Aldehyde Dehydrogenase 1 Isoforms ALDH1A1 and ALDH1A3 are Essential for Myogenic Differentiation

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Abstract

Background Satellite cells (SC) constitute the stem cell population of skeletal muscle tissue and are determinants for myogenesis. Aldehyde Dehydrogenase 1 (ALDH1) enzymatic activity correlates with myogenic properties of SCs and, recently, we could show co-localization of its isoforms ALDH1A1 and ALDH1A3 in SCs of human skeletal muscle. ALDH1 is not only the pacemaker enzyme in retinoic acid signaling and differentiation, but also protecting cell maintenance against oxidative stress products. However, the molecular mechanism of ALDH1 in SC activation and regulation of myogenesis has not yet been characterized. In regard of ALDH1A1 and ALDH1A1 expression in myogenesis human RH30 and murine C2C12 myoblast cell lines were investigated using Western Blot, Immunofluorescence and Aldefluor Assay.

Results Here, we show, that isoforms ALDH1A1 and ALDH1A3 are pivotal factors in the process of myogenic differentiation, since ALDH1A1 knock-out and ALDH1A3 knock-out, respectively, impaired differentiation potential. Recombinant re-expression of ALDH1A1 and ALDH1A3, respectively, in corresponding ALDH1-isoform knock-out cells recovered their differentiation potential. Most interestingly, the chemical inhibition of enzymatic activity by disulfiram leads to ALDH1A1 and ALDH1A3 protein upregulation and subsequent myogenic differentiation.

Conclusion Our findings indicate that ALDH1A1 and ALDH1A3 proteins are important for myogenic differentiation and, therefore, seem to be essential activators and regulators of SCs.

Background

Satellite cells (SC) constitute the stem cell population of skeletal muscle tissue and are the resource for muscle growth and regeneration. SC action is induced by various stimuli, such as signaling for adaptive growth after physical activity and for regenerative processes after insult, inflammation or oxidative stress (Forcina et al. 2019; Sin et al. 2013).

Markers for quiescent and activated SCs have been established in experimental studies (Cobb 2013; Shen et al. 2003), that allow the tracing of proliferation and differentiation processes. Although the influence of environmental stimuli on myogenic growth and myotube formation has been addressed in several studies (Pownall et al. 2002; Mukund and Subramaniam 2020), the molecular mechanism of SC activators and regulators has yet not been fully understood.

The enzyme Aldehyde Dehydrogenase 1, in particular its isoforms ALDH1A1 and ALDH1A3, has been identified as an interesting actor in SC activation and myogenic differentiation. Recently, we were able to demonstrate the co-localization of isoforms ALDH1A1 and ALDH1A3 in SCs of human skeletal muscle tissue (Rihani et al., unpublished data). ALDH1 oxidizes retinaldehyde into retinoic acid (RA) in the vitamin A signaling pathway and regulates differentiation (Wang et al. 2010, Soprano et al. 2007). In addition, ALDH1 is detoxifying aldehydes resulting from oxidative stress and protects cells against mitochondria damage and apoptosis (Schieber and Chandel 2014). ALDH1 enzymatic activity has been

identified as a hallmark of myoblast subpopulations with high myogenic capacity and resistance against oxidative stress (Vella et al. 2011).

These findings indicate that ALDH1 seems to play an important role in SC maintenance and myogenesis, since it coordinates antioxidative cell protection and the regulation of differentiation by RA signaling. For this reason, detailed analysis of ALDH1 function in myogenic processes would be interesting.

Here we show, that isoforms ALDH1A1 and ALDH1A3 are key factors in the process of myogenic differentiation, since ALDH1A1 knock-out and ALDH1A3 knock-out, respectively, lost their differentiation potential. Recombinant re-expression of ALDH1A1 and ALDH1A3, respectively, in ALDH1-isoform knock-out cells recovered myogenic differentiation. Most interestingly, the chemical inhibition of enzymatic activity leads to ALDH1A1 and ALDH1A3 protein upregulation and consequently to myogenic differentiation.

Results

Morphological alteration in myogenesis

Proliferating murine C2C12 and human RH30 myoblasts showed a characteristic roundish shape with small protrusions (Fig. 1A left). Myotube formation was induced by serum-withdrawal and the typical multinucleic elongated shape could be observed within 6 days (Fig. 1A right). Differentiated C2C12 and RH30 cells demonstrated in Western Analysis upregulated levels of ALDH1A1 and ALDH1A3 (Fig. 2A).

Enzymatic ALDH1 activity in myogenesis was analyzed using Aldefluor assay (data not shown). Both cell lines demonstrated an increase of ALDH1 enzyme activity of 31.9% in differentiated C2C12 and 43.7% in differentiated RH30. We next inhibited generic ALDH activity by chemical inhibition using Disulfiram (DSF) in proliferating C2C12 and RH30 cells. Against our expectation, we observed myotube formation within 4 days of treatment despite no serum withdrawal was applied (Fig. 1A middle). Western Analysis showed comparable up-regulation of ALDH1A1 and ALDH1A3 protein levels in differentiated C2C12 and RH30 cells after DSF treatment and serum withdrawal (Fig. 2A). In addition, differentiated cells of serum-withdrawal and DSF-treatment demonstrated in immunofluorescent staining increased immunoreaction to antibodies directed against ALDH1A1 and ALDH1A3 (Fig. 3).

Expression of ALDH1A1 and ALDH1A3 in differentiation

Since ALDH1 protein levels seemed to be important for myotube formation, we then transfected C2C12 and RH30 cells with ALDH1A1-GFP and ALDH1A3-GFP fusion vectors. Transfected clones showed a strong overexpression of the fusion products by Western analysis (Fig. 2B) and, most importantly, spontaneously differentiated without serum withdrawal (Fig. 1B). In contrast, recombinant knock-out by CRISPR-Cas9 of ALDH1A1 and ALDH1A3, respectively, in C2C12 and RH30 completely inhibited myotube formation in the standard paradigm of serum withdrawal (Fig. 1C, 4 A, Fig. 5). Consecutive Aldefluor Assay demonstrated 1% ALDH1 activity in knock-out cells.

Rescue by re-transfection of ALDH1a1 and ALDH1a3 in knockout cells

In order to confirm the functional role of ALDH1 in differentiation, we re-expressed ALDH1A1 and ALDH1A3 proteins by transfection using ALDH1A1-GFP and ALDH1A3-GFP fusion vectors in corresponding knock-out cells. Successful expression of proteins could be demonstrated by Western blots (Fig. 4B upper line). As expected, the re-expression of ALDH1A1 and ALDH1A3 rescued the defective differentiation phenotype of the knock-outs. Re-transfected knock-out cells showed spontaneous differentiation by morphology (Fig. 1D) and Western analysis (Fig. 4B lower line).

Conclusion

The process of skeletal muscle growth relies on satellite cells (SCs), that constitute the skeletal muscle stem cell population. Various kinds of environmental stimuli like physical activity, insult, inflammation or oxidative stress affect myogenesis (Vella et al. 2011). However, SC activators and their functional role in skeletal muscle development have not yet been characterized.

Previously, the enzyme Aldehyde Dehydrogenase 1 (ALDH1) was associated as regulator of differentiation through retinoic acid (RA) signaling and as scavenger of oxidative stress products. Hence, ALDH1 is not only a functional part of growth behavior, but also a protector of cell maintenance. Moreover, highest ALDH1 activity was identified in a subpopulation of myogenic progenitors with a remarkable myogenic capacity (Vauchez et al. 2009).

In the present study, we investigated the myogenic behavior of human RH30 and murine C2C12 muscle cells with regard to ALDH1 activity and function. Increased ALDH1 activity and high protein levels of its isoforms ALDH1A1 and ALDH1A3, respectively, could be demonstrated in differentiated cells. Chemical inhibition of ALDH1 enzymatic activity caused accumulated protein levels of ALDH1A1 and ALDH1A3 and induced differentiation. These results were confirmed by recombinant overexpression of ALDH1A1 and ALDH1A3, respectively, that also lead to myotube formation, and genomic knock-out cells showed deficient differentiation. Recovery of differentiation ability in knock-out models could be restored by transfection of recombinant ALDH1A1 and ALDH1A3, respectively. We used human RH30 and murine C2C12 myoblast cell lines, which are widely accepted as an appropriate model system for the investigation of satellite cell biology.

Our data are in line with previous studies that demonstrated high ALDH activity in myogenic progenitors (Vella et al. 2011; Vauchez et al. 2009). More recently, several studies showed the importance of ALDH1 for skeletal muscle homeostasis in longevity(Papaconstantinou et al. 2015) and in protection against ageing effects (Etienne et al. 2020). Although these experiments demonstrated the importance of ALDH1 activity in muscle cell preservation(Jean et al. 2011) and myogenesis, functional aspects in

differentiation have not been addressed in detail. Importantly, the effect of a functional blockade by chemical inhibition of ALDH1 on cell differentiation could solve the question about the role of its enzymatic activity. We, therefore, inhibited ALDH1 by DSF, but contrary to our expectation RH30 and C2C12 cells showed myogenic differentiation without serum withdrawal. This effect was most likely due to a reactive upregulation of protein levels, since differentiated cells after DSF showed high ALDH1A1 and ALDH1A3. Next, we knocked-out ALDH1A1 and ALDH1A3, respectively, and could demonstrate, that the absence of its proteins blocked differentiation. These results were corroborated by transfection experiments, since ALDH1A1 and ALDH1A3 overexpression by recombinant re-expression in prior knock-out cells recovered the differentiation phenotype.

The exact mechanisms involved in ALDH1 transcriptional and post-translational regulation are not characterized in detail. Recently, we showed regulation of ALDH1A3 in glioma cells by autophagy in response to chemotherapy (Wu et al. 2018). Interestingly, protein levels were upregulated in a reactive manner comparable with the results of DSF inhibition in myoblast cells. Moreover, autophagy has been demonstrated as an important biological mechanism in maintaining the regenerative potential of satellite cells (Wen and Klionsky 2016). Therefore, ALDH1 seems to be a key factor for the understanding of skeletal muscle biology.

Our findings indicate that ALDH1A1 and ALDH1A3 proteins are important for myogenic differentiation and, therefore, seem to be essential activators and regulators of SCs.

Methods

Cell culture

Human RH30 and murine C2C12 myoblast cell lines were purchased from American Type Culture Collection (ATCC) and cultivated in antibiotic-free standard high-glucose medium without pyruvate (Life Technologies, Darmstadt, Germany). Both cell lines showed irreversible myotube formation by serum-withdrawal (10% fetal bovine serum versus 2% horse serum). For microscopic analysis an inverted microscope (Nikon, Düsseldorf, Germany; TS100) was used and captures were taken with a NIS-Elements Camera (Nikon, Düsseldorf, Germany). For chemical inhibition of ALDH1 enzymatic activity Disulfiram (DSF; Sigma, Munich, Germany) was applied with a concentration of 3 µM of DSF.

Antibodies and reagents

Primary antibodies for Western Blot and immunofluorescent staining were anti-α-Aktin (Santa Cruz Biotechnology, Heidelberg, Germany; monoclonal, J1917, mouse, dilution IF 1:500), anti-ALDH1A1 (Abcam, England, UK; monoclonal, EP1933Y, rabbit, dilution: WB 1:1000, IF 1:500; polyclonal, rabbit, dilution: WB 1:1000, IF 1:500), anti-ALDH1A3 (Abcam, England, UK; polyclonal, rabbit, dilution: WB 1:1000, IF 1:500; Novus Biologicals, Abingdon, UK; polyclonal, rabbit, dilution: WB 1:1000), anti-GFP XP (Cell signaling, Frankfurt, Germany; monoclonal, D5.1, rabbit, dilution: WB 1:1000), anti-Myogenin (Sigma, Munich, Germany; polyclonal, rabbit, dilution: WB 1:500, IF 1:500), anti- MF20/ Myosin 4 (Thermo Fisher,

Munich, Germany; monoclonal, 4301341, mouse, dilution: WB 1:1000), anti-Myosin Fast (Sigma, Munich, Germany; monoclonal, MY-32, dilution: WB 1:500) and anti-Vinculin (Abcam, England, UK; polyclonal, dilution WB: 1:20000).

Secondary antibodies for Western Blot were goat anti-rabbit, HRP-linked (Abcam, England, UK; dilution 1:5000), goat anti-mouse, HRP-linked (Cell signaling, Frankfurt, Germany; 1:3000); and for immunofluorescent staining: donkey anti-rabbit (Thermo Fisher, Munich, Germany; AlexaFluor 568, dilution 1:500), donkey anti-mouse (Thermo, Fisher, Munich, Germany; AlexaFluor 546, dilution 1:500).

Western Analysis

Samples were separated in SDS-PAGE and subsequently transferred to PVDF-membranes. To prevent unspecific binding of first antibodies the membrane was incubated for 45 minutes in 5% blocking-buffer (roti-block and milk, respectively). Subsequently, primary antibodies were adjusted as described above and incubated overnight at 4 °C, rolling. Anti-Vinculin was included for loading control. HRP-conjugated secondary antibodies were applied as indicated above and visualized by ECL substrate following the manufacturer’s recommendations. Image documentation was performed using AI680 Imager (GE Healthcare Life Science, Munich, Germany).

Immunofluorescence

Cells were cultivated and treated on µ-slides (Ibidi GmbH, Gräfelfing, Germany) for immunofluorescent staining. Slides were washed with PBS and cells were fixed in 4% formalin for 20 minutes at room temperature. Blocking buffer provided with donkey-serum was added and incubated for 30 minutes. After washing, primary antibody, diluted in blocking buffer with additive donkey serum, was applied overnight at 4 °C. Secondary antibody was added in a dilution as indicated with an incubation time of 40 minutes. Nucleic counterstaining with DAPI was conducted for 10 minutes. Samples were mounted with Aqua Poly/Mount (PolySciences, Hirschberg an der Bergstrasse, Germany). Immunofluorescence was examined with a Carl Zeiss Axioplan fluorescence microscope (Zeiss, Munich, Germany; HBO 100 Upright Fluorescence Microscope). Captures were conducted with an AxioCam and AxioVision 4.8 software.

Aldefluor assay

Enzymatic ALDH-activity analysis (Aldefluor assay) was performed according to the manufacturer`s guidelines (Stem Cell Technologies, Cologne, Germany). Cells were incubated for 30 minutes at 37 °C in Aldefluor assay buffer containing ALDH substrate bodipy-aminoacetaldheyde (1 µmol/l per 1.000.000 cells). As negative control, a fraction of each cell sample incubated under identical conditions with additional ALDH inhibitor diethylaminobenzaldehyde (DEAB). Flow cytometer Calibur (BD Biosciences, Heidelberg, Germany) was applied ALDH-positive population detection.

Genomic ALDH1-isoform knockout using CRISPR-Cas9

In C2C12 and RH30 genomic knockout of ALDH1A1 or ALDH1A3 isoforms was conducted following the protocol of Ran et al. 2013 and Wu et al. 2020. Oligonucleotides for consecutive single guide RNA

(sgRNA) generation were designed using the online-tool at Massachusetts Institute of Technology (https://zlab.bio/guide-design-resources). Plasmid pSpCas9(BB)-2A-GFP (PX458) with GFP-insert for fluorescence activated cell sorting (FACS) was a gift from Feng Zhang (Addgene plasmid # 48138). In order to achieve high purification, single cell colony cloning was performed on the next day. After two weeks ALDH1A1 knock out (1A1 ko) and ALDH1A3 knock out (1A3 ko), respectively, was confirmed in cells grown from single colonies.

Recombinant overexpression of ALDH1-isoforms

For recombinant ALDH1A1 or ALDH1A3 overexpression plasmids containing GFP-tagged open reading frame of ALDH1A1 and ALDH1A3, respectively, were purchased (OriGENE, Herford, Germany; MG208039, MG222097, respectively). Transfection was performed using 2 µg of DNA and Lipofectamine 3000 reagent (Thermo Fisher Scientific, Munich, Germany) following the manufacturer's recommendations. Empty GFP-vector transfection served as control. Transfected cells were selected by G418-treatment (Sigma, Munich, Germany), subsequently protein expression was confirmed by Western blotting using antibodies against ALDH1A1-/ALDH1A3 and GFP as indicated before.

Abbreviations

ALDH Aldehyde Dehydrogenase

Ctrl Control

Diff Differentiation

DSF Disulfiram

FACS Fluorescence Activated Cell Sorting

KO Knock-out

MyoG Myogenin

RA Retinoic Acid

SC Satellite Cell

1A1 Aldehyde Dehydrogenase 1A1

1A3 Aldehyde Dehydrogenase 1A3

Declarations

Ethics approval

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The data and material will not be shared, since all items are able to be purchased and reproduced.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Cell cultivation, experimental implementation, data collection and analysis were performed LR, SF, WW and JS. The first draft of the manuscript was written by LR. All authors commented on previous versions of the manuscript, contributed to the study conception and design, read and approved the final manuscript.

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Figures

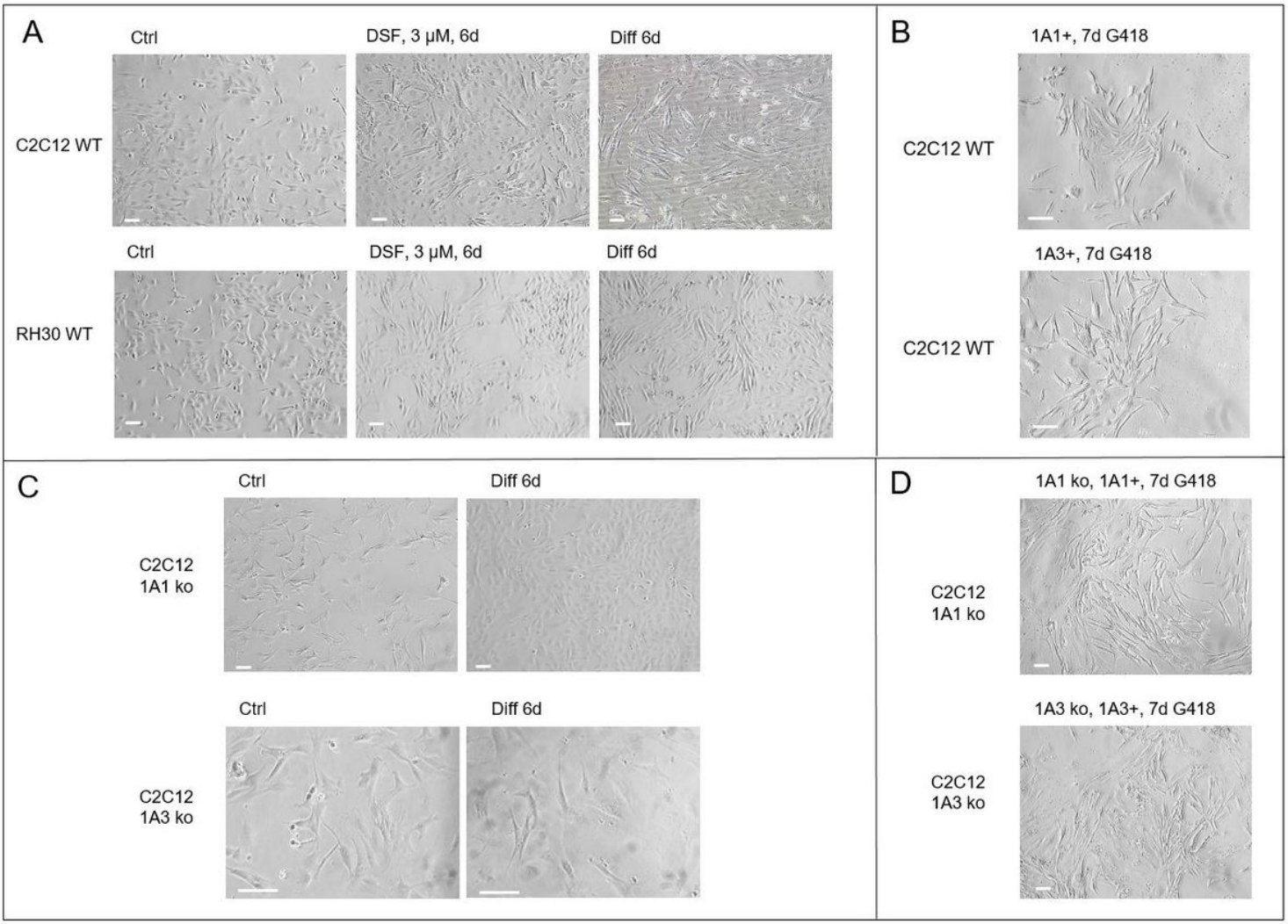

Figure 1

Morphological alterations of wildtype and ALDH1A1/ ALDH1A3 knock-out C2C12 and RH30 cells. (A) Wildtype cells in standard media with characteristic roundish shape (Ctrl), treatment of 3µM DSF for 6 days and serum-withdrawal for 6 days (Diff). Samples of DSF-treatment and Diff depict a typical elongated shape of myotubes. (B) C2C12 cells transfected with recombinant ALDH1A1 and ALDH1A3,

respectively, overexpression plasmid and consecutive G418-resistance selection, cultivated in standard media. Samples spontaneously formed elongated myotubes. (C) Recombinant knock-out of ALDH1A1 and ALDH1A3, respectively, in C2C12 cells. Standard media (Ctrl) and serum-withdrawal (Diff) cultivation. Diff samples show increased confluence and do not show myotube formation. (D) C2C12 ALDH1A1 and ALDH1A3, receptively, knock-out re-transfected with recombinant ALDH1A1 and ALDH1A3, respectively, plasmid and consecutive G418-selection for 7 days. Re-transfected cells depict spontaneous myotube formation.

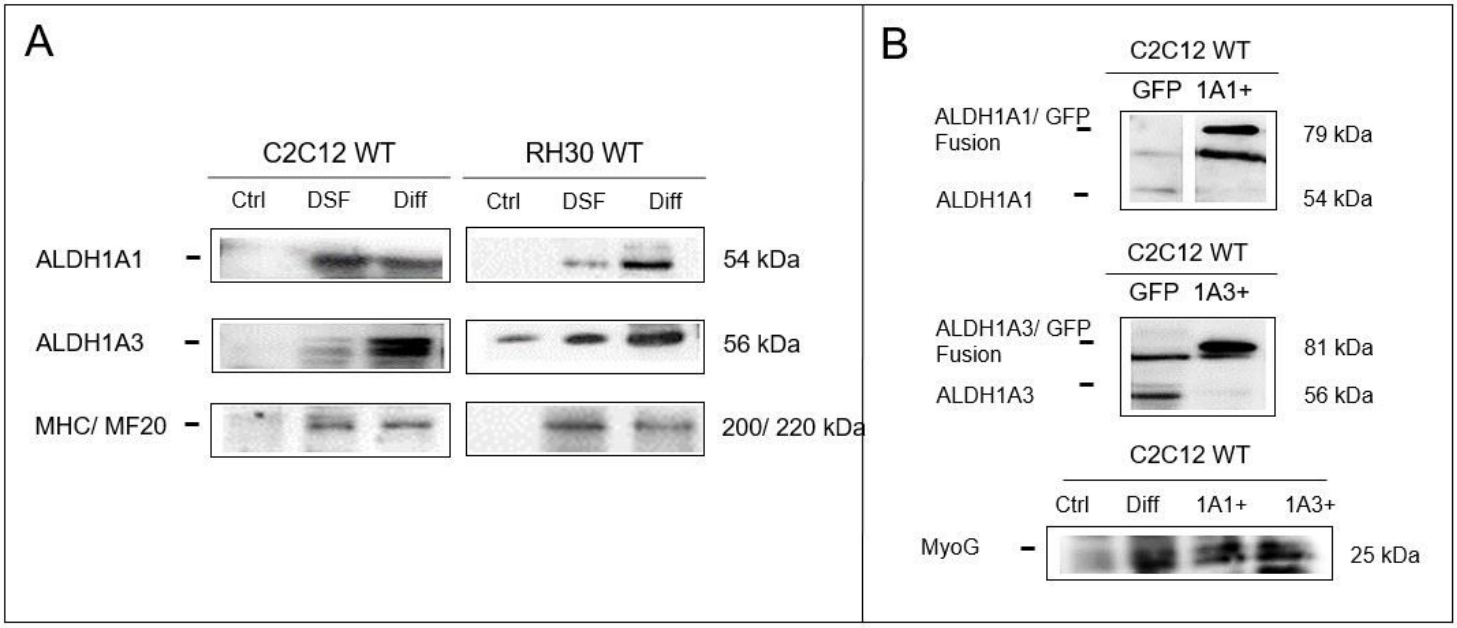

Figure 2

Cropped Western Blot of wildtype C2C12 and RH30 cells. (A) C2C12 and RH30 cells in standard media (Ctrl), DSF-treatment and serum-withdrawal (Diff). Upper Blots: 54 kDa ALDH1A1 protein in DSF and Diff of C2C12 and RH30. Middle Blots: 56 kDa ALDH1A3 protein in C2C12 DSF and Diff and increasing in RH30DSF and Diff. Lower Blots: 200 and 220, respectively, Myosin Heavy Chain (MHC) and MF20 protein as myogenic differentiation markers. Observed in DSF and Diff of C2C12 and RH30 (B) ALDH1A1-GFP/ ALDH1A3-GFP fusion vector and control-GFP transfection in C2C12 cells. Upper Blots: standard 54 kDa ALDH1A1 protein band size in control-GFP and ALDH1A1-GFP fusion product of 79 kDa (25 kDa GFP + 54 kDa ALDH1A1) in transfected C2C12. Middle Blot: 56 kDa of ALDH1A3 protein in control-GFP and ALDH1A3-GFP fusion product of 81 kDa (25 kDa GFP + 56 kDa ALDH1A3) in transfected C2C12. Lower Blot: 25 kDa of myogenic differentiation marker Myogenin (MyoG) observed in non-transfected Diff and C2C12 transfected with recombinant ALDH1A1 and ALDH1A3 vector plasmids.

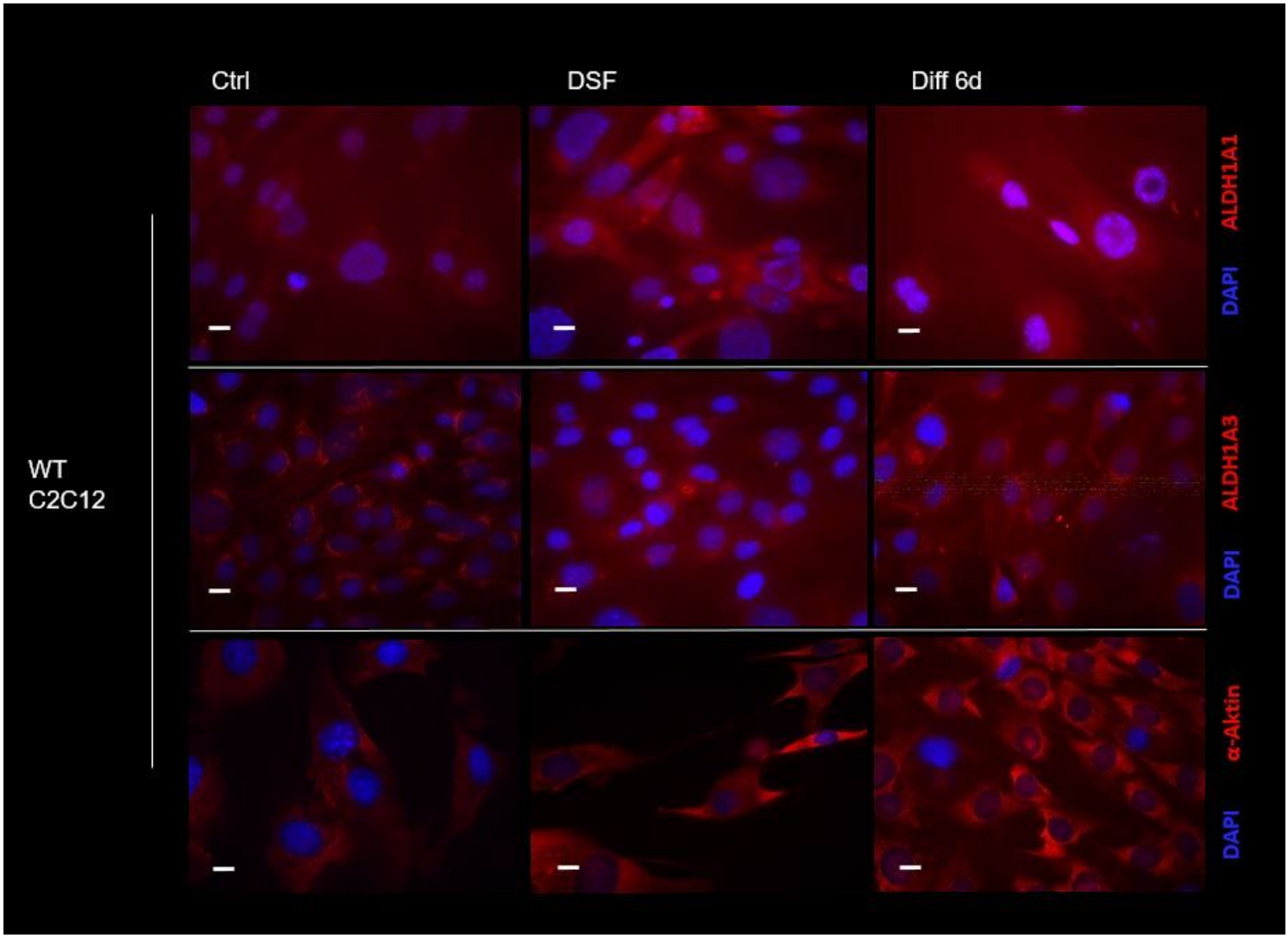

Figure 3

Immunofluorescent staining of wildtype C2C12 cells. Immunofluorescence of C2C12 cells in standard media (Ctrl), DSF-treatment and serum-withdrawal for 6 days (Diff) with additional nucleic DAPI staining. Upper lane: strong cytoplasmic ALDH1A1 immunoexpression in C2C12 DSF and Diff. Middle lane: cytoplasmic ALDH1A3 immunoexpression increased in C2C12 DSF and Diff. Lower lane: strong myogenic differentiation marker α-Aktin immunoexpression in C2C12 DSF and Diff myotubes.

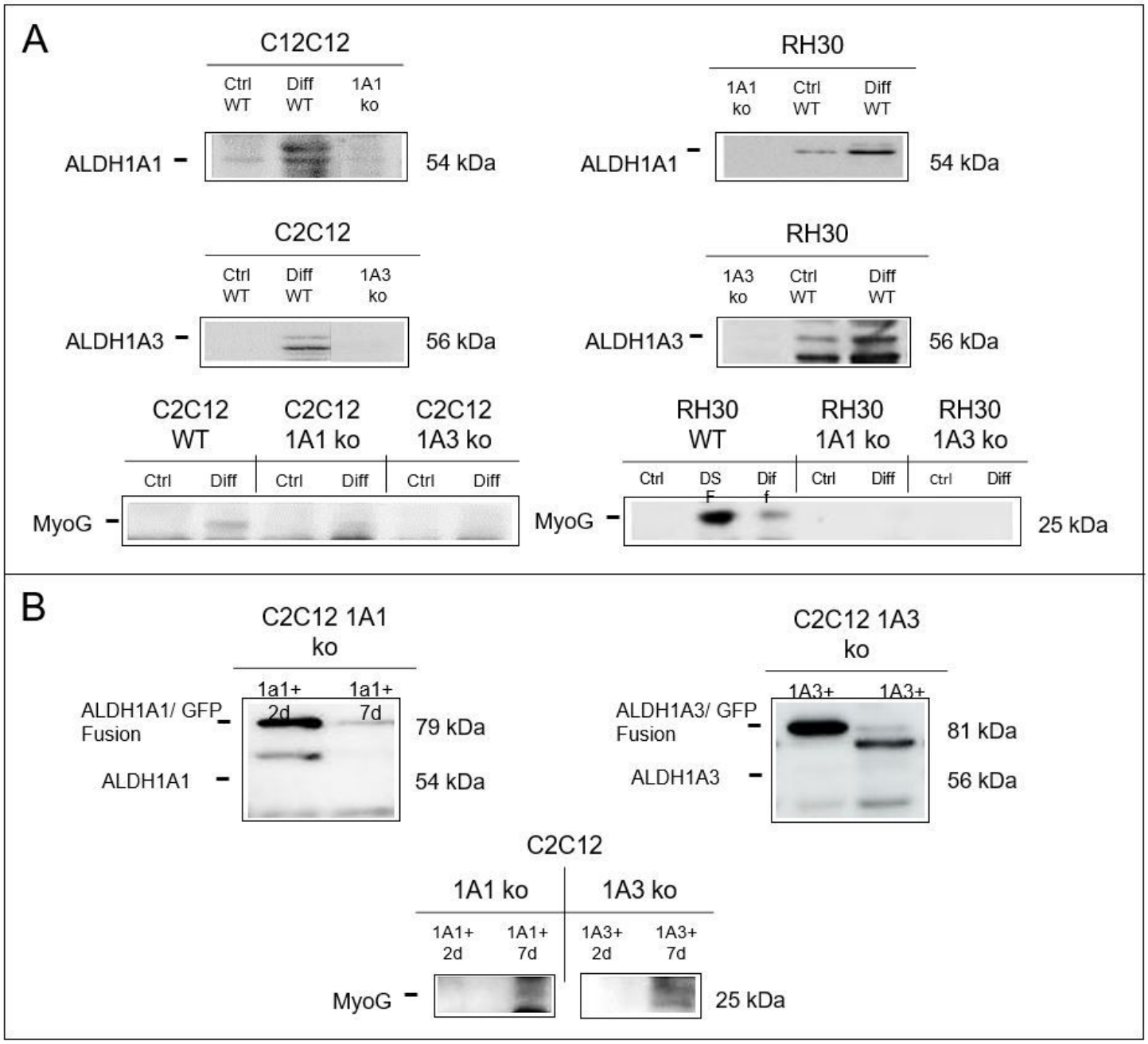

Figure 4

Cropped Western Blot of ALDH1A1/ ALDH1A3 knock-out C2C12 and RH30 cells. Western Blots of C2C12 and RH30 ALDH1A1 and ALDH1A3 knock-outs (1A1 ko, 1A3 ko, respectively) with additional non-depleted control samples (A) Upper Blots: C2C12 and RH30 controls of standard media and serum-withdrawal (Diff) and 1A1 ko cells in standard media. 54 kDa ALDH1A1 protein increased in Diff controls, strong knock-down in ALDH1A1 ko cells. Middle Blots: C2C12 and RH30 controls of standard media and serum-withdrawal (Diff) and 1A3 ko cells in standard media. 56 kDa ALDH1A3 protein upregulated in controls, knock-out in ALDH1A3 ko cells. Lower Blots: 25 kDa protein of myogenic differentiation marker Myogenin (MyoG). Observed in DSF and Diff of C2C12 and RH30 controls. No protein in C2C12 and RH30 1A1 ko and 1A3 ko cells, respectively, in standard media and serum-withdrawal. (B) Re-transfection of C2C12

1A1 ko and 1A3 ko cells with corresponding recombinant ALDH1A1-GFP/ ALDH1A3-GFP fusion vectors. Upper Blots: standard 54 kDa ALDH1A1 protein band size not illustrated. ALDH1A1-GFP fusion product of 79 kDa (25 kDa GFP + 54 kDa ALDH1A1) in re-transfected C2C12 1A1 ko cells. Stable re-expression depicted 7 days post transfection in former 1A1 ko cells. Middle Blots: 56 kDa of ALDH1A3 protein not observed. ALDH1A3-GFP fusion product of 81 kDa (25 kDa GFP + 56 kDa ALDH1A3) in re-transfected C2C12 1A3 ko cells. Stable re-expression demonstrated 7 days post transfection in former 1A3 ko cells. Lower Blots: 25 kDa of myogenic differentiation marker Myogenin (MyoG) depicted in re-transfected C2C12 1A1 and 1A3 ko cells 7 days after re-expression of recombinant ALDH1A1 and ALDH1A3.

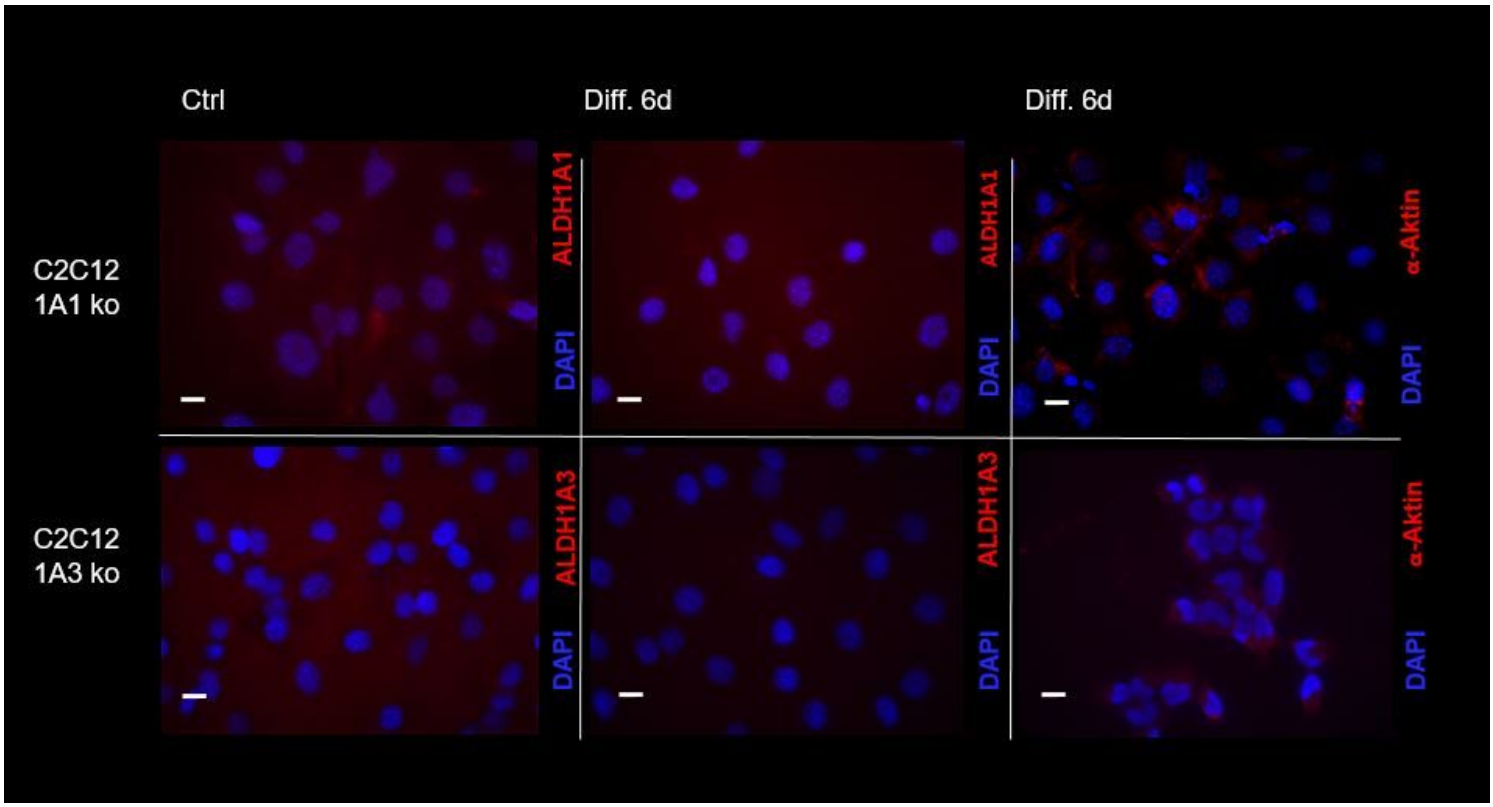

Figure 5

Immunofluorescent staining of ALDH1A1/ ALDH1A3 knock-out C2C12 cells. Immunofluorescence of C2C12 1A1 and 1A3 ko, respectively, cells in standard media (Ctrl) and serum-withdrawal for 6 days (Diff) with additional nucleic DAPI staining. Upper lane: no cytoplasmic ALDH1A1 immunoexpression in C2C12 1A1 ko cells. Last capture depicts depleted α-Aktin immunoexpression in C2C12 1A1 ko cells. Lower lane: no cytoplasmic ALDH1A3 immunoexpression in C2C12 1A3 ko cells. Last capture illustrates depleted α-Aktin immunoexpression in C2C12 1A3 ko cells.

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