto a 7.5% or 10% polyacrylamide gels and subjected to electrophoresis.  
53  
54 25 The proteins were then transferred onto PVDF membranes which were blocked using a blocking  
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3 1 solution at room temperature for 1 hour prior to incubation with the primary antibodies listed in  
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5 2 Supplementary Table 2. After incubation with the HRP-conjugated secondary antibodies for 1 h at  
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7 3 room temperature (Secondary antibodies are listed in Supplementary Table 2). The protein bands  
8  
9 4 were detected using ECL detection systems. Imaging acquisition has been done with the Alliance  
10  
11 5 MINI HD9 AUTO Western Blot Imaging System (UVItec Limited, Cambridge) and analysed with  
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13 6 the related software (Bellipanni, Murakami, & Weinberg, 2010).  
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3 **1 Results**

4 *HDAC8 is expressed in skeletal muscle and its expression correlates with an advance differentiated*  
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6  
7 *state of muscle cells.*

8  
9 Several expression profiles of *HDAC8* suggested that it has a ubiquitous expression in human  
10 tissues, with higher expression in particular organs such as brain, pancreas, kidney, prostate, liver  
11 and smooth muscles. *HDAC8* transcript and protein have been detected both in the nucleus and  
12 cytosol, suggesting that *HDAC8* might have a variable localization within the cell, depending on the  
13 cell type and its post-translational modifications such as phosphorylation (Buggy et al., 2000; de  
14 Ruijter, van Gennip, Caron, Kemp, & van Kuilenburg, 2003; Hu et al., 2000; Waltregny et al.,  
15 2004). Using immunofluorescence assays we detected for the first time an expression of *HDAC8* in  
16 normal human skeletal muscle with a predominant nuclear localization of the protein, as shown by  
17 the co-localization of *HDAC8* and Lamin B (Figure 1A-C).

18  
19 In parallel, we cloned the zebrafish orthologue of human *HDAC8* (Chr 7: 51,656,099-51,710,015),  
20 and by whole mount *in situ* hybridization analyses (WISH) we confirmed the expression of *hdac8*  
21 in skeletal muscle of zebrafish embryos at different developmental stages (Figure 1D-F'). In  
22 zebrafish the expression of *hdac8* varied among the developmental stages analysed (24, 36 and 48  
23 hours post fertilization, hpf), and was increased at 36 hpf when the first myogenic wave have  
24 already occurred (Stellabotte, Dobbs-McAuliffe, Fernandez, Feng, & Devoto, 2007) (Figure 1D-F').

25  
26 We therefore investigated a possible correlation between *HDAC8* expression and skeletal muscle  
differentiation progression. We first examined its expression in murine C2C12 skeletal myogenic  
cells, which represent a highly suitable model for analysis of myogenic differentiation. C2C12  
myoblasts proliferate in growth medium with high serum concentration (10% FBS) until they reach  
confluence, while differentiation into multinucleated myotubes is triggered shifting to  
differentiation medium with low serum concentration (2% horse serum). *Hdac8* transcript, analysed  
by qRT-PCR techniques, was present in C2C12 cells in growth medium and in differentiation  
medium at 1 days but its expression was significantly increased at 7 and 9 days of differentiation

1 (Figure 1G). In zebrafish the expression of *hdac8* analysed by qRT-PCR techniques confirmed the  
2 results previously shown by WISH, as the transcript is increased after the first myogenic wave when  
3 differentiation was accomplished with an expression peak at 36 hpf (Figure 1H). To further confirm  
4 the correlation between *HDAC8* expression and an advanced stage of differentiation, we choose two  
5 different subclones of the rhabdomyosarcoma cell line RD which differ in the differentiation  
6 potency: the RD/18 cells are able to reach a terminal differentiation while the RD/12 cells do not  
7 fully differentiate (Lollini et al., 1991). The expression of *HDAC8* was significantly increased at 11  
8 days of differentiation with an increment of 4 fold in RD/18 and about 1 fold in RD/12 (Figure 1I).

9 *HDAC8 activity regulates skeletal muscle differentiation in zebrafish and C2C12 myoblasts.*

10 To investigate a possible function of HDAC8 in differentiating skeletal muscles we took advantage  
11 of the well characterized PCI inhibitor that blocks HDAC8 deacetylase activity (Balasubramanian et  
12 al., 2008). We administrated PCI to zebrafish embryos *in-vivo* and C2C12 cells *in-vitro*. Zebrafish  
13 embryos were treated with a concentration of 150  $\mu$ M of PCI from the 50% stage of epiboly, a  
14 developmental stage in which the mesodermal layer, from which skeletal muscle derives, is  
15 positioning in the gastrula. At 48 hpf, zebrafish embryos presented morphological defects in the  
16 Central Nervous System and muscles, the regions where *hdac8* transcript was more expressed as  
17 shown in Figure 1. The PCI treated embryos could be divided in three phenotypical classes based  
18 on the severity of the CNS and muscle phenotype: class I showed a phenotype comparable to the  
19 control embryos treated with the solvent DMSO, class II presented a mild phenotype and class III  
20 presented a severe phenotype (Figure 2A-D, class quantification in E). We performed a dose-  
21 response assay demonstrating that the observed phenotypes were correlated to the doses of PCI  
22 treatment (Suppl. Figure S1). The sarcomeric myosins, that are expressed in differentiated and  
23 functional muscle, were diminished in PCI-treated embryos in comparison to controls analysed by  
24 immunohistochemistry and Western blot techniques (Figure 2 F-I). Interestingly, same  
25 morphological defects and myosin reduction were obtained in zebrafish embryos injected with the

1  
2  
3 1 *hdac8* morpholino (*hdac8*-MO) that blocks Hdac8 protein production. These data indicate that the  
4  
5 2 skeletal muscle differentiation impairment was specific due to Hdac8 loss-of-function (Suppl.  
6  
7 3 Figure S2). Moreover, at 24 hpf the embryos treated with PCI did not present myogenic impairment  
8  
9 4 confirming that Hdac8 activity is not necessary during early skeletal muscle differentiation (Suppl.  
10  
11 5 Figure S3).

12  
13 6 Also *in-vitro*, PCI treatment blocked differentiation of C2C12 myoblasts in comparison to DMSO  
14  
15 7 treated cells. Under differentiating conditions, wild-type C2C12 cells fused into multinucleated  
16  
17 8 myotubes. By contrast, when challenged to differentiate in low-serum medium in presence of PCI,  
18  
19 9 C2C12 cells remained mononucleated and maintained an undifferentiated phenotype. We assessed  
20  
21 10 that the differentiation of PCI treated cells was impaired in comparison to DMSO treated cells as  
22  
23 11 the levels of sarcomeric myosins analysed by Western blot technique were diminished (Figure 2J-  
24  
25 12 K).

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31 14 *HDAC8 regulates skeletal muscle differentiation through the activation of the canonical Wnt*  
32  
33 15 *pathway.*

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35  
36 16 In order to gain mechanistic insights into how HDAC8 regulates skeletal muscle differentiation, we  
37  
38 17 hypothesized that it modulates the canonical Wnt pathway, a well-known regulator of skeletal  
39  
40 18 muscle development and differentiation (Rudnicki & Williams, 2015). Indeed, in a hepatocellular  
41  
42 19 model, it has been demonstrated that HDAC8 positively regulates the  $\beta$ -catenin/TCF signalling  
43  
44 20 acting in concert with EZH2 to epigenetically repress Wnt antagonists (Tian et al., 2015).  
45  
46 21 Therefore, we analysed the activation status of the canonical Wnt pathway in zebrafish embryos and  
47  
48 22 C2C12 myoblasts treated with PCI. The phosphorylated and active form of  $\beta$ -catenin was  
49  
50 23 diminished by Western blot analyses in PCI treated zebrafish embryos in comparison to controls  
51  
52 24 treated with the DMSO. By contrast, the levels of total  $\beta$ -catenin were even increased (Figure 3A,  
53  
54 25 quantification in B and C). This last result is not surprising since we have seen similar up-regulation  
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1 of  $\beta$ -Catenin in zebrafish embryos with impaired activity of the canonical Wnt pathway (Valenti et  
2 al., 2015). To verify the efficiency of the PCI-mediated Hdac8 inhibition that is responsible of the  
3 Wnt pathway down-regulation, we analysed the acetylation status of Smc3, a known Hdac8 target  
4 (Deardorff et al., 2012). Acetylated Smc3 (Smc3ac) levels were increased following PCI treatment  
5 of the embryos, confirming the block of Hdac8 activity (Figure 3D, quantification in E). Same  
6 results were obtained in the C2C12 cells in differentiation medium treated with PCI: Western blot  
7 analyses confirmed the lower expression of active  $\beta$ -catenin in comparison to total  $\beta$ -catenin  
8 (Figure Figure 3F, quantification in G and H) and increased levels of Smc3ac following PCI  
9 treatment (Figure 3I, quantification in J).

10 The Wnt pathway in zebrafish can be activated through chemical treatments such as LiCl  
11 (Pistocchi, Fazio, et al., 2013). Therefore, to further demonstrate that skeletal muscle differentiation  
12 impairment observed with PCI-mediated Hdac8 inhibition was specifically due to Wnt pathway  
13 down-regulation, we re-activated the pathway adding LiCl in PCI-treated zebrafish embryos. The  
14 morphological defects presented by PCI-treated embryos at 36 hpf (embryos with morphological  
15 defects: 50/70) were partially rescued by LiCl addition (embryos with morphological defects:  
16 20/70) (Figure 4A-C). Moreover, the levels of sarcomeric myosins analysed by Western blot  
17 techniques were rescued in embryos treated with PCI+LiCl in comparison to embryos treated only  
18 with PCI (Figure 4D, quantification in E). We also verify the efficiency of LiCl treatment by  
19 measuring the active  $\beta$ -catenin levels (Figure 4D, quantification in F).

## 1 Discussion

2 In previous works HDAC8 was shown to be expressed in smooth muscle cells in association with  
3 SMA and cortactin (Jia Li et al., 2014) and its silencing by RNA interference (RNAi) impairs the  
4 contraction of smooth muscle cultured cells (Waltregny et al., 2005). However, the role and  
5 mechanism of HDAC8 action in smooth muscle tissues are largely unknown. In this work, we  
6 described for the first time the expression and role of HDAC8 in the skeletal muscle. Firstly, we  
7 demonstrated that HDAC8 is expressed in human and zebrafish skeletal muscle; then we analysed  
8 the expression of *HDAC8* during muscle differentiation in the murine C2C12 skeletal muscle cells,  
9 during zebrafish muscle development and in two types of rhabdomyosarcoma cells with various  
10 degree of invasiveness correlating to their ability to differentiate (RD/12 and RD/18). We decided  
11 to include these cells in the expression analyses as it has been reported that HDACi synergize with  
12 current anticancer drugs to induce apoptosis in rhabdomyosarcoma although the authors observed a  
13 switch to myogenic differentiation (Vleeshouwer-Neumann et al., 2015; Di Pompo et al., 2015).  
14 Interestingly, we correlate the expression of *HDAC8* with an advanced differentiation state of  
15 skeletal muscles. Indeed, both in C2C12 cells and zebrafish, *HDAC8* expression is weak in the  
16 initial phases and increases later during the muscle differentiation process. These data are even  
17 more striking in the rhabdomyosarcoma cells, where the RD/18 cell line cultured in the  
18 differentiation medium for 11 days shows a greater increase in the HDAC8 expression compared to  
19 the RD/12 cell line maintained in the same conditions. This increase correlates with the  
20 differentiation capacity of the two cell lines.

21 For functional analyses, we treated the C2C12 cells and the zebrafish embryos with the HDAC8  
22 inhibitor PCI-34051. In zebrafish, we also performed loss-of-function studies by injecting the  
23 oligonucleotide antisense morpholino designed against *hdac8* to compare and confirm the results  
24 obtained with the PCI-34051 treatment. Both in the cellular and zebrafish models with reduced  
25 HDAC8 activity, we observed an impairment in muscle differentiation following the initial  
26 myoblast commitment, in line with the kinetic of *HDAC8* expression previously analysed. In the



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3 1 C2C12 cells, myoblasts were formed but failed to fuse in myotubes and to express the markers of  
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5 2 differentiation; in zebrafish, the levels of functional myosins were reduced after 24 hpf but the  
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7 3 myogenic program started, as demonstrated by the proper expression of the MRFs MyoD and Myog  
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9 4 and by the presence of myosin proteins. Interestingly, it has been already shown that the levels of  
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11 5 myogenin were not affected by myoblast exposure to HDACi (Iezzi, Cossu, Nervi, Sartorelli, &  
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13 6 Puri, 2002), suggesting that HDACi selectively activate late muscle markers. It is also reported a  
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15 7 dual action for HDACi on muscle differentiation, depending on the stage of administration:  
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17 8 previous studies reported that HDACi have different effects by promoting or inhibiting myogenesis  
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19 9 (Steinbach, Wolffe, & Rupp, 1997) and this discrepancy might be explained by the stage-specific  
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21 10 effects of HDACi exposure. In zebrafish embryos, we performed the Hdac8 inhibition by adding the  
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23 11 PCI-34051 inhibitor after the shield stage (6 hpf) to prevent gross morphological defects in the  
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25 12 initial phase of gastrulation when mesoderm is defined.

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28 13 The block on muscle differentiation observed following HDAC8 inhibition is correlated with the  
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30 14 down-regulation of the canonical Wnt pathway. Several works demonstrate that the formation of  
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32 15 skeletal muscle is tightly modulated by Wnt signalling for self-renewal and muscle differentiation  
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34 16 and its dysregulation leads to perturbation of muscle fibers. Chemical modulation of the Wnt/ $\beta$ -  
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36 17 catenin pathway in differentiating myoblasts, using the activator LiCl, increases both the number  
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38 18 and size of C2C12 myotubes while inhibitors of Wnt/ $\beta$ -catenin signalling result in a significant  
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40 19 decrease in myotube length (Abraham, 2016). Indeed, the Wnt target  $\beta$ -catenin interacts directly  
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42 20 with MyoD, enhancing its binding to E box elements and its transcriptional activity of muscle  
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44 21 specific genes. This transactivation is inhibited when  $\beta$ -catenin is deficient or the interaction  
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46 22 between MyoD and  $\beta$ -catenin is disrupted (Kim, Mei 2008). We demonstrate that the reduction of  
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48 23 myosins observed in PCI-34051 treated embryos was caused by a decrease in activated  $\beta$ -catenin  
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50 24 levels. A mechanism by which HDAC8 regulates the canonical Wnt pathway has been recently  
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52 25 described in human NAFLD-associated hepatocellular carcinoma (HCC) by Tian and colleagues  
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1 (Tian, Mok, Yang, & Cheng, 2016). HDAC8 physically interacts with the polycomb protein  
2 enhancer of zeste homolog 2 (EZH2) and contributes to the activation of Wnt/ $\beta$ -catenin signalling.  
3 Further analyses are necessary to demonstrate whether this mechanism is conserved also in skeletal  
4 muscle and acetylome profile following PCI inhibition may uncover HDAC8-related targets.  
5 Dis-regulation of canonical Wnt signalling has been reported in different muscle pathologies, such  
6 as Duchenne Muscular Dystrophy (DMD) (Trensz, Haroun, Cloutier, Richter, & Grenier, 2010),  
7 FascioScapuloHumeral Muscular Dystrophy (FSHD) (Block et al., 2013) and OculoPharyngeal  
8 Muscular Dystrophy (OPMD) (Abu-Baker et al., 2013). Inhibition of canonical Wnt signalling by  
9 Dkk in a mouse model for DMD (mdx), was shown to reduce fibrosis (Trensz et al., 2010). HDACi  
10 are recently emerged as potential pharmacological strategies for cancer treatment, and several of  
11 them are already approved by the international Drug Administration agencies. The increasing  
12 interest and use of HDACi has led to the development of class-specific inhibitors, such as the PCI-  
13 34051, which helps us to uncover the functional role of HDAC8 in skeletal muscle differentiation  
14 and, in the future, might ameliorate the phenotype in pathological conditions. Based on the  
15 numerous beneficial effects of HDACi in skeletal muscle under pathological conditions, we believe  
16 that they are promising therapeutics.

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3 **1 Competing interests**

4  
5 2 All authors declare that they have no conflict of interest. Declaration of interest: none.  
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9 4 **Author contributions:** conceived and designed the experiments: AP<sup>1</sup>, AM<sup>1</sup>, PV<sup>1</sup>. Performed the  
10 experiments on human samples: CB<sup>2,5</sup>, MM<sup>2</sup>. Performed the experiments in zebrafish: MS<sup>1</sup>, SE<sup>1</sup>,  
11 LB<sup>1</sup>, AP<sup>1</sup>, AP<sup>1</sup>, LF<sup>1</sup>. Performed the experiments in C2C12 cells: LF<sup>1</sup>, AMF<sup>4,6</sup>, FP<sup>3</sup>, FF<sup>3</sup>. Performed  
12 the experiments in rhabdomyosarcoma cells: FP<sup>3</sup>, FF<sup>3</sup>. Analyzed the data on human sample: CB<sup>2,5</sup>,  
13 MM<sup>2</sup>. Analysed the data in zebrafish: MS<sup>1</sup>, SE<sup>1</sup>, LB<sup>1</sup>, AP<sup>1</sup>, AP<sup>1</sup>, LF<sup>1</sup>, PR<sup>1</sup>, AG<sup>7,8</sup>, GB<sup>7,8</sup>. Analysed  
14 the data in C2C12 cells: LF<sup>1</sup>, PR<sup>1</sup>, FP<sup>3</sup>, FF<sup>3</sup>, FB, AMF<sup>4,6</sup>, GC<sup>4</sup>. Wrote the paper: AP<sup>1</sup>. Supervised  
15 paper drafting: AP<sup>1</sup>, AG<sup>7,8</sup>, GB<sup>7,8</sup>. Supervised the research project AP<sup>1</sup>.  
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3 **1 Figures legends**

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5 **2 Figure 1: HDAC8 is expressed in human, murine and zebrafish skeletal muscle and its**  
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7 **3 expression correlates with differentiation potency.** (A-C) HDAC8 protein expression in normal  
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9 4 human skeletal muscles. Immunofluorescence staining of HDAC8 (A), Lamin B (B) and merge of  
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11 5 the two signals (C). The localization of HDAC8 in human skeletal muscle is predominantly nuclear  
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13 6 as shown by the co-localization with the Lamin B protein. (D-F) *hdac8* mRNA expression in  
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15 7 zebrafish. WISH analyses of *hdac8* transcript localization in skeletal muscle of zebrafish embryos at  
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17 8 24 hpf (D), 36 hpf (E) and 48 hpf (F). Transverse histological sections of the previously hybridized  
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19 9 embryos show the localization of *hdac8* transcript in the myotome (D',E',F'). (G) *Hdac8* qRT-PCR  
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21 10 analyses on murine C2C12 myoblasts at different stages of differentiation. *Hdac8* expression is  
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23 11 increased at 7-9 days after the induction of the differentiation when differentiation is accomplished.  
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25 12 (H) *hdac8* qRT-PCR analyses on RNA from 24, 36 and 48 hpf zebrafish embryos. *hdac8* expression  
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27 13 is increased at 36 hpf when the first myogenic wave is completed. (I) *HDAC8* qRT-PCR analyses  
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29 14 on RD/12 and RD/18 rhabdomyosarcoma cells. At 11 days after the induction of differentiation,  
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31 15 *HDAC8* is more expressed in RD/18 cells that are able to fully differentiate in comparison to RD/12  
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33 16 cells. Scale bar represents 50  $\mu\text{m}$  in (A-C) and 100  $\mu\text{m}$  (D-F'). Asterisks represent \*\* $p < 0.01$ ,  
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35 17 \*\*\* $p < 0.001$ , Student's t test.  
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42 **19 Figure 2: Inhibition of HDAC8 activity reduces skeletal muscle differentiation in zebrafish**  
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44 **20 embryos and murine C2C12 myoblasts.** (A-E) *In-vivo* treatment of zebrafish embryos with DMSO  
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46 21 or PCI. Different phenotypical classes with increased severity (B-D; quantification in E) with PCI  
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48 22 treatment compared to the control embryos treated with the DMSO (A). (F-G)  
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50 23 Immunohistochemical staining (IHC) and (H-I) western blot analyses of sarcomeric myosins.  
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52 24 Sarcomeric myosins are reduced in PCI treated embryos at 48 hpf (G) in comparison to controls  
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54 25 (F). Western blot analyses (H; quantification in I) confirmed all MyHC reduction in PCI treated  
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56 26 embryos in comparison to controls. (J-K). Inhibition of HDAC8 activity impaired C2C12  
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3 1 differentiation. Western blot analyses (*J*; quantification in *K*) confirmed all MyHC reduction in PCI  
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5 2 treated C2C12 cells in comparison to DMSO treated. Scale bars indicates 100  $\mu\text{m}$  in (*A*, *F*).  
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7 3 Asterisks represent  $*p<0.05$ , Student's t test.  
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11 5 **Figure 3: HDAC8 activates canonical Wnt signalling.** (*A-C*) Canonical Wnt signalling was  
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13 6 decreased with the PCI treatment in zebrafish embryos. (*A*) Active  $\beta$ -catenin was decreased in PCI  
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15 7 treated embryos in comparison to DMSO controls while total  $\beta$ -catenin was increased by Western  
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17 8 blot analyses and relative quantifications (*B-C*). (*D-E*) The efficacy of PCI treatment was verified  
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19 9 by the acetylation status of the Hdac8 target Smc3. (*D*) Smc3ac levels were increased in PCI treated  
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21 10 embryos in comparison to DMSO controls, quantification in (*E*). (*F-H*) Canonical Wnt signalling  
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23 11 was decreased with the PCI treatment in C2C12 cells in differentiation medium. (*F*) Active  $\beta$ -  
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25 12 catenin was decreased in PCI treated C2C12 cells in comparison to those treated with DMSO, while  
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27 13 total  $\beta$ -catenin was increased by Western blot analyses and relative quantifications (*G-H*). (*I-J*) The  
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29 14 efficacy of PCI treatment in the C2C12 was verify by the acetylation status of the Hdac8 target  
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31 15 Smc3. (*I*) Smc3ac levels were increased in PCI treated C2C12 in comparison to DMSO controls,  
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33 16 quantification in (*J*). Asterisks represent  $*p<0.05$ ,  $***p<0.001$ , Student's t test.  
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40 18 **Figure 4: The HDAC8-mediated positive regulation of Wnt signalling is responsible for**  
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42 19 **skeletal muscle differentiation.** (*A-C*) Morphological defect presented by PCI-treated embryos  
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44 20 were rescued by LiCl addition. (*D-F*) Skeletal muscle differentiation was rescued when the Wnt  
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46 21 pathway was restored by LiCl in PCI treated zebrafish embryos. (*D*) Sarcomeric myosins, analysed  
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48 22 by Western blot techniques, decreased in PCI treated embryos and returned comparable to those  
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50 23 treated with DMSO when Wnt pathway was rescued adding LiCl (quantification in *E*). The efficacy  
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52 24 of LiCl treatment was verify measuring the active  $\beta$ -catenin by Western blot techniques  
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54 25 (quantification in *F*). Asterisks represent  $*p<0.05$ , Student's t test.  
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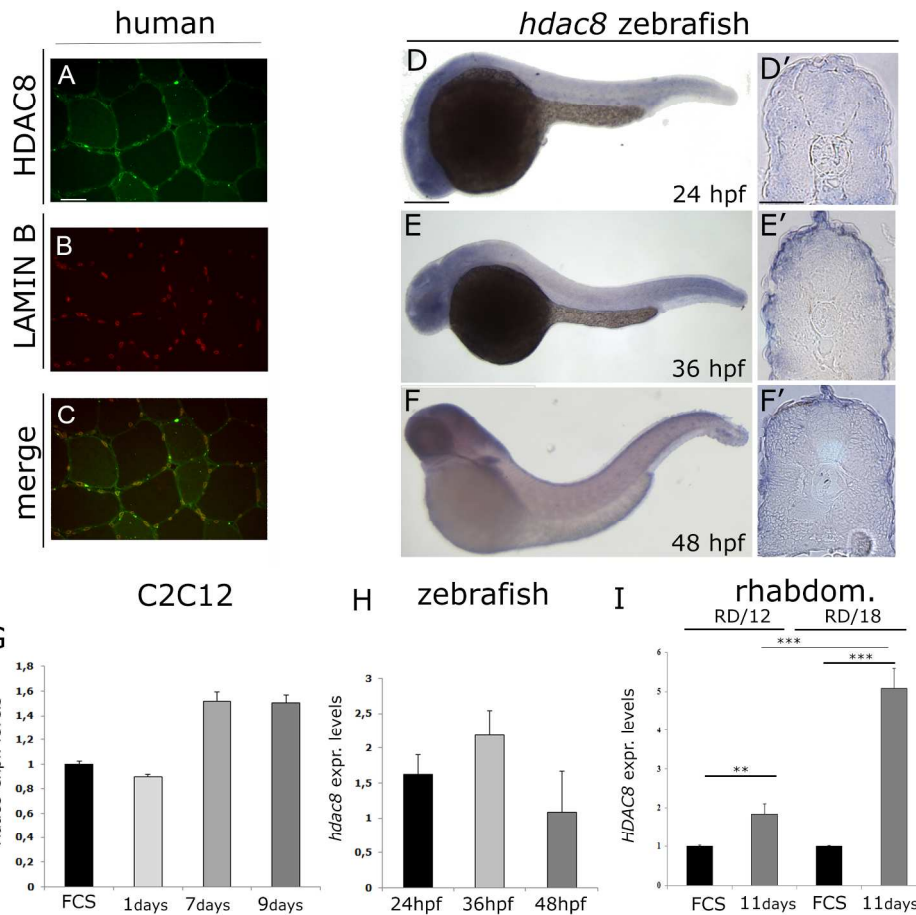


Figure 1: HDAC8 is expressed in human, murine and zebrafish skeletal muscle and its expression correlates with differentiation potency. (A-C) HDAC8 protein expression in normal human skeletal muscles. Immunofluorescence staining of HDAC8 (A), Lamin B (B) and merge of the two signals (C). The localization of HDAC8 in human skeletal muscle is predominantly nuclear as shown by the co-localization with the Lamin B protein. (D-F) *hdac8* mRNA expression in zebrafish. WISH analyses of *hdac8* transcript localization in skeletal muscle of zebrafish embryos at 24 hpf (D), 36 hpf (E) and 48 hpf (F). Transverse histological sections of the previously hybridized embryos show the localization of *hdac8* transcript in the myotome (D',E',F'). (G) *Hdac8* qRT-PCR analyses on murine C2C12 myoblasts at different stages of differentiation. *Hdac8* expression is increased at 7-9 days after the induction of the differentiation when differentiation is accomplished. (H) *hdac8* qRT-PCR analyses on RNA from 24, 36 and 48 hpf zebrafish embryos. *hdac8* expression is increased at 36 hpf when the first myogenic wave is completed. (I) HDAC8 qRT-PCR analyses on RD/12 and RD/18 rhabdomyosarcoma cells. At 11 days after the induction of differentiation, HDAC8 is more expressed in RD/18 cells that are able to fully differentiate in comparison to RD/12 cells. Scale bar represents 50  $\mu$ m in (A-C) and 100  $\mu$ m (D-F'). Asterisks represent \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , Student's t test.

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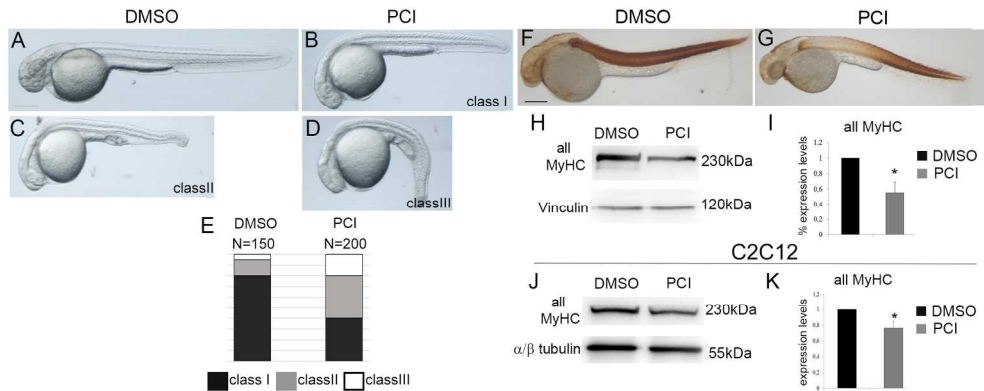


Figure 2: Inhibition of HDAC8 activity reduces skeletal muscle differentiation in zebrafish embryos and murine C2C12 myblasts. (A-E) In-vivo treatment of zebrafish embryos with DMSO or PCI. Different phenotypical classes with increased severity (B-D; quantification in E) with PCI treatment compared to the control embryos treated with the DMSO (A). (F-G) Immunohistochemical staining (IHC) and (H-I) western blot analyses of sarcomeric myosins. Sarcomeric myosins are reduced in PCI treated embryos at 48 hpf (G) in comparison to controls (F). Western blot analyses (H; quantification in I) confirmed all MyHC reduction in PCI treated embryos in comparison to controls. (J-K). Inhibition of HDAC8 activity impaired C2C12 differentiation. Western blot analyses (J; quantification in K) confirmed all MyHC reduction in PCI treated C2C12 cells in comparison to DMSO treated. Scale bars indicates 100  $\mu$ m in (A, F). Asterisks represent  $*p < 0.05$ , Student's t test.

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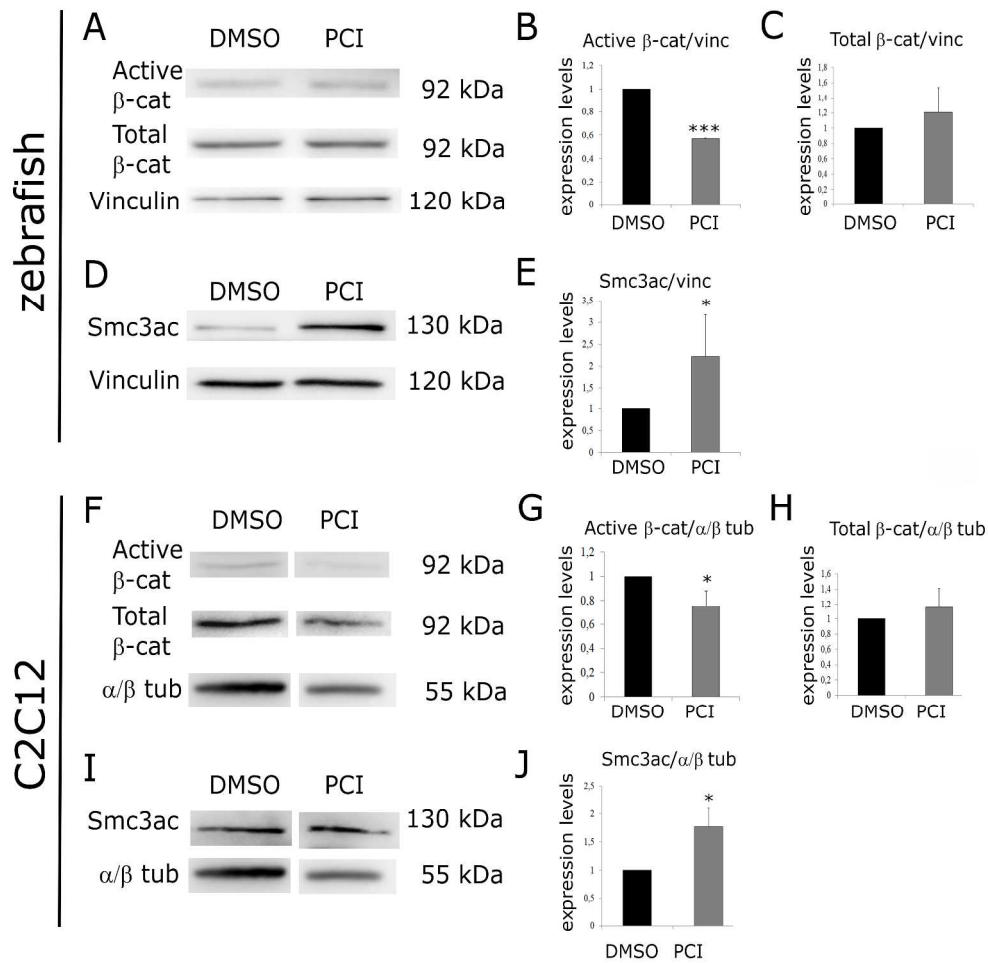


Figure 3: HDAC8 activates canonical Wnt signalling. (A-C) Canonical Wnt signalling was decreased with the PCI treatment in zebrafish embryos. (A) Active β-catenin was decreased in PCI treated embryos in comparison to DMSO controls while total β-catenin was increased by Western blot analyses and relative quantifications (B-C). (D-E) The efficacy of PCI treatment was verified by the acetylation status of the Hdac8 target Smc3. (D) Smc3ac levels were increased in PCI treated embryos in comparison to DMSO controls, quantification in (E). (F-H) Canonical Wnt signalling was decreased with the PCI treatment in C2C12 cells in differentiation medium. (F) Active β-catenin was decreased in PCI treated C2C12 cells in comparison to those treated with DMSO, while total β-catenin was increased by Western blot analyses and relative quantifications (G-H). (I-J) The efficacy of PCI treatment in the C2C12 was verify by the acetylation status of the Hdac8 target Smc3. (I) Smc3ac levels were increased in PCI treated C2C12 in comparison to DMSO controls, quantification in (J). Asterisks represent \* $p < 0.05$ , \*\*\* $p < 0.001$ , Student's t test.

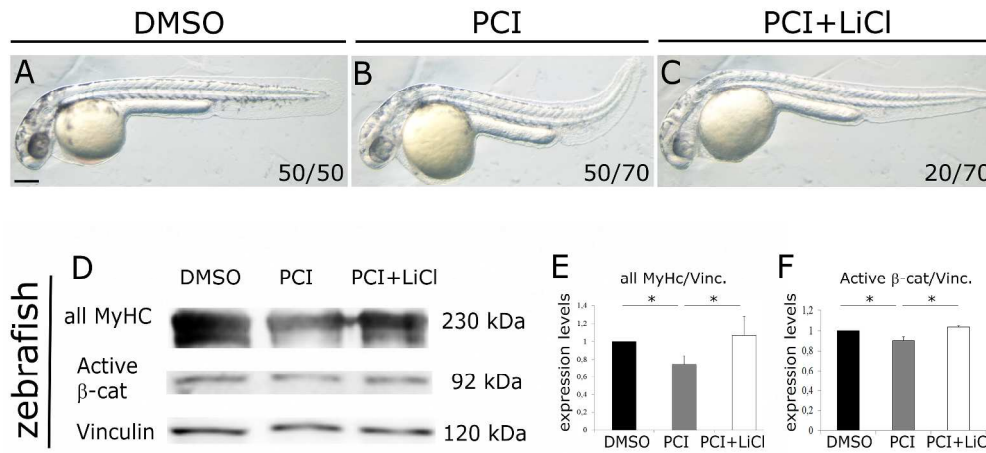


Figure 4: The HDAC8-mediated positive regulation of Wnt signalling is responsible for skeletal muscle differentiation. (A-C) Morphological defect presented by PCI-treated embryos were rescued by LiCl addition. (D-F) Skeletal muscle differentiation was rescued when the Wnt pathway was restored by LiCl in PCI treated zebrafish embryos. (D) Sarcomeric myosins, analysed by Western blot techniques, decreased in PCI treated embryos and returned comparable to those treated with DMSO when Wnt pathway was rescued adding LiCl (quantification in E). The efficacy of LiCl treatment was verify measuring the active  $\beta$ -catenin by Western blot techniques (quantification in F). Asterisks represent  $*p < 0.05$ , Student's t test.