p38α Regulates Expression of DUX4 in a Model of Facioscapulohumeral Muscular Dystrophy

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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,
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; Tuwai 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; Segalès et al., 2016b). p38α abrogation in mouse myoblasts inhibits fusion and myotube formation in vitro (Zetser et al., 1999; Perdiguerro 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 down-regulation of DUX4.

### Materials and Methods

#### Cell Lines and Cell Culture.

Immortalized myoblasts from FSHD (AB1080/FSHD26 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 culture 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 trirreactent 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 oligodeoxynucleotide (cat. 18091050; Thermofisher). Pre amplification 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′-GCCGCCCAGGT ACCA-3′, Reverse: 5′-CAGCAGTCCTCTTGCA-3′, and Probe: 5′- /56-FAM/CAGTGGCGA/ ZEN/KCGG/3IABkFQ/-3′; and HMBS HS00609297m1VIC). 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. 441882; Thermofisher).

To measure DUX4 target gene expression in a 96-well plate format, cells were lysed into 25 μl Realtime Ready lysis buffer (0724831001; Roche) containing 1% RNase inhibitor (0335399001; 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 10 μl using...
RNase-free water. One microliter of this reaction was used as input for a 5-μl qPCR reaction using the Taqman Multiplex Master Mix (4484262; ThermoFisher). The following Taqman probes were purchased from ThermoFisher: MBD3L2 (Hs01028718_m1, FAM-MGB; ThermoFisher), TRIM43 (Hs01028718_m1, FAM-MGB; ThermoFisher), LEUTX (Hs00537549_m1, FAM-MGB; ThermoFisher), KHDC1L (Hs01024323_g1, FAM-MGB; ThermoFisher, (Bosnakovski et al., 2019)], zinc finger and SCAN domain containing 4 (ZSCAN4), and RNA Polymerase II Subunit A (POLR2A). Amplification was detected in a Quantstudio 7 Flex instrument from 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 discovery 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 MgCl₂, 10 mM HEPES, 1% Triton X-100 at pH = 7.4). Nuclei were pelleted by centrifugation at 2000g for 4 minutes at 4°C. Nuclei were resuspended in ice-cold wash buffer (320 mM sucrose, 5 mM MgCl₂, 10 mM HEPES at pH = 7.4) and pelleted by centrifugation at 2000g for 4 minutes at 4°C. Nuclei were suspended in 150 μl of RIPA buffer at 4°C (+150 mM

**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 homolog; WT, wild type; ZSCAN4, zinc finger and SCAN domain containing 4; S/N, signal to noise ratio; rep, replicate.
NaCl). Extracts were diluted 1:1 with assay buffer, and 10 μl per well was added to 384-well precoated/blotted 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. 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 during 1 hour 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 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% confluence and added differentiation medium lacking growth factors (Fig. 1A) (Brewer et al., 2008; Krom et al., 2012; Thorley et al., 2016).
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, IC50 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 factors.
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) (Perdiguerò et al., 2007). We observed that p38α/β inhibitors reduced phosphorylated HSP27 levels with similar IC₅₀ values to that of MBΔ3L2 (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 MBΔ3L2 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 MBΔ3L2 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 (Perdiguerò 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, MBΔ3L2.

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; Perdiguerò 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 quantify 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,
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 IC50 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.

**p38α 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.

**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 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. Wilcoxon test, P value **0.002, ***0.0002. DAPI, 4′,6-diamidino-2-phenylindole; WT, wild type.
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 approaches 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 p38α/β.

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Authorship Contributions

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Segales J, Perdiguero E, and Muino-Cánoves P (2016) Regulation of muscle stem cell functions: a focus on the p38 MAPK signaling pathway. Front Cell Dev Biol 4:91.
Shude SC, Zhong JW, Campbell AE, Conery ML, Jagannathan S, Wong C-J, Morello TD, van der Maarel SM, and Tapscott SJ (2017) DUX4-induced dsRNA and MYC mRNA stabilization activate apoptotic pathways in human cell models of facioscapulohumeral dystrophy. PLoS Genet 13:e1006598.
Simone C, Forcades SV, Hill DA, Imbalzano AN, Latella L, and Puri PL (2004) p38 pathway targets SWI-SNF chromatin-remodeling complex to muscle-specific loci. Nat Genet 36:738–743.
Snider L, Geng LN, Lemmers RJ, Kýba M, Ware CB, Nelson AM, Tawil R, Filipova GN, van der Maarel SM, Tapscott SJ, et al. (2010) Facioscapulohumeral dystrophy: incomplete suppression of a retrotansposed gene. PLoS Genet 6:e1001181.
Statland JM, Odrzywolski R, Shah B, Henderson D, Fricke AF, van der Maarel SM, Tapscott SJ, and Tawil R (2015) Immunohistochemical characterization of facioscapulohumeral muscular dystrophy muscle biopsies. J Neuromuscul Dis 2:291–299.
Statland JM and Tawil R (2014) Risk of functional impairment in Facioscapulohumeral muscular dystrophy. Muscle Nerve 49:520–527.
Tasca G, Pescatori M, Menforte M, Miraabella L, Iannaccone E, Frasciante R, Cubeddu T, Laschena F, Ottaviani P, and Ricci E (2012) Different molecular signatures in magnetic resonance imaging-staged facioscapulohumeral muscular dystrophy muscles. PLoS One 7:e38779.
Tawil R, Kissel JT, Heatwole C, Pandya S, Gronseth G, and Benatar M; Guideline Development, Dissemination, and Implementation Subcommittee of the American Academy of Neurology; Practice Issues Review Panel of the American Association of Neuromuscular & Electrophysiology; the path to consensus on pathophysiology. Eur J Hum Genet (2016a) Double SMCHD1 variants in FSHD2: the synergistic effect of two kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence. J Pharmacol Exp Ther 360:281–288.
van den Boogaard ML, Lemmers RJLF, Bagel J, Wehlgemuth M, Auranen M, Mitsuhashi S, van der Vliet PJ, Strasheim KJ, van den Akker RFF, Kriket M, et al. (2016b) Mutations in DNMT3B modify epigenetic regulation of the D4Z4 repeat and the penetrance of facioscapulohumeral dystrophy. Am J Hum Genet 98:1020–1029.
van den Heuvel A, Mahfouz A, Kloet SL, Bajog J, van Engelen BGM, Tawil R, Tapscott SJ, and van der Maarel SM (2013) Single-cell RNA sequencing in facioscapulohumeral muscular dystrophy disease etiology and development. Hum Mol Genet 22:1064–1075.
van Overveld PGM, Lemmers RJFL, Sandkuijl LA, Enthoven L, Winokur ST, Bakels F, Padberg GW, van Ommen G-JB, Frants RR, and van der Maarel SM (2003) Hypomethylation of D4Z4 in 4q-linked and non-4q-linked facioscapulohumeral muscular dystrophy. Nat Genet 35:315–317.
Wallace LM, Saad NY, Pyke NK, Fowler AM, Eidahl JO, Demire JS, Griffin DA, Herman AC, Sahenk Z, Rodino-Klapac LR, et al. (2017) Pre-clinical and efficacy studies to target expression of AAV-mediated RNAi therapy for FSHD. Mol Ther Methods Clin Dev 4:121–130.
Wang LH, Friedman SD, Shaw D, Snider L, Wong C-J, Budech CB, Poliaichik SL, Gove NE, Lewis LM, Campbell AE, et al. (2019) MRI-informed muscle biopsies correlate MRI with pathology and DUX4 target gene expression in FSHD. Hum Mol Genet 28:476–486.
Whiddon JL, Langford AT, Wong C-J, Zhong JW, and Tapscott SJ (2017) Conservation and innovation in the DUX4-family gene network. Nat Genet 49:935–940.
Whitmarsh AJ (2010) A central role for p38 MAPK in the early transcriptional response to stress. BMC Biol 8:47.
Wissing ER, Boyer JG, Kwong JQ, Sargent MA, Karch J, McNally EM, Otsu K, and Molkentin JD (2014) P38α MAPK underlies muscular dystrophy and myofiber death through a Rax-dependent mechanism. Hum Mol Genet 23:5542–5552.
Yao Z, Snider L, Bajog J, Lemmers RJLF, Van Der Maarel SM, Tawil R, and Tapscott SJ (2014) DUX4-induced gene expression is the major molecular signature in FSHD skeletal muscle. Hum Mol Genet 23:5542–5552.
Zarubin T and Han J (2005) Activation and signaling of the p38 MAP kinase pathway. Cell Res 15:11–18.
Zeng W, de Groot JC, Chen Y-Y, Chien R, Kong X, Gregson HC, Winokur ST, Pyle A, Robertson KD, Schmieszew JI, et al. (2009) Specific loss of histone H3 lysine 9 trimethylation and HP1α/cohesin binding at D4Z4 repeats is associated with facioscapulohumeral dystrophy (FSHD). PLoS Genet 5:e1000559.
Zetser A, Grediw D, Z, and Bengal E (1999) p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation. Participation of the MeF2c transcription factor. J Biol Chem 274:5193–5200.

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