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p38α Regulates Expression of DUX4 in a Model of Facioscapulohumeral Muscular Dystrophy[S]

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ABSTRACT

Facioscapulohumeral muscular dystrophy (FSHD) is caused by the loss of repression at the *D4Z4* locus leading to aberrant double homeobox 4 (DUX4) expression in skeletal muscle. Activation of this early embryonic transcription factor results in the expression of its target genes causing muscle fiber death. Although progress toward understanding the signals driving DUX4 expression has been made, the factors and pathways involved in the transcriptional activation of this gene remain largely unknown. Here, we describe the identification and characterization of p38α as a novel regulator of DUX4 expression in FSHD myotubes. By using multiple highly characterized, potent, and specific inhibitors of p38α/β, we show a robust reduction of DUX4 expression, activity, and cell death across patient-derived FSHD1 and FSHD2 lines. RNA-seq profiling reveals that a small number of genes are differentially expressed upon p38α/β inhibition, the vast majority of which are DUX4 target genes. Our results reveal a novel and apparently critical role for p38α in the aberrant activation of DUX4 in FSHD and support the potential of p38α/β inhibitors as effective therapeutics to treat FSHD at its root cause.

SIGNIFICANCE STATEMENT

Using patient-derived facioscapulohumeral muscular dystrophy (FSHD) myotubes, we characterize the pharmacological relationships between p38α/β inhibition, double homeobox 4 (DUX4) expression, its downstream transcriptional program, and muscle cell death. p38α/β inhibition results in potent and specific DUX4 downregulation across multiple genotypes without significant effects in the process of myogenesis in vitro. These findings highlight the potential of p38α/β inhibitors for the treatment of FSHD, a condition that today has no approved therapies.

Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is a rare and disabling condition with an estimated worldwide population prevalence of between 1 in 8000 and 20,000 (Deenen et al., 2014; Statland and Tawil, 2014). Most cases are familial and inherited in an autosomal dominant fashion, and about 30% of cases are known to be sporadic. FSHD is characterized by progressive skeletal muscle weakness affecting the face, shoulders, arms, and trunk, which is followed by weakness of the distal lower extremities and pelvic girdle (Tawil et al., 2015). There are currently no approved treatments for this condition.

FSHD is caused by aberrant expression of the double homeobox 4 (*DUX4*) gene, a homeobox transcription factor in the skeletal muscle of patients. This gene is located within the *D4Z4* macrosatellite repeats on chromosome *4q35*. DUX4 is not expressed in adult skeletal muscle when the number of repeat units (RUs) is >10, and the locus is properly silenced (Lemmers et al., 2010). In most patients with FSHD (FSHD1), the *D4Z4* array is contracted to 1–9 RUs in one allele. Loss of these repetitive elements leads to derepression of the *D4Z4* locus and ensuing aberrant DUX4 expression in skeletal muscle (de Greef et al., 2009; Wang et al., 2019). In FSHD2, patients manifest similar signs and symptoms as described above but genetically differ from FSHD1. These patients have longer *D4Z4* arrays but exhibit similar derepression of the locus caused by mutations in *SMCHD1*, an important factor in the proper deposition of DNA methylation across the genome (Jones et al., 2014, 2015; Calandra et al., 2016; Jansz et al., 2017; Dion et al., 2019). Similarly, modifiers of the disease,

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ABBREVIATIONS: abs(FC), absolute fold change; BET, bromodomain and extraterminal motif protein; DNMT3B, DNA methyltransferase 3B; DUX4, double homeobox 4; FDR, false discovery rate; FSHD, facioscapulohumeral muscular dystrophy; HMBS, hydroxymethylbilane synthase; HSP27, heat shock protein 27; MAPK, mitogen-activated protein kinase; MBD, methyl-CpG binding protein-like; MHC, myosin heavy chain; MSD, Mesoscale Diagnostics; MYOG, myogenin; POLR2A, RNA polymerase II subunit A; qPCR, quantitative polymerase chain reaction; RT, reverse transcription; RU, repeat unit; siRNA, small interfering RNA; SLC34A2, solute carrier 34A2; SMCHD1, structural maintenance of chromosomes flexible hinge domain containing 1; TRIM, tripartite motif–containing.

such as *DNMT3B*, are thought to also participate in the establishment of silencing of this region (van den Boogaard et al., 2016b).

DUX4 expression in skeletal muscle as a result of the *D4Z4* repeat contraction or *SMCHD1* mutations leads to activation of a downstream transcriptional program that causes FSHD (Yao et al., 2014; Jagannathan et al., 2016; Shadle et al., 2017). Major target genes of DUX4 are members of the DUX family itself and other homeobox transcription factors. Additional target genes include highly homologous gene families, including the preferentially expressed in melanoma family (PRAMEF), tripartite motif–containing (TRIM), and methyl-CpG–binding protein-like (*MBD3L*) (Geng et al., 2012; Tawil et al., 2014; Yao et al., 2014; Shadle et al., 2017). Expression of DUX4 and its downstream transcriptional program in skeletal muscle cells is toxic, leading to dysregulation of multiple pathways and resulting in impairment of contractile function and cell death (Bosnakovski et al., 2014; Tawil et al., 2014; Himeda et al., 2015; Homma et al., 2015; Rickard et al., 2015; Statland et al., 2015).

Several groups have made progress toward understanding the molecular mechanisms regulating DUX4 expression (van den Boogaard et al., 2016a,b; Campbell et al., 2017, 2018; Cruz et al., 2018; Oliva et al., 2019). However, factors that drive transcriptional activation of DUX4 in patients with FSHD are still largely unknown. By screening our annotated chemical probe library to identify disease-modifying small-molecule drug targets that reduce DUX4 expression in FSHD myotubes, we have identified multiple chemical scaffolds that inhibit p38α and β mitogen-activated protein kinase (MAPK). We found that inhibitors of p38α kinase or its genetic knockdown reduce DUX4 and its downstream gene expression program in FSHD myotubes, thereby impacting the core pathophysiology of FSHD.

Members of the p38 MAPK family composed of α, β, γ, and δ, isoforms are encoded on separate genes and play a critical role in mediating cellular responses to extracellular signals (Whitmarsh, 2010). In many inflammatory, cardiovascular, and chronic disease states, p38 MAPK stress-induced signals can trigger maladaptive responses that aggravate rather than alleviate the disease process (Krementsov et al., 2013; Martin et al., 2015). Similarly, in skeletal muscle, a variety of extracellular signals, including exercise, insulin exposure, myoblast differentiation, and reactive oxygen species as well as apoptosis, have all been shown to induce the p38 kinase pathways (Zarubin and Han, 2005; Keren et al., 2006). Downstream substrates of p38 MAPK include other kinases, downstream effectors like heat shock protein 27 (HSP27), and modulation of transcription factor activity culminating in gene expression changes (Kyriakis and Avruch, 2001; Cuenda and Rousseau, 2007).

p38α is the most abundantly expressed isoform in skeletal muscle, and it plays an important role controlling the activity of transcription factors that drive myogenesis (Simone et al., 2004; Knight et al., 2012; Segalés et al., 2016b). p38α abrogation in mouse myoblasts inhibits fusion and myotube formation in vitro (Zetser et al., 1999; Perdiguero et al., 2007). However, conditional ablation of p38α in the adult mouse skeletal muscle tissue appears to be well-tolerated and alleviates phenotypes observed in models of other muscular dystrophies (Wissing et al., 2014).

Here, we show that selective p38α/β inhibitors potently decrease the expression of DUX4, its downstream gene program, and cell death in FSHD myotubes across a variety of FSHD1 and FSHD2 genotypes. Using RNA-seq and high-content image analysis, we also demonstrated that myogenesis is not affected at concentrations that result in downregulation of DUX4.

Materials and Methods

Cell Lines and Cell Culture. Immortalized myoblasts from FSHD (AB1080FSHD26 C6) and healthy individuals (AB1167C20FL) were generated and obtained from the Institut Myologie, France. In short, primary myoblast cultures were obtained from patient samples and immortalized by overexpression of telomerase reverse transcriptase (TERT) and cyclin-dependent kinase 4 (CDK4) (Krom et al., 2012). Primary myoblasts were isolated from FSHD muscle biopsies and were obtained from University of Rochester.

Immortalized myoblasts were expanded on gelatin-coated dishes (ES-006-B; EMD Millipore) using skeletal muscle cell growth media (C-23060; Promocell) supplemented with 15% FBS (16000044; ThermoFisher). Primary myoblasts were also expanded on gelatin-coated plates but using media containing Ham's F-10 Nutrient Mix (11550043; ThermoFisher), 20% FBS, and 0.5% chicken embryo extract (100-163P; Gemini Bio-product). For differentiation, immortalized or primary myoblasts were grown to confluency in matrigel-coated plates (356234; Corning), and growth media was exchanged for differentiation media (Nb4-500; Brainbits) after a PBS wash. DMSO (vehicle) or compounds (previously dissolved in DMSO at 10-mM stock concentrations) were added at the desired concentration at the time differentiation media was exchanged and maintained in the plates until harvesting or analysis.

Small-Molecule Compounds and Antisense Oligonucleotides. SB239063, Pamapimod, LY2228820, and Losmapimod were purchased from Selleck Chem (S7741, S8125, S1494, and S7215). Ten-millimolar stock solutions in DMSO were maintained at room temperature away from light. DUX4 antisense oligonucleotides (gapmer) were purchased from Qiagen and were designed to target exon 3 of DUX4. The lyophilized oligos were resuspended in PBS at 25-mM final concentration and kept frozen at −20°C until used. This antisense oligonucleotide was added to cells in growth media 2 days before differentiation and maintained during the differentiation process until harvesting.

Detection of DUX4 and Target Gene Expression by Reverse-Transcription Quantitative Polymerase Chain Reaction. RNA from myotubes was isolated from C6 FSHD cells differentiated in six-well plates using 400 μl of trireagent and transfer to Qiagen qiashredder column (cat. 79656). An equal amount of 100% ethanol was added to flow through and transferred to a Direct-zol microcolumn (cat. 2061; Zymo research), and the manufacturer's protocol, including on-column DNA digestion, was followed. RNA (1 μg) was converted to cDNA using Superscript IV priming with oligodeoxythymine (cat. 18091050; Thermofisher). Preamplification of DUX4 and housekeeping gene *HMBS* was performed using preamp master mix (cat. 4384267; Thermofisher) as well as 0.2× diluted taqman assays (IDT DUX4 custom; Forward: 5′-GCCGGCCCAGGT ACCA-3′, Reverse: 5′-CAGCGAGCTCCCTTGCA-3′, and Probe: 5′-/56-FAM/CAGTGCGCA/ZEN/CCCCG/3IABkFQ/-3′; and *HMBS* HS00609297m1-VIC). After 10 cycles of preamplification, reactions were diluted 5-fold in nuclease-free water, and quantitative polymerase chain reaction (qPCR) was performed using Taqman Multiplex Master Mix (cat. 4461882; Thermofisher).

To measure DUX4 target gene expression in a 96-well plate format, cells were lysed into 25 μl Realtime Ready lysis buffer (07248431001; Roche) containing 1% RNAse inhibitor (03335399001; Roche) and 1% DNase I (AM2222; ThermoFisher) for 10 minutes while being shaken on a vibration platform shaker (1000; Titramax) at 1200 rpm. After homogenization, lysates were frozen at −80°C for at least 30 minutes and thawed on ice. Lysates were diluted to 100 μl using

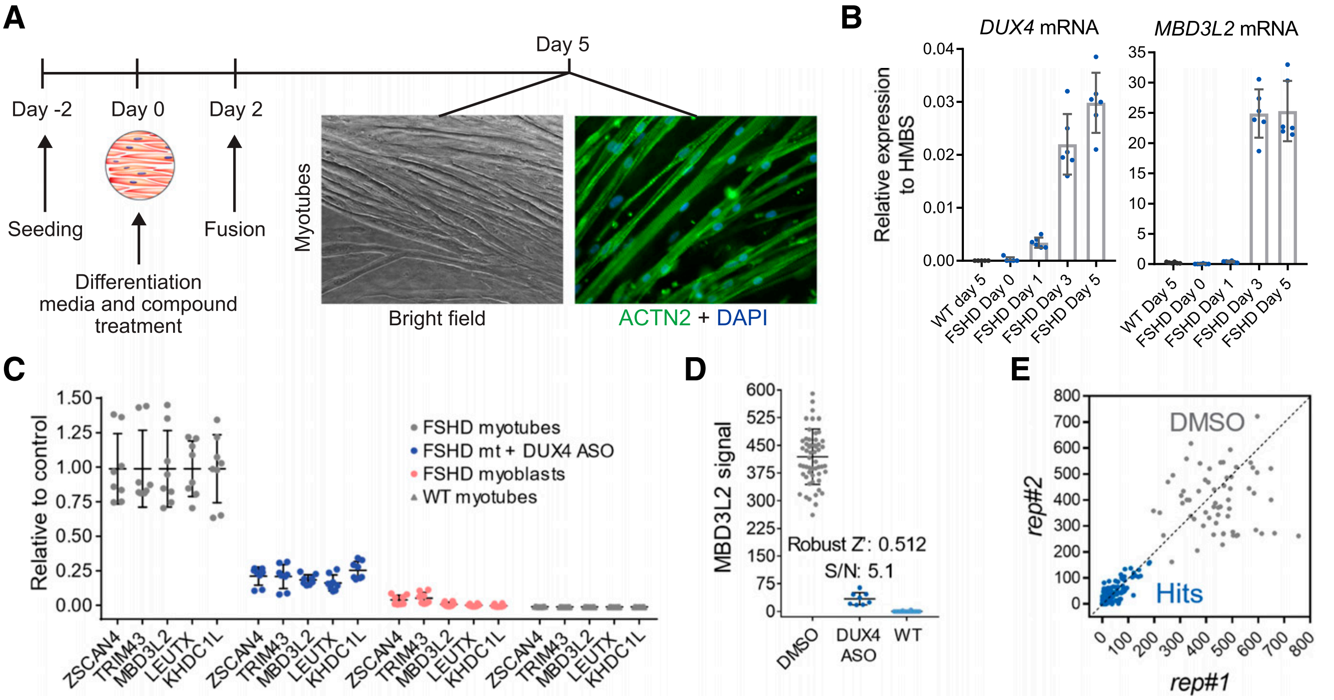

Fig. 1. Description of an assay for the identification of inhibitors of DUX4 expression. (A) Schematic describing the cellular assay used to identify small molecules that result in the inhibition of DUX4 expression and activity. In short, immortalized FSHD myoblasts (C6, 6.5 D4Z4 RUs) were seeded in 96-well plates 2 days before differentiation was induced. After myoblasts reached confluence, media was replaced, and compounds for treatment were added. At day 2, fusion was observed, and at day 5, differentiated myotubes were harvested for gene expression analysis or fixed for immunostaining. Representative image of the α-actinin staining in differentiated myotubes. (B) DUX4 expression is rapidly induced after differentiation of immortalized FSHD myotubes in vitro. To measure DUX4 transcript, C6 FSHD myotubes were grown in 12-well plates similarly to (A), and cells were harvested on day 5 for RNA extraction. RT-qPCR was used to determine expression of *DUX4* mRNA and its downstream gene *MBD3L2* (normalized using *HMBS* as housekeeping). These transcripts were not detected in wild-type immortalized myotubes derived from healthy volunteers. (C) Canonical DUX4 target genes are specifically detected in FSHD myotubes and are downregulated when *DUX4* is knocked down using a specific antisense oligonucleotide. RT-qPCR analysis was used to detect expression in immortalized myoblasts/myotubes. Antisense oligonucleotide knockdown in FSHD myotubes (mt) was carried out during the 5 days of differentiation. Bars indicate mean ± S.D. (D) A 96-well plate cell-based assay was optimized to screen for inhibitors of DUX4 expression. An assay measuring *MBD3L2* by RT-qPCR was selected because of robust separation and specificity reporting DUX4 activity. *MBD3L2* signal was normalized using *POLR2A* as a housekeeping gene. Bars indicate mean ± S.D. (E) Hits identified in small-molecule screen potently reduced the activity of DUX4. *x*- and *y*-axis show the normalized *MBD3L2* signal obtained from the two replicate wells analyzed. ASO, antisense oligonucleotide; KHDC1L, K homology domain containing 1 like; LEUTX, leucine twenty homeobox; WT, wild type; ZSCAN4, zinc finger and SCAN domain containing 4; S/N, signal to noise ratio; rep, replicate.

RNase-free water. One microliter of this reaction was used for reverse transcription and preamplification of cDNA in a 5-μl one-step reaction using the reverse-transcription (RT) enzyme from Taqman RNA-to-Ct (4392938; ThermoFisher) and the Taqman Preamp Master Mix (4391128; ThermoFisher) according to manufacturer's specifications. This preamplification reaction was diluted 1:4 using nuclease-free water; 1 μl of this reaction was used as input for a 5-μl qPCR reaction using the Taqman Multiplex Master Mix (4484262; ThermoFisher). Amplification was detected in a Quantstudio 7 Flex instrument from ThermoFisher. The following Taqman probes were purchased from ThermoFisher: *MBD3L2* Taqman Assay [Hs00544743_m1, FAM-MGB; ThermoFisher, (Bosnakovski et al., 2019)], zinc finger and SCAN domain containing 4 (*ZSCAN4*) Taqman Assay (Hs00537549_m1, FAM-MGB; ThermoFisher), leucine twenty homeobox (*LEUTX*) Taqman Assay (Hs01028718_m1, FAM-MGB; Thermo Fisher), *TRIM43* Taqman Assay (Hs00299174_m1, FAM-MGB; ThermoFisher), K homology domain containing 1 like (*KHDC1L*) Taqman Assay (Hs01024323_g1, FAM-MGB; ThermoFisher), and RNA Polymerase II Subunit A (*POLR2A)* Taqman Assay (Hs00172187_m1, VIC-MGB; ThermoFisher).

Detection of HSP27 by Electrochemiluminescence. Total and phosphorylated HSP27 was measured using a commercial MesoScale Discovery assay, Phospho (Ser82)/Total HSP27 Whole Cell Lysate Kit (K15144D; MesoScale Discovery). Myotubes were grown in 96-well plates using conditions described above and were lysed using 25 μl of 1× Mesoscale Diagnostics (MSD) lysis buffer with protease and phosphatase inhibitors. The lysates were incubated at room temperature for 10 minutes with shaking at 1200 rpm using Titramax 1000. Lysates were stored at −80°C until all timepoints were collected. Lysates were then thawed on ice, and 2 μl were used to perform a BCA protein assay (23225; ThermoFisher). Ten microliters of lysate were diluted 1:1 in 1× MSD lysis buffer and added to the 96-well Mesoscale assay plate. Manufacturer instructions were followed, and data were obtained using a MesoScale Discovery SECTOR S 600 instrument.

Myotube Nuclei Isolation and Detection of DUX4 by Electrochemiluminescence. DUX4 was measured using a novel MesoScale Discovery assay developed at Fulcrum Therapeutics. Anti-DUX4 monoclonal capture antibody (clone P2B1) was coated overnight at 5 μg/ml in 0.1 M sodium bicarbonate (pH = 8.4) onto a Mesoscale 384-well plate (L21XA). The plate was blocked with 5% bovine serum albumin/PBS for at least 2 hours. Human FSHD myotubes grown in 100-mm plates in the conditions described above were harvested 4 days postdifferentiation using TrypLE express solution (12605-010; Gibco) and neutralized with growth media, and the myotubes were pelleted by centrifugation. Myotubes were resuspended in ice-cold nuclei extraction buffer (320 mM sucrose, 5 mM $MgCl_2$, 10 mM HEPES, 1% Triton X-100 at pH = 7.4). Nuclei were pelleted by centrifugation at 2000*g* for 4 minutes at 4°C. Nuclei were resuspended in ice-cold wash buffer (320 mM sucrose, 5 mM $MgCl_2$, 10 mM HEPES at pH = 7.4) and pelleted by centrifugation at 2000*g* for 4 minutes at 4°C. Nuclei were suspended in 150 μl of RIPA buffer at 4°C (+150 mM

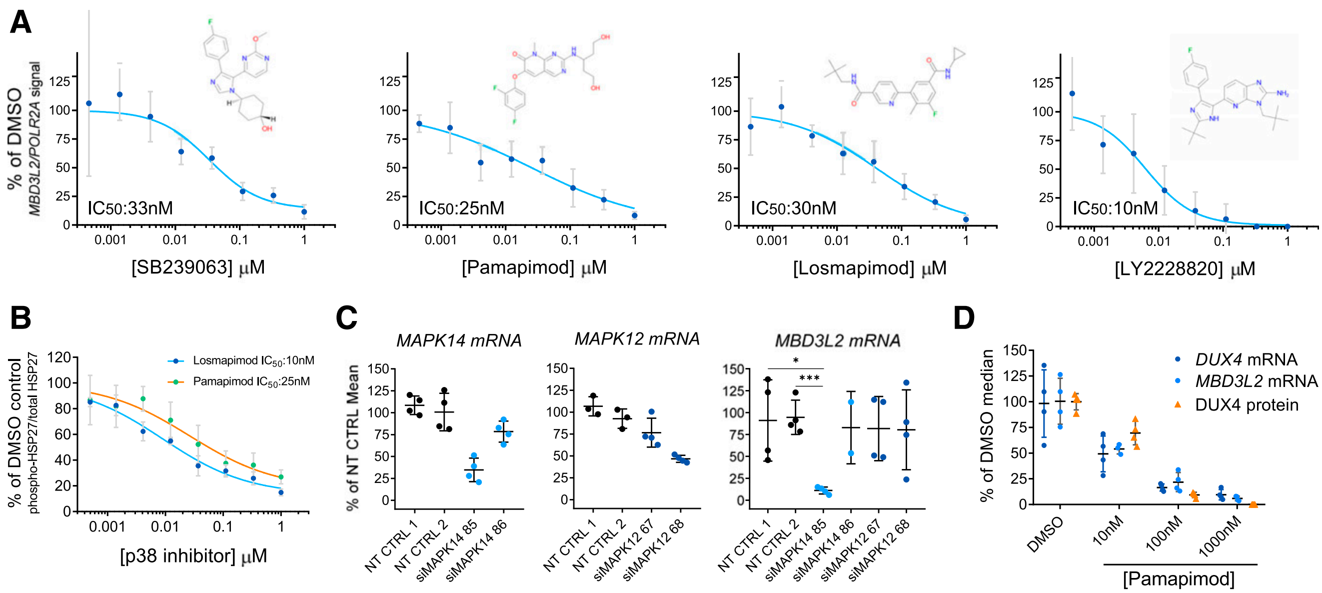

Fig. 2. Small-molecule inhibitors of p38 α-reduced expression of DUX4 in FSHD myotubes. (A) Diverse inhibitors of p38α/β reduce the expression of *MBD3L2* in differentiating FSHD myotubes. Concentration-dependent responses were observed with all tested inhibitors. Four replicates per concentration were tested to measure reduction of *MBD3L2* in immortalized C6 FSHD myotubes, and bars indicate mean ± S.D. (B) p38α/β pathway inhibition in C6 FSHD myotubes. The ratio between phosphorylated HSP27 to total HSP27 was measured by an immunoassay (MSD) after 12 hours of treatment of C6 FSHD myotubes with the indicated inhibitors. IC_{50} observed for phosphorylated HSP27 (p-HSP27) were comparable to those obtained for reduction of *MBD3L2* expression. Bars indicate mean ± S.D. for four replicate wells. (C) Knockdown of p38α (*MAPK14*) results in reduction of *MBD3L2* expression. Immortalized C6 myoblasts were electroporated with siRNAs specific for *MAPK14* (p38α) and *MAPK12* (p38γ) plated and differentiated for 3 days. Expression of the indicated transcripts was measured using RT-qPCR and normalized against *POLR2A*. Reduction of *MBD3L2* expression was observed when >50% knockdown of *MAPK14* was achieved. Bars indicate mean ± S.D. (D) p38α/β inhibition results in the reduction of DUX4 expression. After inhibition, correlated reduction of *DUX4* mRNA, protein and downstream gene *MBD3L2* was observed. To measure DUX4 protein, a novel immunoassay was developed using previously described antibodies (see *Materials and Methods* and Supplemental Fig. 4). Bars indicate mean ± S.D., *t* test *P* value * <0.01, *** 0.0002. CTRL, control; si, small interfering; NT, non-targeting.

NaCl). Extracts were diluted 1:1 with assay buffer, and 10 μl per well was added to 384-well precoated/blocked MSD plate and incubated for 2 hours. Anti–DUX4-sulfo conjugate (clone E5-5) was added to each well and incubated for 2 hours. Plates were washed, and 40 μl per well of 1× Read T buffer was added. Data were obtained using a MesoScale Discovery SECTOR S 600 instrument.

Quantitative Immunofluorescent Detection of Myosin Heavy Chain, Solute Carrier 34A2, and Cleaved Caspase-3. Myotubes were grown and treated as described above. At day 5 after differentiation was induced, cells were fixed using 4% paraformaldehyde in PBS during 10 minutes at room temperature. Fixative was washed, and cells were permeabilized using 0.5% Triton X-100 during 10 minutes at room temperature. After washing, fixing, and permeabilizing, the cells were blocked using 5% donkey serum in PBS/0.05% Tween 20 during 1 hour at room temperature. Primary antibodies against myosin heavy chain (MHC) (MF20, MAB4470; R&D systems), solute carrier 34A2 (SLC34A2) (66445; Cell signaling), and active Caspase-3 (9661; Cell signaling) were diluted 1:500 in PBS containing 0.1% Triton X-100 and 5% donkey serum and incubated with cells for 1 hour at room temperature. After four washes, secondary antibodies were added (A32723 and R37117; ThermoFisher) in a 1:2000 dilution and incubated during 1 hour at room temperature. During the last 5 minutes of incubation, a 1:2000 dilution of DAPI (4′,6-diamidino-2-phenylindole) was added before proceeding with final washes and imaging. Images were collected using the CellInsight CX7 (ThermoFisher). Images were quantified using HCS Studio Software. Differentiation was quantified by counting the percentage of nuclei in cells expressing MHC from the total of the well. SLC34A2 and active Caspase-3 signal were quantified by colocalization of cytoplasmic cleaved Caspase-3 within MHC-expressing cells.

Knockdown of *MAPK12* and *MAPK14* in FSHD Myotubes. Exponentially dividing immortalized C6 FSHD myoblasts were harvested and counted. Fifty thousand myoblasts were electroporated using a 10-μl tip in a neon electroporation system (ThermoFisher). Conditions used were determined to preserve viability and achieved maximal electroporation (pulse V = 1100 V, pulse width = 40 and pulse # = 1). After electroporation, cells were plated in growth media, and media was changed for differentiation 24 hours after. Three days after differentiation, cells were harvested and analyzed for knockdown and effects in *MBD3L2* using the RT-qPCR assay described before. siRNAs used were obtained from ThermoFisher (4390843, 4390846, s3585, s3586, s12467, s12468).

Gene Expression Analysis by RNA-seq. RNA from myotubes grown in six-well plates in conditions described above was isolated using the RNeasy Micro Kit from Qiagen (74004). Quality of RNA was assessed by using a Bioanalyzer 2100, and samples were submitted for library preparation and deep sequencing to the molecular biology core facility at the Dana Farber Cancer Institute. After sequencing, raw reads of fastq files from all samples were mapped to hg38 genome assemblies using ArrayStudio aligner. Raw read count and FPKM (fragments per kilobase of exon model per million reads mapped) were calculated for all the genes, and DESeq2 was applied to calculate differentially expressed genes using general linear model. Statistical cutoff of absolute fold change [abs(FC) > 4, FDR < 0.001] were applied to identify differentially expressed protein coding genes. The data in this publication is accessible through GEO Series accession number GSE153301 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153301).

Results

Identification of Inhibitors of DUX4 Expression. To model FSHD in vitro, we differentiated patient-derived FSHD1 immortalized myoblasts into skeletal muscle myotubes. We allowed myoblasts to reach >70% confluency and added differentiation medium lacking growth factors (Fig. 1A) (Brewer et al., 2008; Krom et al., 2012; Thorley et al., 2016).

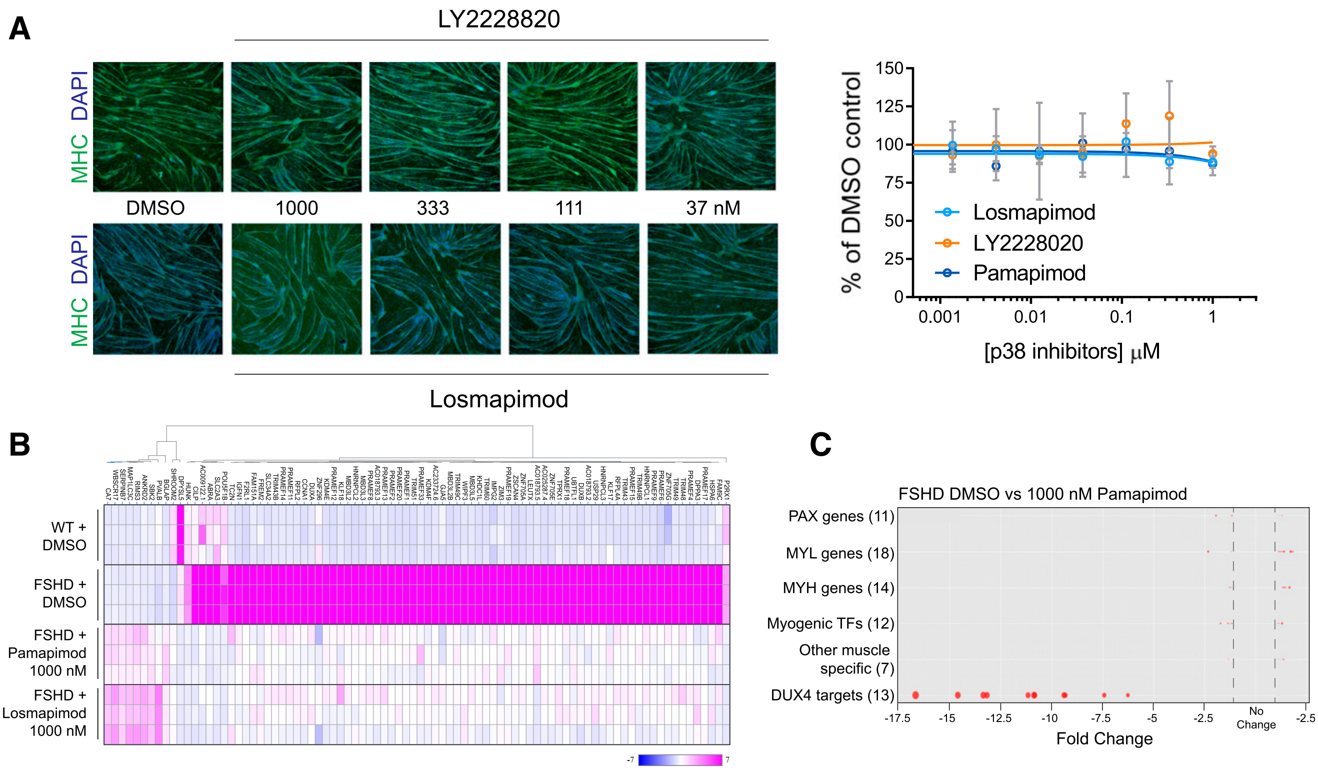

Fig. 3. Inhibition of the p38α/β pathway results in normalized gene expression in FSHD myotubes without affecting the differentiation process in vitro. (A) Quantification of myotube differentiation after p38α/β inhibition. Two inhibitors were used to demonstrate the effects of p38α/β inhibition in a high-content imaging assay to quantify the number of nuclei that properly underwent differentiation by activation of expression of myofiber specific proteins (i.e., MHC). No changes were observed in the morphology of C6 myotubes treated for 5 days. Bars indicate mean ± S.D. (B) Heat map representing fold change of expression levels of differentially expressed genes after p38α/β inhibition in FSHD myotubes for 5 days. Eighty-six genes showed significant changes in expression after treatment with two different inhibitors [abs(FC) > 4; FDR < 0.001]. Each condition was tested in triplicate represented as rows in the heatmap. (C) DUX4 target genes are specifically downregulated by p38 inhibition. *x*-Axis indicates the fold changes observed in members of the gene families indicated. Diameter of dots represent *P* value. DAPI, 4′,6-diamidino-2-phenylindole; PAX, paired box; WT, wild type; MYL, Myosin Light Chain; MYH, Myosin Heavy Chain; TF, transcription factor.

After 1 day of differentiation, we detected DUX4 expression by RT-qPCR, and its expression increased throughout the course of myogenic fusion and formation of postmitotic, multinucleated FSHD myotubes (Fig. 1B). Because of the stochastic and low expression levels of DUX4 in FSHD cells, we measured DUX4-regulated genes as an amplified readout of the expression and activity of DUX4. These include *ZSCAN4* (zinc finger and SCAN domain containing 4), *MBD3L2*, *TRIM43*, *LEUTX* (leucine twenty homeobox), and *KHDC1L* (K homology domain containing 1 like), which are among the most commonly described DUX4 targets (Geng et al., 2012; Tasca et al., 2012; Yao et al., 2014; Jagannathan et al., 2016; Chen et al., 2016b; Whiddon et al., 2017; Wang et al., 2019). These genes were downregulated after *DUX4* antisense oligonucleotide treatment of FSHD myotubes and were nearly undetectable or completely absent in FSHD myoblasts or wild-type myotubes (Fig. 1C). We concluded that the assays used to detect these transcripts were specific because their expression was solely dependent on DUX4 expression in differentiating myotubes. Although a number of DUX4-dependent transcripts have been previously described, we selected an assay to specifically detect *MBD3L2* for high-throughput screening because it displayed the best signal window of differential expression in our in vitro system comparing FSHD to healthy wild-type myotubes (Fig. 1D). With this assay, we identified several small molecules that reduced *MBD3L2* expression after 5 days of differentiation and treatment and showed good reproducibility across replicates (Fig. 1E). Validating our results, we found several molecules identified previously to reduce DUX4 expression, including BET inhibitors and β-adrenergic agonists exemplified in Supplemental Fig. 1 (Campbell et al., 2017; Cruz et al., 2018). However, when treating differentiating FSHD myotubes in our assay, we observed a reduction in fusion as indicated by visual inspection and by the reduction of *MYOG* expression with BET inhibitors. Importantly, we identified multiple scaffolds that inhibit p38α and β and strongly inhibit the expression of *MBD3L2* without affecting differentiation.

p38α Signaling Participates in the Activation of DUX4 Expression in FSHD Myotubes. Potent and selective inhibitors of p38α/β have been previously explored in multiple clinical studies for indications associated with the role of p38α in the regulation of the expression of inflammatory cytokines and cancer (Coulthard et al., 2009). We tested several p38α/β inhibitors of different chemical scaffolds in our assays, which showed significant inhibition of *MBD3L2* expression (Fig. 2A). Importantly, IC_{50} obtained for *MBD3L2* reduction were comparable to reported values by other groups in unrelated cell-based assays that measured p38α/β inhibition, suggesting the specificity for the assigned target (Underwood et al., 2000; Campbell et al., 2014; Fehr et al., 2015). p38α and β kinases phosphorylate a myriad of substrates, including downstream kinases like MAPK–activated protein kinase 2 (MAPKAPK2 or MK2), which phosphorylates effector molecules, such as HSP27 as well as a variety of transcription

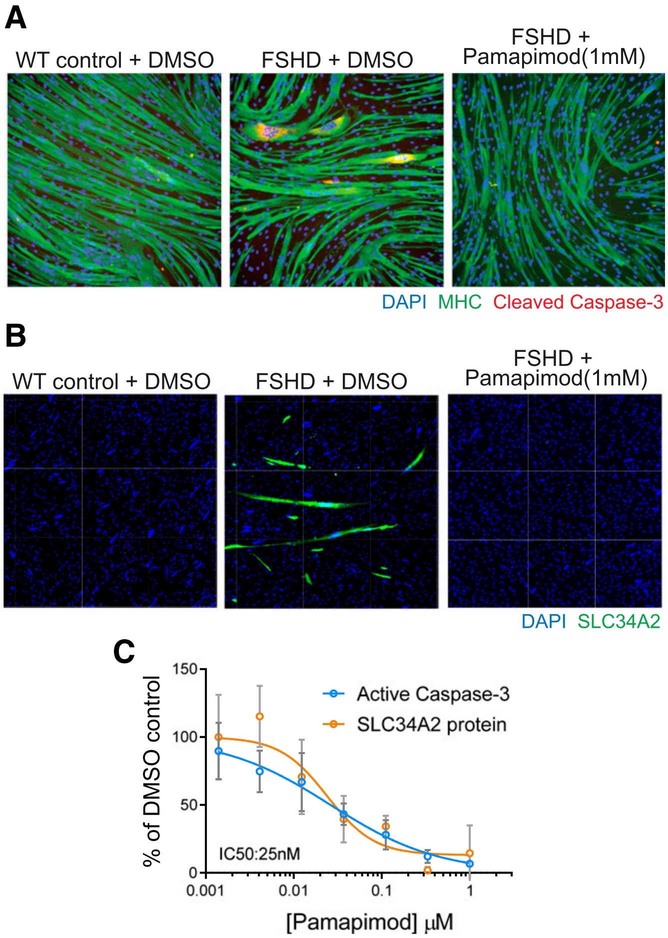

Fig. 4. Inhibition of the p38α/β pathway reduced the activation of programmed cell death in differentiating FSHD myotubes. (A) A high-content imaging assay was developed to measure cleaved caspase-3 in differentiating myotubes. C6 FSHD myotubes were differentiated and treated for 5 days as indicated above and stained to measure MHC, cleaved caspase-3, and nuclei. Representative images show that cleaved caspase-3 was only detected in FSHD myotubes and not in wild-type controls or after inhibition of the p38 pathway. Six replicates were imaged, and cleaved caspase-3 signal under MHC staining was quantified. (B) Stochastic expression of DUX4 target gene *SLC34A2* in C6 FSHD myotubes. Expression of SLC34A2 was measured by immunostaining in similar conditions as image above. No expression was detected in wild-type control or p38 inhibitor–treated myotubes. Signal of SLC34A2 under MHC staining was quantified in two replicates. (C) Concentration-dependent inhibition of the expression of DUX4 target genes is highly correlated to the inhibition of programmed cell death in C6 myotubes. Bars indicate mean ± S.D. DAPI, 4′,6-diamidino-2-phenylindole; WT, wild type.

factors, including myogenic transcription factors like myocyte enhancer factor 2C (Zetser et al., 1999; Simone et al., 2004; Knight et al., 2012; (Segalés et al., 2016)). To determine p38α/β-signaling activity in differentiating myoblasts, we measured the levels of phosphorylation of HSP27. As reported previously, we observed increased p38 signaling rapidly upon addition of differentiation media (Supplemental Fig. 2) (Perdiguero et al., 2007). We observed that p38α/β inhibitors reduced phosphorylated HSP27 levels with similar IC_{50} values to that of *MBD3L2* (Fig. 2B). To further validate our findings, we electroporated FSHD myoblasts with siRNAs against p38α and γ, the most abundant p38 MAPKs in skeletal muscle. After 3 days of differentiation, transient knockdown of p38α showed robust inhibition of expression of *MBD3L2* in FSHD myotubes (Fig. 2C), and no significant effects in fusion were observed (Supplemental Fig. 3). We observed that close to 50% reduction of *MAPK14* (p38α) mRNA was sufficient to inhibit *MBD3L2* expression without impacting myogenesis, and this level of reduction may account for the differences on myogenesis observed between this study and those previously reported using p38 mouse knockout myoblasts (Perdiguero et al., 2007).

Our results suggest the p38α pathway is an activator of DUX4 expression in FSHD muscle cells undergoing differentiation. To further understand the reduction in DUX4, we measured the expression of DUX4 transcript and protein upon inhibition of p38α and β. To measure protein, we developed a highly sensitive assay based on the electrochemiluminescent detection of DUX4 on the MSD platform using two previously generated antibodies (Supplemental Fig. 4). We observed that p38α/β inhibition resulted in a highly correlated reduction of DUX4 transcript and protein (Fig. 2D). We concluded this led to the reduction in the expression of DUX4 target gene, *MBD3L2*.

p38α and β Inhibition Normalizes Gene Expression of FSHD Myotubes without Impacting the Myogenic Differentiation Program. We further examined the effect of p38α and β selective inhibition on myotube formation because this pathway has been linked to muscle cell differentiation (Simone et al., 2004; Perdiguero et al., 2007; Wissing et al., 2014; Segalés et al., 2016a,b). We developed a quantitative assay to measure cell fusion and myotube formation to assess skeletal muscle differentiation in vitro. In this assay, we stained immortalized FSHD myotubes cells using antibodies against MHCs and quantified the number of nuclei detected inside MHC-stained region. This provided a way to quantitate the number of cells that successfully underwent the process of in vitro myogenesis. p38α/β inhibition by LY2228820 and GW856553X (losmapimod) did not impact differentiation of myoblasts into skeletal muscle myotubes. Treated cells fused properly at all tested drug concentrations to levels comparable to the DMSO control (Fig. 3A).

We also further assessed gene expression changes in FSHD myotubes upon p38α/β inhibition. We performed RNA-seq analysis of FSHD and WT myotubes after 4 days of treatment with vehicle or p38α/β inhibitors. Inhibition of the p38-signaling pathway during differentiation did not induce significant transcriptome changes and resulted in fewer than 100 differentially expressed genes [abs(FC) $>$ 4; FDR $<$ 0.001]. About 90% of these differentially expressed genes were known DUX4-regulated transcripts and were all downregulated after p38α and β inhibition (Fig. 3B). This set of DUX4-regulated genes overlapped significantly with genes upregulated in muscle biopsies in patients with FSHD (Wang et al., 2019). Moreover, key driver genes of myogenic programs, such as *MYOG*, myocyte enhancer factor, and paired box genes, and markers of differentiation, such as myosin subunits and sarcomere proteins, were not affected by p38 inhibition (Fig. 3C).

Inhibition of DUX4 Expression Results in the Reduction of Cell Death in FSHD Myotubes. DUX4 activation and downstream DUX4-regulated target gene expression in muscle cells is toxic, leading to oxidative stress, changes in sarcomere organization, and apoptosis, culminating in reduced contractility and muscle tissue replacement by fat (Block et al., 2013; Bosnakovski et al., 2014; Tawil et al., 2014; Homma et al., 2015; Rickard et al., 2015; Choi et al., 2016). In particular,

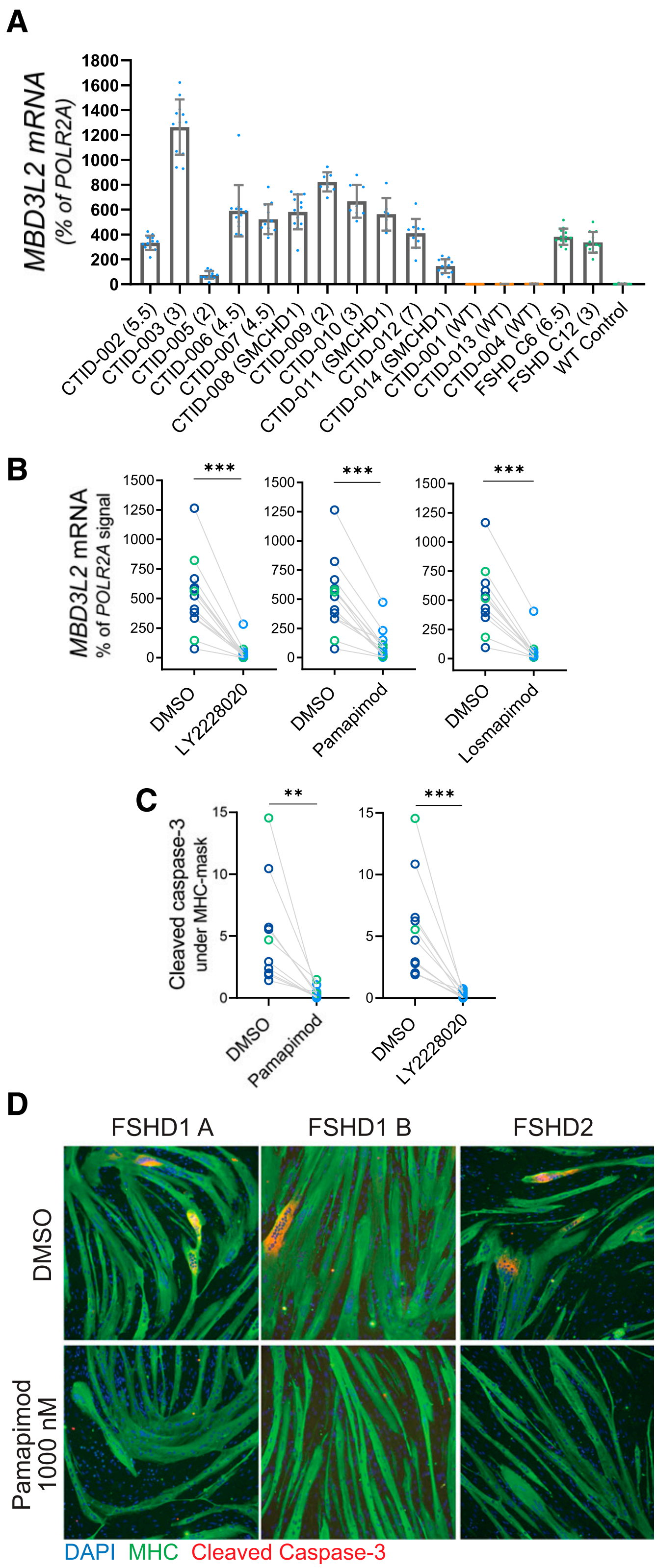

Fig. 5. p38α/β inhibition results in the reduction of DUX4 activity and cell death across a variety of genotypes of FSHD1 and FSHD2 primary myotubes. (A) Levels of *MBD3L2* expression across different primary and immortalized myotubes determined RT-qPCR. DUX4 activity is only detected in FSHD1/2 lines after 4 days of differentiation. Bars indicate mean ± S.D., and repeat number is indicated in parenthesis in FSHD1 lines and SMCHD1 mutation for FSHD2 lines used. (B) Inhibition of the p38α/β pathway results in potent reduction of *MBD3L2* expression activation across the entire set of FSHD primary cells tested. Three different inhibitors were used, and each circle indicates a different FSHD cell line tested. FSHD1 in blue and FSHD2 in green. Expression levels were measured by RT-qPCR in six replicates. (C and D) p38α/β pathway inhibition reduces activation of programmed cell death across primary FSHD cell lines with different genotypes. Stochastic activation of caspase-3 in a small number of FSHD myotubes was detected by immunostaining and quantified in all lines. Six replicates were used to quantify signal of cleaved caspase-3 under MHC-stained myotubes. Wilconox test, P value **0.002, ***0.0002. DAPI, 4′,6-diamidino-2-phenylindole; WT, wild type.

apoptotic cells have been detected in skeletal muscle of patients with FSHD, thereby supporting the hypothesis that programmed cell death is caused by aberrant DUX4 expression and contributes to FSHD pathology (Sandri et al., 2001; Statland et al., 2015). To test this hypothesis in vitro, we evaluated the effect of p38α/β inhibition on apoptosis in FSHD myotubes. We used an antibody recognizing caspase-3 cleavage products by immunofluorescence to quantify changes in the activation of programmed cell death. Cleavage of caspase-3 is a major step in the execution of the apoptosis-signaling pathway, leading to the final proteolytic steps that result in cell death (Fuentes-Prior and Salvesen, 2004; Dix et al., 2008). We detected activated caspase-3 in FSHD but not in wild-type myotubes and observed a stochastic pattern of expression of DUX4 in FSHD as previously reported (Fig. 4A) (Snider et al., 2010; Jones et al., 2012; van den Heuvel et al., 2019). Levels of cleaved caspase-3 were reduced in a concentration-dependent manner with an IC_{50} similar to what we observed for inhibition of the p38 pathway and DUX4 expression (Fig. 4B). Moreover, we measured SLC34A2, a DUX4 target gene product using a similar immunofluorescence assay (Fig. 3B). This protein was expressed in a similar stochastic pattern observed for active caspase-3, and its expression was also reduced by p38α/β inhibition (Fig. 4, B and C). Our results demonstrate that DUX4 inhibition in FSHD myotubes results in a significant reduction of apoptosis.

p38 α and β Inhibition Results in Downregulation of DUX4 Expression and Suppression of Cell Death Across Multiple FSHD1 and FSHD2 Genotypes. FSHD is caused by the loss of repression at the *D4Z4* locus leading to DUX4 expression in skeletal muscle due to the contraction in the *D4Z4* repeat arrays in chromosome 4 or by mutations in *SMCHD1* and other modifiers, such as *DNMT3B*. Primary FSHD myotubes were used to study the in vitro efficacy of p38α/β inhibitors across different genotypes. We tested eight FSHD1 primary myoblasts with 2-7 *D4Z4* repeat units and three FSHD2 cell lines with characterized *SMCHD1* mutations. Upon differentiation, the primary cells tested expressed a wide range of *MBD3L2* levels (Fig. 5A, number of D4Z4 repeat units or SMCHD1 mutation indicated in parenthesis), comparable to what we and others have observed in other FSHD myotubes (Jones et al., 2012). However, we observed significant inhibition of the DUX4 program expression after treatment with multiple p38α/β inhibitors in all primary myotubes tested from patients with FSHD1 and FSHD2 (Fig. 5B). Furthermore, this reduction in the DUX4 program resulted in concomitant reduction of cleaved caspase-3 (Fig. 5C) without any measurable effects on myotube differentiation (Fig. 5D). Our results suggest that the p38α/β pathway critically regulates the activation of DUX4 independently of the mutation driving its expression in FSHD muscle cells.

Discussion

Recent studies have advanced the understanding of the mechanisms that normally lead to the establishment and maintenance of repressive chromatin at the *D4Z4* repeats. Similar to other repetitive elements in somatic cells, chromatin at this locus is decorated by DNA methylation and other histone modifications associated with gene silencing, such as H3K27me3 and H3K9me3 (van Overveld et al., 2003; Zeng et al., 2009; Cabianca et al., 2012; van den Boogaard et al., 2016b). Factors involved in the deposition of these modifications like *SMCHD1* and *DNMT3B* have been identified by genetic analysis of affected FSHD populations (Lemmers et al., 2012; Calandra et al., 2016; van den Boogaard et al., 2016b). Other factors that associate with the *D4Z4* locus like nucleosome remodeling deacetylase (NuRD) and chromatin assembly factor 1 (CAF1) have been identified by biochemical approaches (Campbell et al., 2018). However, sequence-specific transcriptional activators of *DUX4* have remained elusive not only in skeletal muscle but also in the regulation of *DUX4* in the developing embryo, where this factor is normally expressed. Because of the effects of expression of DUX4 in FSHD and the apparent tissue-specific expression of DUX4 in skeletal muscle, it has been hypothesized that myogenic regulatory elements upstream of the *D4Z4* repeats participate in the expression of *DUX4* in FSHD (Himeda et al., 2014), yet this finding has not led to the identification of other factors that can specifically activate *DUX4*.

In this study, by modeling FSHD in vitro and screening a library of probe molecules using a highly sensitive and specific assay to detect a DUX4 target gene, we identified p38α as a novel activator of *DUX4* expression in patient-derived FSHD cells. This signaling kinase directly phosphorylates transcription factors involved in myogenesis and may signal directly to activate *DUX4* expression in differentiating myoblasts. Using highly selective and potent small molecules extensively characterized previously, we have studied the pharmacological relationships between the inhibition of this signaling pathway and the inhibition of the expression of DUX4, its downstream gene program expression, and its consequences in muscle cells from patients with FSHD. These relationships are maintained across multiple FSHD genotypes, including FSHD1 and FSHD2, indicating that this mechanism acts independent of the genetic lesion present in these patients. Our studies show a specific effect of p38α and β inhibition in downregulation of the DUX4 program and normalization of gene expression compared with cells from healthy donors. Notably, no effects in differentiation were detected at the tested concentrations of p38 inhibitor.

Other recent efforts to identify targets for the treatment of FSHD have reported similar studies in which the investigators followed the expression of *MBD3L2* as a readout for DUX4 expression or by using a reporter driven by the activity of DUX4 in immortalized FSHD myotubes in vitro (Campbell et al., 2017; Cruz et al., 2018). Our results have reproduced their previous identification of β-adrenergic agonists and BET inhibitors as inhibitors of DUX4 expression. However, these molecules also caused downregulation of the transcription factor *MYOG* expression or affected myoblasts fusion at concentrations similar to the half-maximal inhibitory concentration for DUX4 expression inhibition in our model (Supplemental Fig. 1B, lack of fusion indicated by arrow). Similarly, we also observed that inhibition of phosphodiesterases resulted in DUX4 downregulation, suggesting that cyclic AMP levels during differentiation are also important for its expression as previously reported (Cruz et al., 2018). It remains to be deciphered how all these pathways interconnect to regulate DUX4 expression during the process of in vitro differentiation and, most importantly, in the skeletal muscle tissue of patients with FSHD. In vitro models like the one used in this study, may suffer from diverse limitations. Differences in media, extracellular matrix used as coating in culture plates, and timing in treatments might result in deviation of pharmacological effects observed. However, an independent study recently described in a different in vitro model that p38α/β inhibitors inhibit expression of DUX4, further validating findings reported here. Importantly, in this study, they showed that p38α/β inhibitors are efficacious in downregulating expression of DUX4 in a xenograft mouse model of FSHD, supporting the idea that this mechanism is a viable therapeutic target in the FSHD muscle. Other approaches to identify therapeutics for FSHD have explored inhibition of the effects downstream of DUX4 activation. These efforts have resulted in the identification of potential targets like P300/CBP (cAMP response element-binding protein) and the hypoxia response pathway, which could help in protecting muscle cells against the toxic effects of DUX4 expression (Bosnakovski et al., 2014). In addition, other groups have directly targeted DUX4 by using antisense oligonucleotides and gene therapy approaches and have demonstrated preclinical efficacy in animal models (Chen et al., 2016a; Ansseau et al., 2017; Wallace et al., 2017).

In humans, previous clinical studies evaluating p38α/β inhibitors in non-FSHD indications under an anti-inflammatory therapeutic hypothesis were tested extensively and shown to be safe and well-tolerated. However, they never met efficacy endpoints in diseases such as rheumatoid arthritis, chronic obstructive pulmonary disease, and acute coronary syndrome (Hill et al., 2008; Damjanov et al., 2009; Hammaker and Firestein, 2010; Barbour et al., 2013; MacNee et al., 2013; Norman, 2015; Patnaik et al., 2016). Here, we present further evidence from in vitro studies that support the therapeutic hypothesis of treatment of FSHD at its root cause, prevention, or reduction of aberrant expression of DUX4 via inhibition of p38 α/β.

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Authorship Contributions

Participated in research design: Rojas, Valentine, Accorsi, Maglio, Shen, Robertson, Kazmirski, Rahl, Tawil, Cadavid, Thompson, Ronco, Chang, Cacace, Wallace.

Conducted experiments: Rojas, Valentine, Accorsi, Maglio, Shen, Robertson.

Contributed new reagents or analytic tools: Valentine, Accorsi, Kazmirski, Tawil.

Performed data analysis: Rojas, Valentine, Accorsi, Robertson.

Wrote or contributed to the writing of the manuscript: Rojas, Wallace.

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Title:

P38α Regulates Expression of DUX4 in a model of Facioscapulohumeral Muscular Dystrophy

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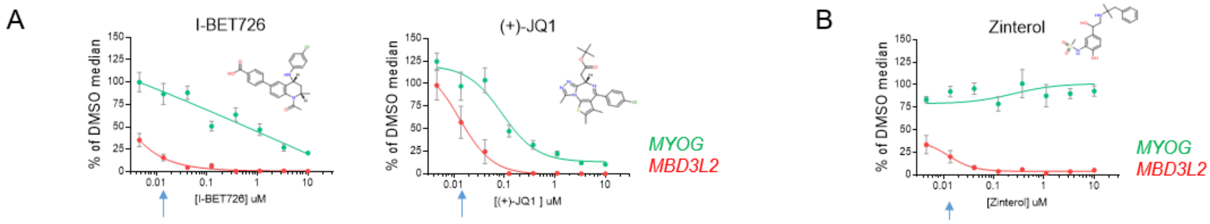
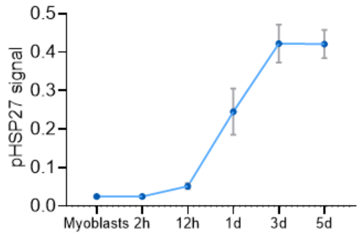
SUPPLEMENTARY FIGURES

Supplemental Figure 1.



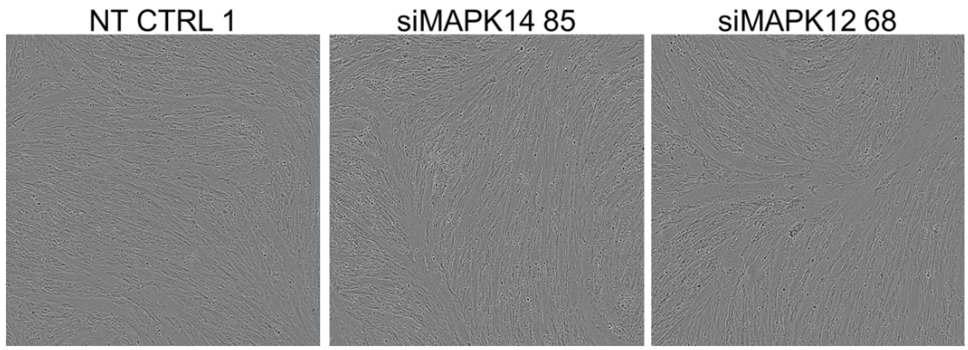
Bromodomain containing proteins inhibitors (A) and β-adrenergic agonist reduced the expression of *MBD3L2* in a concentration dependent manner as previously described (Campbell *et al.*, 2017)Arrow indicates concentration at which effects in differentiation started to be observed by visual inspection.

Supplemental Figure 2.



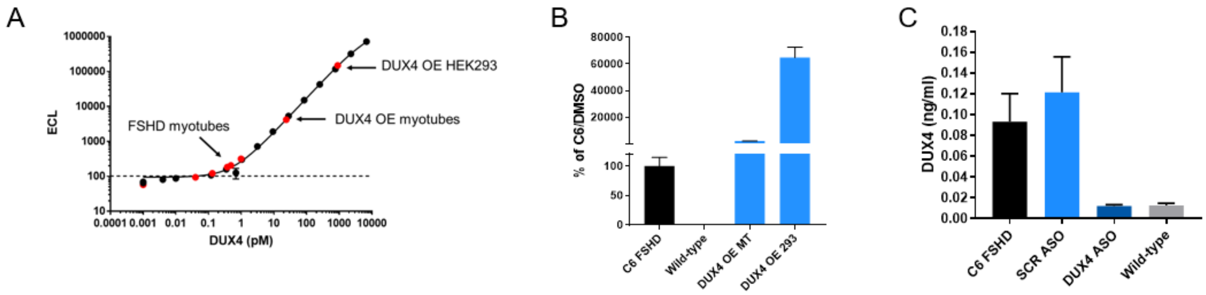
Levels of phosphorylated-HSP27 increase during myogenic differentiation in C6 FSHD myotubes.

Supplemental Figure 3.



Differentiation of C6 FSHD myotubes was not affected by *MAPK12* and *MAPK14* partial knockdown that resulted in *MBD3L2* level reduction.

Supplemental Figure 4.



Specific detection of DUX4 protein in mesoscale electro-chemiluminescent immunoassay (A) Recombinant GST-DUX4 calibrator curve. (B) C6 FSHD or wild type 5-day differentiated myotubes, DUX4 overexpressed 1-day differentiated myotubes infected with DUX4 bacmam, DUX4 overexpressed in 293 cells transfected with CMV-DUX4 plasmid. (C) C6 FSHD myotubes treated with scrambled or DUX4 anti-sense oligonucleotide or wild type control.