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Clinically advanced p38 inhibitors suppress DUX4 expression in cellular and animal models of facioscapulohumeral muscular dystrophy

Jonathan Oliva[1], Scott Galasinski[2], Amelia Richey[1], Amy E. Campbell[3], Marvin J. Meyers[4], Neal Modi[1], Jun Wen Zhong[4], Rabi Tawil[5], Stephen J. Tapscott[3,6] and Francis M. Sverdrup[1]

[1]Department of Biochemistry and Molecular Biology, Saint Louis University, Saint Louis, MO 63104, USA

[2]Ultragenyx Pharmaceutical Inc., Novato, CA 94949, USA

[3]Human Biology Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA

[4]Department of Chemistry, Saint Louis University, Saint Louis, MO 63104, USA

[5]Department of Neurology, University of Rochester Medical Center, Rochester, NY 14642, USA

[6]Department of Neurology, University of Washington, Seattle, WA 98105, USA

Running Title: p38 inhibitors suppress DUX4 expression in FSHD

Address correspondence to:

Francis M. Sverdrup

Saint Louis University

Doisy Research Rm # 417

1100 South Grand Blvd.

Saint Louis, MO 63104

TEL: 314-977-5189

FAX: 314-977-9205

Email: fran.sverdrup@health.slu.edu

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Non-standard abbreviations:

FSHD	facioscapulohumeral muscular dystrophy
ASO	antisense oligonucleotide
MAPK	mitogen activated protein kinase
Cts	cycle times

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Abstract

Facioscapulohumeral muscular dystrophy (FSHD) is characterized by mis-expression of the DUX4 developmental transcription factor in mature skeletal muscle where it is responsible for muscle degeneration. Preventing expression of DUX4 mRNA is a disease-modifying therapeutic strategy with the potential to halt or reverse the course of disease. We previously reported that agonists of the beta-2 adrenergic receptor suppress *DUX4* expression by activating adenylate cyclase to increase cAMP levels. Efforts to further explore this signaling pathway led to the identification of p38 MAP kinase as a major regulator of *DUX4* expression. In vitro experiments demonstrate that clinically advanced p38 inhibitors suppress *DUX4* expression in FSHD type 1 (FSHD1) and FSHD2 myoblasts and differentiating myocytes in vitro with exquisite potency. Individual siRNA-mediated knockdown of either p38alpha or p38beta suppresses *DUX4* expression, demonstrating that each kinase isoform plays a distinct requisite role in activating *DUX4*. Finally, p38 inhibitors effectively suppress *DUX4* expression in a mouse xenograft model of human FSHD gene regulation. These data support the repurposing of existing clinical p38 inhibitors as potential therapeutics for FSHD. The surprise finding that p38 alpha and beta isoforms each independently contribute to DUX4 expression offers a unique opportunity to explore the utility of p38 isoform-selective inhibitors to balance efficacy and safety in skeletal muscle. We propose p38 inhibition as a disease-modifying therapeutic strategy for FSHD.

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Significance Statement

Facioscapulohumeral muscular dystrophy (FSHD) currently has no treatment options. This work provides evidence that repurposing a clinically advanced p38 inhibitor may provide the first disease-modifying drug for FSHD by suppressing toxic DUX4 expression, the root cause of muscle degeneration in this disease.

Introduction

Facioscapulohumeral dystrophy (FSHD) is one of the most prevalent muscular dystrophies (Padberg et al., 1995; Deenen et al., 2014), yet there is currently no treatment available and few clinical trials of promising therapies are ongoing. Thus, there is a desperate need to identify drug targets to stop or reverse progression of the disease. As described in its name, FSHD typically presents as facial, shoulder and upper arm weakness, which eventually progresses to involve nearly all skeletal muscle groups (Tawil et al., 2014). Although most individuals develop symptoms during their second or third decade of life, a small number have a more acute pediatric onset (Goselink et al., 2017). FSHD is caused by mis-expression of the double homeobox 4 (DUX4) transcription factor in skeletal muscle. *DUX4* is encoded by a retrogene located within each unit of the D4Z4 macrosatellite repeat array on chromosome 4q35 and is normally expressed in pre-implantation embryos where it activates an early developmental program that marks the cleavage stage of embryogenesis (Tawil et al., 2014; De Iaco et al., 2017; Hendrickson et al., 2017; Whiddon et al., 2017).

In normal skeletal muscle, *DUX4* is silenced likely through repeat-mediated epigenetic repression (van Overveld et al., 2003; Zeng et al., 2009; Snider et al., 2010; Daxinger et al., 2015; Das and Chadwick, 2016). In patients with FSHD, deletion of a subset of D4Z4 repeats (FSHD type 1, FSHD1) or damaging variants in epigenetic regulators of the D4Z4 array (FSHD type 2, FSHD2) (Lemmers et al., 2010; Lemmers et al., 2012; van den Boogaard et al., 2016) lead to inefficient D4Z4 repression in somatic cells, which, when combined with a permissive chromosome 4 haplotype that provides a polymorphic polyadenylation site, results in the ectopic expression of DUX4 in muscle cells (Lemmers et al., 2010; Snider et al., 2010; Tawil et al., 2014). DUX4 mis-expression in skeletal muscle causes many cellular phenotypes, ultimately leading to cell death (Winokur et al., 2003; Kowaljow et al., 2007; Bosnakovski et al., 2008; Snider et al., 2009; Wallace et al., 2011; Geng et al., 2012; Young et al., 2013; Feng et al., 2015; Rickard et al., 2015; Shadle et al., 2017).

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Because of its causative role in FSHD, targeting DUX4 is an obvious therapeutic approach to halt or reverse disease progression (Himeda et al., 2015). Blocking transcription of *DUX4* mRNA with small molecule drugs is an attractive option because all downstream pathological mechanisms are inherently disrupted and small molecules are generally amenable to oral dosing, avoiding delivery complications associated with RNAi or gene editing technologies. However, subsequent to chromatin decompaction associated with FSHD, the mechanisms responsible for bursts of *DUX4* mRNA synthesis are still poorly understood and limited small molecule drug targets have been identified (Block et al., 2013; Campbell et al., 2017; Teveroni et al., 2017; Cruz et al., 2018).

Previously, we reported on the discovery of beta-2 adrenergic receptor agonists as modulators of DUX4 expression (Campbell et al., 2017). β2 adrenergic signaling has been extensively characterized in skeletal muscle, so we began systematically testing small molecule inhibitors that could potentially interfere with the pathway (for review: (Joassard et al., 2013)). One set of kinases that are activated by β2 adrenergic signaling in a PKA dependent manner are the p38 mitogen-activated protein (MAP) kinases (MAPK) (Moule and Denton, 1998; Zheng et al., 2000; Aggeli et al., 2002; McAlees and Sanders, 2009). p38 MAPKs are classically involved in the cellular response to stressful stimuli including inflammatory cytokines and have been heavily pursued by pharmaceutical companies for diseases with inflammatory components, such as rheumatoid arthritis, resulting in an abundance of chemical tools from p38α selective to pan-p38 inhibitors (Xing, 2015). In exploring the hypothesis that activation of p38 played a role in β2-agonist repression of *DUX4*, we determined that rather than blocking the ability of β2-agonists to suppress DUX4, p38 inhibitors instead potently inhibited the expression of *DUX4* in the absence of β2 agonism. Here, we identify p38α and p38β MAP kinases as having important roles in the pathogenic expression of *DUX4* in FSHD and demonstrate that clinically advanced p38 inhibitors decrease expression of *DUX4* and DUX4 target genes in FSHD patient-derived muscle cells at inhibitor levels that do not negatively affect muscle differentiation. In a mouse pharmacology model of FSHD gene

regulation, these inhibitors suppress *DUX4* at blood levels that are efficacious in published pre-clinical models of inflammatory diseases and that have been routinely achieved in human clinical trials. These findings suggest that existing clinical p38 inhibitors are repurposing candidate drugs for FSHD therapeutic development.

Materials and Methods

Ethics Statement

This research used pre-existing de-identified human cell lines from approved repositories. These studies were determined to not be Human Subject Research by the Saint Louis University Institutional Review Board. Primary human myoblast cell lines were obtained from the Fields Center at the University of Rochester (http://www.urmc.rochester.edu/fields-center.aspx) and immortalized by retroviral transduction of cyclin-dependent kinase 4 and human telomerase reverse transcriptase (MB200, FSHD2) (Snyder et a.l, 2010; Stadler et al., 2011). An immortalized FSHD1 cell line (54-2) and isogenic control line from a mosaic male patient (54-6, non FSHD) were also used (Krom et al., 2012).

Cell culture

Immortalized myoblasts were grown in Ham's F-10 Nutrient Mix (Gibco, Waltham, MA, USA) supplemented with 20% Corning USDA Approved Source Fetal Bovine Serum (Corning, Corning, NY, USA), 100 U/100 μg penicillin/streptomycin (Gibco), 10 ng/ml recombinant human fibroblast growth factor (Promega Corporation, Madison, WI, USA) and 1 μM dexamethasone (Sigma-Aldrich). Differentiation of myoblasts into myotubes was achieved by switching confluent myoblast monolayers into DMEM:F-12 Nutrient Mixture (1:1, Gibco) supplemented with 2% KnockOut Serum Replacement (Gibco), 100 U/100 μg penicillin/streptomycin, 10 μg/ml insulin and 10 μg/ml transferrin (KSR media) for 40 hours.

Compounds

Individual compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA), Tocris Bioscience (Bio-Techne Corporation, Minneapolis, MN), MedChemExpress (Monmouth Junction, NJ) or Selleck

Chemicals (Houston, TX), dissolved in 100% DMSO as concentrated stocks and stored at -20 ºC until use. For in vitro experiments, concentrated DMSO stocks were first diluted in 100% DMSO to 2000-fold the final concentration, then diluted 2000-fold into culture media before addition to cells. Experiments were performed in triplicates with standard deviations represented. For determination of the 50% inhibitory concentration (IC_{50}) for each compound, eleven-point concentration response curves were generated by first creating 3-fold serial dilutions of compounds from concentrated stocks in 100% DMSO in 96-well plates. Serial dilutions were then further diluted 2000-fold into culture medium before addition to cells. IC_{50}s were determined by nonlinear regression using a four-parameter logistic equation (GraphPad Prism Software Inc., San Diego, CA; http://www.graphpad.com). Data are presented as IC_{50}s with two significant digits.

DUX4 activity assay

DUX4 activity assay was performed as detailed in our previous studies (Campbell et al., 2017). Briefly, control 54-6 (non-FSHD) myoblasts in 1 well of a 6-well plate were co-transfected with 75 ng of the *DUX4* expression vector pCS2-DUX4 (Geng et al., 2012) and 2.925 µg of pGL3-promoter vector (Promega) using Lipofectamine 3000 (Thermo Fisher Scientific) following the manufacturer's instructions. Three hours after transfection, cells were trypsinized and distributed to 42 wells of a 96-well plate. Two hours later (a time at which there is a low but detectable level of DUX4 target gene expression), 6 wells were harvested to represent the 'baseline' gene expression state while DMSO control or compounds were added to the remaining wells as indicated in the figures. Twenty hours later, the remaining wells were harvested to represent the 'endpoint' gene expression state. DUX4 activity was determined by normalizing DUX4 target gene mRNA levels at the 25 hour 'endpoint' to the levels at the 5 hour 'baseline' and setting that value to 100 in the absence of drug.

mRNA expression analyses

For screening of compounds in 96-well plates for IC_{50} determinations and for the DUX4 activity assay, cell lysates were prepared using Cells-to-Ct Bulk Lysis Reagents (Invitrogen, Carlsbad, CA, USA). For other gene expression analyses, total RNA was extracted from whole cells using the E.Z.N.A. Total RNA

Kit or xenograft tissues using the E.Z.N.A. Tissue RNA Kit (OMEGA Bio-Tek, Norcrass, GA) and RNA/DNA was isolated from xenograft tissue using E.Z.NA. DNA/RNA kit (OMEGA Bio-Tek, Norcrass, GA) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out on a QuantStudio 5 (Applied Biosystems, Foster City, CA, USA). For all gene detection except for *DUX4*, TaqMan Gene Expression Assays (Applied Biosystems) and TaqMan Fast Virus 1-Step Master Mix (Invitrogen) were used. For *DUX4* expression, isolated RNA was treated with DNase I (Thermo Fisher Scientific) and reverse transcribed into cDNA using Superscript IV (Thermo Fisher Scientific) using oligo (dT) primers (Invitrogen) following the manufacturer's protocol. qRT-PCR was performed using a custom Taqman primer/probe set and TaqMan Gene Expression Master Mix (Applied Biosystems). The relative expression levels of target genes were normalized to that of the reference gene ribosomal protein L30 (*RPL30*) which was included in multiplex (two gene) PCR reactions, using the delta-delta-Ct method (Livak and Schmittgen, 2001) after confirming equivalent amplification efficiencies of reference and target molecules.

Small interfering RNA (siRNA) transfections

Duplex siRNAs were obtained from Thermo Fisher Scientific (Silencer Select). Transfections of siRNAs into myoblasts were carried out, in triplicate, using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded at $1x10^5$ cells/well in 12-well plates and transfected ~20 hours later with 2 µl Lipofectamine RNAiMAX and 10 pmol of either gene-specific siRNAs or a scrambled non-silencing control siRNA diluted in 100 µl Opti-MEM Reduced Serum Medium. For myoblast knockdowns, cells were harvested for RNA analysis 72 hours later. For myotube experiments, twenty-four to forty-eight hours following transfection, cell were switched to differentiation medium and harvested for RNA analysis 40 hours later. Where noted, a double transfection protocol was followed to ensure efficient depletion of pre-existing proteins. Cells were transfected a second time 24 hours after the first transfection, switched to differentiation medium 24 hours later and harvested for RNA analysis 40 hours after switching to differentiation medium.

Western blotting

Samples from siRNA knockdown experiments were obtained by lysing cells by addition of SDS-PAGE loading buffer. Reduced and boiled samples were run on NuPage 4-12% Bis-Tris precast polyacrylamide gels (Life Technologies, Carlsbad, CA) and transferred to Immobilon-FL polyvinylidene difluoride membrane (Millipore, Burlington, MA). After blocking in 0.2% I-Block (Thermo Fisher Scientific, Waltham, MA) in TBST for 1 hour at room temperature, membranes were incubated with appropriate primary antibodies diluted at 1:1000 in 1X TBST overnight at 4 ºC. Membranes were then incubated with Li-Cor near-infrared fluorescently labeled secondary antibodies diluted at 1:15,000 in 1X TBST for 1 hour at room temperature. Blots were scanned and analyzed with the Li-Cor Odyssey CLx Imaging System (Li-Cor Biosciences, Lincoln, Nebraska). Quantitation of bands was normalized to α-tubulin using Image Studio software (Li-Cor Biosciences, Lincoln, Nebraska).

Antibodies

The following antibodies were used: α-Tubulin mouse mAb (926-42213; Li-Cor Biosciences Lincoln, Nebraska); p38-alpha MAPK polyclonal Rabbit Ab (9218S; Cell Signaling Technology, Danvers, MA); p38β MAPK (C28C2) Rabbit mAb (2339; Cell Signaling Technology, Danvers, MA); IRDye 680 goat anti-mouse secondary, (926-68070; Li-Cor Biosciences Lincoln, Nebraska); and IRDye 800 goat anti-rabbit secondary, (926-32211; Li-Cor Biosciences Lincoln, Nebraska).

Animals

Male NOD-Rag immunodeficient mice (strain #007799 NOD.CgRag1^{tm1Mom} Il2rg^{tm1Wjl}/SzJ; Jackson Laboratories) were used for xenograft model of FSHD because of the absence of T, B, and NK cells in this strain makes them suitable for xenograft transplantation (Silva-Barbosa et al., 2005). All protocols were approved by the Institutional Animal Care and Use Committee of Saint Louis University.

Plasma drug levels

Terminal plasma samples were diluted with control naïve mouse plasma as appropriate to bring samples into the standard curve range (1–1000 ng/ml) or were run undiluted. Enalapril was used as an internal standard prior to extraction at a 100 ng/ml final concentration. Samples were vortexed for 5 minutes to fully incorporate the internal standard. Afterwards, 180 ml of acetonitrile was added, vortexed for 5

minutes, and centrifuged for 5 minutes at 4ºC and greater than 2100g. The supernatant was transferred to a 96-well sample plate and heat sealed for liquid chromatography-tandem mass spectrometry analysis using a system consisting of an LC-20AD pump (Shimadzu, Kyoto, Japan), an HTC PAL autosampler (Leap Technologies, Carrboro, NC), and a Sciex API-4000 mass spectrometer in ESI mode (AB Sciex, Foster City, CA). The MRM transitions for losmapimod were m/z: 283 >267. An Amour C18 reverse-phase column (2.1 mmx30 mm, 5 um; Analytical Sales and Services, Pompton Plains, NJ) was used for chromatographic separation. Mobile phases were 0.1% formic acid (aqueous) and 0.1% formic acid in acetonitrile (organic) with a flow rate of 0.35 ml/ min. The starting phase was 10% acetonitrile for 0.9 minutes, increased to 90% acetonitrile over 0.4 minutes, maintained for an additional 0.2 minutes, returned to 10% acetonitrile over 0.4 minutes, and then held for 2 minutes. Peak areas were integrated using Analyst 1.5.1 (AB Sciex, Foster City, CA).

Cell and barium chloride preparations for injection

Barium chloride was dissolved in 0.9% saline to a concentration of 2.4% w/v. The barium chloride solution was then sterile filtered (0.2um filter, Thermo Scientific). Growing FSHD myoblasts were detached with 0.25% trypsin 2.21mM EDTA (Corning) and resuspended in growth media for enumeration. Cells were then centrifuged at 900g for 5 minutes and suspended in 30 ml of sterile saline. This saline rinse was repeated one more time before cells were suspended at ~6.67 x 10^7 cells/ml in saline. The cells were stored on ice and injected within 1-2 hours of preparation. Just prior to injection cells are combined with an equal volume of barium chloride solution yielding 1.2% w/v barium chloride solution with 3.33 x 10^7 cells per ml.

Barium chloride xenograft pharmacology model of FSHD

NOD-Rag immunodeficient mice were anesthetized with 3–3.5 % isoflurane to effect and the injection site was shaved and cleaned with betadine. Barium chloride cell suspensions were injected using a 26-gauge needle into three sites along the tibialis anterior muscle. Injections were 10 µl/site in volume amounting to 30 µl and 1 x10^6 cells injected in total. Mice recovered for 1-2 hours prior to administration

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of test compounds. PH-797804 and losmapimod were dissolved in DMSO prior to addition of the remaining vehicle components. PH-797804 was administered b.i.d. at 5 ml/kg subcutaneous in 10% DMSO, 40% PG, and 50% saline vehicle. Losmapimod was administered b.i.d. at 10 ml/kg P.O. in 10% DMSO and 90% 0.5% methylcellulose. At the termination of the study, mice were euthanized via CO_2 asphyxiation and blood and tissue samples were collected for bioanalytical or qPCR analysis. For FSHD endpoints, the entire xenograft muscle was harvested, weighed and homogenized in lysis buffer as described (see mRNA expression analyses). Lysis buffer was then transferred so that 30 mg of tissue was used in the RNA isolation procedure and 10 mg of tissue was used in the DNA/RNA procedure to prevent column clogging.

Taqman gene expression assay ID numbers

Taqman assays were purchased from Applied Biosystems (Thermo Fisher Scientific). MBD3L2, Hs00544743_m1; LEUTX, Hs01028718_m1; MYH2, Hs00430042_m1; MAPK11, Hs00177101_m1; MAPK14, Hs01051152_m1; MYOG, Hs01072232_m1; RPL30, Hs00265497_m1; ZSCAN4, Hs00537549_m1; DUX4, primers GCCGGCCCAGGTACCA and CAGCGAGCTCCCTTGCA with probe 6FAM-CAGTGCGCACCCCG-MGBNFQ.

siRNA assay IDs AND target sequences

All siRNAs were purchased with Silencer Select chemistry (Ambion, Life Technologies). MAPK14, s3585, CCTAAAACCTAGTAATCT; MAPK11, s11155, GCGACTACATTGACCAGCT; Negative control: Silencer Select Negative Control #1.

Statistical analysis

All statistics were performed using GraphPad 4 software. One-way ANOVA was used for multi-group comparisons; Dunnett's Post Test was used to compare individual groups to the control. An unpaired two-tailed t-test was used for two sample comparison. P values and statistical comparison used are listed with each figure.

Results

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p38 Inhibitors Suppress DUX4 in FSHD Myotubes and Myoblasts

We have previously screened several small molecule compound libraries to identify BET inhibitors and agonists of the beta-2 adrenergic receptor as inhibitors of *DUX4* expression in FSHD muscle cells (Campbell et al., 2017). Efforts to further explore the signaling pathways regulating *DUX4* expression in FSHD led us to screen p38 MAP kinase inhibitors in a high throughput assay for *DUX4*. Detection of *DUX4* mRNA in FSHD muscle cells for drug screening purposes is challenging for many reasons (Snider et al., 2009; Snider et al., 2010; Geng et al., 2012; Feng et al., 2015). Therefore, *DUX4* was measured indirectly by quantitating mRNA levels for the DUX4-regulated gene *MBD3L2,* which is a sensitive and highly specific marker of *DUX4* expression as it is not expressed in normal muscle cultures or tissue (Geng et al., 2012; Yao et al., 2014; Campbell et al., 2017). Differentiating cultures of MB200 (FSHD2) myoblasts that express elevated levels of *DUX4* and *DUX4*-target gene mRNA upon differentiation into multinucleated myotubes (myocytes) were utilized. Figure 1A shows the concentration-response curve for *MBD3L2* RNA levels in MB200 (FSHD2) myotubes differentiated in the presence of the clinical p38α/β inhibitor PH-797804 for 40 hours. The levels of *MBD3L2* RNA induced during differentiation are suppressed >95% in a concentration-dependent manner with a potent IC_{50} of 0.41 nM, in line with the reported IC_{50} of 2.3 nM for PH-797804 on p38α enzymatic activity (Selness et al., 2011). Since the p38 family of kinases is known to play important roles in the myogenic program (Wu et al., 2000), it was important to distinguish effects on *DUX4* expression from effects on myocyte differentiation. We noted that only at high concentrations of PH-797804 (> 100 nM) was there a noticeable delay in myotube formation. Key markers of myocyte differentiation (myogenin, *MYOG*, and myosin heavy chain, *MYH2*) were likewise only inhibited at concentrations greater than 100 nM, and even at 1-10 μM the inhibition was incomplete, reaching a maximum of ~60% inhibition (Fig. 1A). To further demonstrate that the effects of p38 inhibition on DUX4-target gene expression were independent of myocyte differentiation, cultures of proliferating FSHD myoblasts were treated with PH-797804. Figure 1B shows that multiple DUX4 targets are suppressed with treatment in both 54-2 (FSHD1) and MB200 (FSHD2) proliferating

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myoblast cultures, consistent with suppression of *DUX4* expression being independent of the differentiation state of the cells. To confirm that p38 inhibition by PH-797804 had a direct effect on *DUX4*, we performed experiments in both FSHD1 and FSHD2 myocytes treated with several drug concentrations for 40h during differentiation. Figure 1C (54-2, FSHD1) and Figure 1D (MB200, FSHD2) demonstrate that p38 inhibitor PH-797804 treatment reduces RNA levels for *DUX4* and DUX4 targets *MBD3L2*, *ZSCAN4* and *LEUTX* at drug concentrations that have negligible effects on markers of differentiation *MYOG* and *MYH2*. These data confirm that p38 inhibition directly suppresses *DUX4* expression and consequently the expression of DUX4 target genes is similarly reduced.

To determine if PH-797804 was representative of the class of p38 inhibitors, we screened twelve commercially available p38 inhibitors that had reached clinical testing and two commonly cited chemical probes in differentiating FSHD myocytes. All p38 inhibitors were active with concentration-response curves similar to those of PH-797804, exhibiting an approximate 3-fold order of magnitude difference in their ability to suppress DUX4 target *MBD3L2* expression versus having an effect on differentiation markers (Supplementary Fig. 1). Inhibitors were similarly active in 54-2 (FSHD1) and MB200 (FSHD2) myocytes with IC_{50} values reported in Table 1, suggesting that *DUX4* expression is exquisitely sensitive to p38 inhibition in both genetic backgrounds. Interestingly, inhibitors of the p38 inflammatory pathway target MAPK-activated protein kinase 2 (MAPKAPK2, MK2) (Mourey et al., 2010) were not active at suppressing *DUX4*, nor were inhibitors of MAPK interacting serine/threonine kinase 1/2 (MKNK1/2, MNK1/2) (Reich et al., 2018) (Table 1).

DUX4 Activity

DUX4 protein is likely a target for posttranslational modifications, including acetylation and phosphorylation, that could affect its function. It was possible that p38 inhibition not only suppressed the transcription of *DUX4* mRNA as shown above, but also affected the transcriptional activation function of

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DUX4 by altering its posttranslational modifications. To test this possibility, we used forced expression of DUX4 in normal skeletal muscle myoblasts to determine if p38 inhibition would alter the ability of transiently expressed DUX4 to induce expression of its target genes. 54-6 (non-FSHD) myoblasts were transfected with the pCS2-DUX4 plasmid (Geng et al., 2012), which drives DUX4 expression from the cytomegalovirus promoter, and then treated with various concentrations of two different p38 inhibitors for twenty hours. Since DUX4 protein interacts with the transcriptional co-activator p300 and utilizes its acetyltransferase activity to induce expression of many DUX4 target genes (Choi et al., 2016), we used the p300 acetyltransferase activity inhibitor A-485 (Lasko et al., 2017) as a control. Figure 2A shows that DUX4-induced increases in targets *MBD3L2* and *LEUTX* are reduced by >95% in a concentration-dependent manner by A-485, demonstrating the utility of this assay in measuring DUX4 activity. Interestingly, DUX4 target *ZSCAN4* mRNA levels were not affected by A-485 treatment (Fig. 2A), suggesting that DUX4 activates some target genes independent of p300 acetyltransferase activity. When transfected cells are exposed to PH-797804 during this time frame, there is a slight decrease in DUX4 targets (~35-40% maximal inhibition); however, significant transcriptional activation activity remains that is insensitive to p38 inhibition (Fig. 2B). Additionally, the p38α-biased inhibitor pamapimod (Hill et al., 2008) did not block DUX4-induced target gene expression (Fig. 2C). These data indicate that p38 inhibition has a primary activity of suppressing *DUX4* expression and potentially minor activity of partially reducing DUX4 transactivation function.

siRNA targeting p38α and p38β

The majority of p38 inhibitors to enter clinical testing are p38α/p38β isoform-selective inhibitors with several having bias towards p38α (for review see (Goldstein and Gabriel, 2005; Yong et al., 2009; Norman, 2015). An exception is BIRB796 which inhibits the α, β, γ and δ isoforms (Kuma et al., 2005). Data presented above suggested that either p38α, p38β or both isoforms play a role in promoting *DUX4* expression in FSHD myoblasts and differentiating myocytes. The consistency of inhibition of *DUX4*

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target genes (Table 1) appeared to track with potency of the drugs towards the p38α isoform. To determine the relative roles of each isoform, we utilized siRNAs to knock down individual isoforms in FSHD1 and FSHD2 myoblasts and myotubes. Figure 3A shows that in proliferating 54-2 (FSHD1) myoblast cultures, siRNAs targeting p38α or p38β, individually or in combination, effectively and selectively decrease levels of their respective target mRNAs (left panel). Interestingly, individual knockdown of p38α mRNA resulted in increased levels of p38β mRNA and individual knockdown of p38β mRNA resulted in increased levels of p38α mRNA (Fig. 3A, left panel). Since *DUX4* expression in myoblasts is difficult to measure, we looked at the effects of p38 knockdown on DUX4 target gene expression. Individual knockdown of each isoform or combined knockdown of both resulted in decreased expression of multiple *DUX4* target genes (Fig. 3A, right panel), suggesting that both isoforms are individually required to support *DUX4* mRNA expression. Similar results were obtained using MB200 (FSHD2) myoblasts (Fig. 3B, left and right panels). These results are surprising because instead of having redundant roles, p38α and p38β appear to have independent roles in supporting *DUX4* mRNA synthesis. We extended these experiments to differentiating FSHD myotubes where *DUX4* expression is increased during the differentiation process (Snider et al., 2010; Jones et al., 2012; Krom et al., 2012; Tassin et al., 2013). For these experiments, confluent monolayers of myoblasts were transfected with siRNAs one or two days prior to differentiation in order to allow for efficient depletion of target proteins. Differentiation was then induced for 40 hours until multinucleated myotubes were predominate in the cultures. Similar to results in myoblasts, siRNAs targeting p38α or p38β, individually or in combination, effectively decrease levels of their respective target mRNA in 54-2 (FSHD1) and MB200 (FSHD2) myotubes (Fig. 4A and B, respectively, left panels). Western analysis confirmed that p38α protein levels were decreased only when siRNA targeting p38α was used alone or in combination with siRNA targeting p38β (Suppl. Fig. 2). Similarly, p38β protein levels were decreased only when siRNA targeting p38β was used alone or in combination with siRNA targeting p38α (Suppl. Fig. 2). In myotubes, knockdown of p38β increased the expression of p38α at the mRNA and protein levels, while knockdown of p38α did not significantly

affect p38β levels. Inspection of qPCR cycle times (Cts) indicated that p38β is expressed much less abundantly than p38α in our FSHD myotube cultures at the mRNA level. Nonetheless, individual knockdown of each p38 isoform alone or combined knockdown of both resulted in decreased expression of *DUX4* and multiple DUX4 target genes in FSHD1 and FSHD2 myotubes (Fig. 4A and B, respectively, right panels), although the decrease in *DUX4* did not reach significance with individual knockdown of p38α in 54-2 (FSHD1) myotubes. It is worth noting that knockdown of p38β decreases DUX4 expression even as p38α levels are increased and that combined knockdown of both isoforms does not decrease DUX4 expression below the level generated with knockdown of p38β alone. Additionally, knockdown of the p38γ isoform did not affect DUX4 expression (Suppl. Fig. 3). These combined data in FSHD myoblasts and myotubes suggest that p38α and p38β are both necessary and play non-redundant roles in supporting pathogenic *DUX4* expression in FSHD muscle cells.

p38 Inhibitors are Active in a Xenograft Model of FSHD

To test the ability of clinically advanced p38 inhibitors to suppress *DUX4* expression in vivo, we developed a pharmacology model of human FSHD gene regulation. The primate-specific organization of the *DUX4* gene imbedded within D4Z4 repeats (Clapp et al., 2007; Leidenroth et al., 2012) and the loss of epigenetic repression of *DUX4* within the context of human genetic mutations represent barriers to the development of a transgenic mouse model that recapitulates *DUX4* misexpression in FSHD. In order to most accurately model FSHD epigenetic dysregulation of *DUX4*, we utilized a mouse xenograft approach in immunodeficient mice in which tibialis anterior (TA) muscles are injured by barium chloride injection to induce a regenerative response (Hardy et al., 2016). The regenerating muscle environment is conducive to engraftment of transplanted human myoblasts that respond to cues from the microenvironment, express appropriate markers of differentiation and become part of mature myofibers (Silva-Barbosa et al., 2005). Variations of this model have been used to demonstrate engraftment and differentiation of human FSHD patient-derived myoblasts or muscle biopsies (Chen et al., 2016; Krom et al., 2012; Sakellariou et al.,

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2016). In order to treat FSHD xenograft mice with p38 inhibitors to suppress *DUX4*, it was necessary to determine the time frame of *DUX4* expression after xenotransplantation. Since it is well established that *DUX4* is induced upon differentiation of myoblasts into multinucleated myotubes in vitro in the time frame of 1.5-6 days (Snider et al., 2010; Jones et al., 2012; Krom et al., 2012; Tassin et al., 2013; Balog et al., 2015) and that the corresponding elevated levels of DUX4 protein are sufficient to cause myotube death (Block et al., 2013; Rickard et al., 2015), we profiled *DUX4* expression during the first week after xenotransplantation of MB200 (FSHD2) myoblasts. Total RNA from xenograft mice TA muscles was analyzed using human-specific primers and Taqman probes for *DUX4* and DUX4 target gene RNA levels by qRT-PCR. Supplementary Figure 4 demonstrates that *DUX4* mRNA levels increase over the first few days and peak at about the fourth day after xenotransplantation (left panel). RNA levels for representative DUX4 target gene *MBD3L2* peak at days 5-6 and begin to decline by day 7 (Suppl. Fig. 2, right panel). These data suggest that *DUX4* expression is induced as transplanted FSHD myoblasts begin to differentiate in vivo and that this process peaks within the first week.

We reasoned that treating animals for four days, starting immediately after the xenograft procedure through the peak of *DUX4* expression, was appropriate to evaluate agents intended to suppress *DUX4* expression in FSHD. We treated xenograft mice systemically with two different inhibitors: PH-797804 and losmapimod. Figure 5A shows that treatment of xenograft mice with PH-797804 by subcutaneous injection twice daily resulted in dose-dependent decreases in mRNA levels for *DUX4* (left panel) and DUX4 targets *MBD3L2*, *ZSCAN4* and *LEUTX* (right three panels, respectively). The highest dose of PH-797804 (5 mg/kg body weight) elicited a decrease of approximately 80% for *DUX4* and its targets compared to vehicle. Similarly, treatment of xenograft mice with losmapimod orally twice daily resulted in dose-dependent decreases in *DUX4* and DUX4 target mRNA levels (Fig. 5B). In this case mRNA levels for *DUX4* and its targets were decreased by approximately 80% with losmapimod dosed at 6 mg/kg twice daily and by greater than 90% at a dose of 18 mg/kg twice daily. In preclinical models of inflammation, losmapimod has been efficacious at a dose of 12 mg/kg/day in food (Willette et al., 2009;

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Yeung et al., 2018). In order to understand how suppression of DUX4 was related to plasma drug levels, we measured trough levels of losmapimod in plasma at sacrifice (~14 hour after last dose). Suppl. Fig. 6 shows that terminal (trough) plasma levels of losmapimod in the 6 mg/kg dosing group were still above the in vitro IC_{50} of losmapimod (average of 16 nM in plasma versus an IC_{50} of 2.4 nM in MB200 cells from Table 1).

To further assess the potential utility of losmapimod in FSHD and the effects of p38 inhibitor treatment on the differentiation of transplanted FSHD myoblasts, xenograft mice were treated for fourteen days with losmapimod dosed at 6 mg/kg twice daily. Muscle RNA was analyzed for the late differentiation marker myosin heavy chain (*MYH2*) while terminal plasma samples were used to measure trough drug levels that are reported in Suppl. Fig 6B. Figure 5C shows that compared with the low level present four days after xenotransplantation, levels of *MYH2* RNA increase dramatically in mice treated with vehicle or losmapimod over the period during which fully formed mouse myofibers develop, between 4 days and 14 days after xenotransplantation (left panel) (Hardy et al., 2016). These data indicate that treatment of xenograft mice with losmapimod at a level that represses *DUX4* by 80% is compatible with robust differentiation of the introduced human FSHD myoblasts. Since DUX4 expression during the differentiation process is sufficient to cause cell death (Block et al., 2013; Rickard et al., 2015), we also looked at the survival of human FSHD cells by measuring the human cell DNA copy number relative to mouse cell DNA after 14 days of losmapimod treatment. Figure 5C, right panel, shows that xenograft mice treated with losmapimod had an approximately two-fold increase in human cell copy number relative to mouse. Analysis of the PCR cycle times for the human *TERT* gene suggests an even greater absolute increase in human cell number per xenograft (5.5-fold, Suppl. Fig. 5) with a 2.7-fold increase in mouse cell number per xenograft, though this analysis is semiquantitative. These data suggest that p38 treatment suppresses *DUX4* expression and promotes the survival and incorporation of differentiating human FSHD cells in regenerating mouse muscle.

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Discussion

FSHD is one of the most prevalent muscular dystrophies, yet development of disease-modifying therapies lags far behind that for other dystrophies. The consensus identification of DUX4 protein expression in skeletal muscle as causing the disease has given a clear therapeutic target. Approaches include targeting the *DUX4* mRNA with antisense oligonucleotide (ASO) technology and targeting DUX4 transcriptional activity or the activity of gene products downstream of DUX4 (Himeda et al., 2015; Banerji et al., 2018). These approaches have challenges to overcome, such as muscle delivery for ASO-based therapeutics. Furthermore, there is an incomplete understanding of the pathophysiology of FSHD in relation to the downstream gene targets of DUX4 that mediate its toxic effects. We have focused instead on identifying small molecule drugs capable of suppressing the transcription of *DUX4* mRNA so that no DUX4 protein is produced, an approach that bypasses many of these challenges.

In this study, we demonstrated that *DUX4* mRNA synthesis in FSHD myoblasts and myotubes is exquisitely sensitive to p38α/β inhibitors. The IC_{50}s shown in Table I are in line with published activities determined for isolated p38α or p38β enzymes (Xing, 2015), strongly suggesting that *DUX4* expression is positively regulated by p38α or p38 α/β kinase activity. One desirable characteristic of a drug intended for a degenerative muscular dystrophy is that the treatment not negatively affect the ability of muscle cells to participate in the muscle regenerative process. The suppression of *DUX4* by p38 inhibitors is independent of myocyte differentiation, evidenced by the activity of inhibitors in proliferating myoblasts. Furthermore, in differentiating myotubes *DUX4* is suppressed at drug concentrations that do not affect markers of differentiation. In fact, decreases in markers of muscle differentiation (*MYOG* and *MHY2*) require three orders of magnitude higher drug concentrations than for *DUX4* suppression. Even at high drug concentrations, *MYOG* and *MHY2* decreases are not complete and may be an artifact of in vitro differentiation. Systemic administration of losmapimod was able to decrease DUX4 expression and its downstream targets without decreasing maturation markers after chronic dosing over 14 days.

The sensitivity of *DUX4* in differentiating muscle cells to p38 inhibition at drug concentrations that do not prevent differentiation is surprising given the literature describing the roles of p38 in skeletal muscle. Four p38 isoforms exist in mammals: p38α, p38β, p38γ and p38δ (Cuenda and Rousseau, 2007; Cuadrado and Nebreda, 2010). The α, β and γ isoforms are expressed in skeletal muscle (Wang et al., 2008), and p38α involvement in regulating the skeletal muscle differentiation process is well documented. Activation of p38α/β has been shown to be important in regulating muscle progenitor satellite cell activation from quiescence and re-establishment of the satellite cell pool (Troy et al., 2012). The latter mechanism led to the proposal of using p38 inhibition to help re-establish the muscle stem cell pool in situations where this pool is depleted, such as in aged skeletal muscle (Bernet et al., 2014). p38 has been implicated in key temporally ordered events involving myogenic regulatory transcription factors and the establishment of muscle-specific gene expression (Zetser et al., 1999; Wu et al., 2000; Bergstrom et al., 2002; Penn et al., 2004). Importantly, p38 is involved in several epigenetic mechanisms including recruitment of methyltransferase and nucleosome remodeling complexes to muscle-specific gene promoters (Simone et al., 2004; Rampalli et al., 2007) and inactivation of methyltransferases at specific genes (Chatterjee et al., 2016). In fact, p38α binds to and regulates many promoters during myogenesis (Segales et al., 2016).

So how can inhibition of p38α/β selectively reduce *DUX4* without disrupting muscle differentiation? Several observations suggest this is possible. First, *DUX4* expressions is exquisitely sensitive to p38 inhibition, with much less inhibitor required than in the case of blocking differentiation. For example, SB203580 (Kumar et al., 1997) inhibited *DUX4* in differentiating FSHD myocytes with IC50s of 17 nM (FSHD1) and 9.8 nM (FSHD2), while muscle differentiation gene markers were partially inhibited at IC50s of > 1500 nM (Suppl. Fig 1). Secondly, mice with muscle-specific deletion of p38α grow and function normally, albeit with smaller muscles and delayed myofiber growth and maturation (Brien et al.,

2013). Additionally, muscle-specific p38α knockout mice exhibit an increase in the muscle satellite cell pool, consistent with the previously mentioned role of p38 in regulating satellite cells activation and restricting excess myoblast proliferation (Troy et al., 2012) and the proposal that p38 inhibition may be a therapeutic option in muscle degenerative disorders in which the stem cell niche appears to be diminished (Bernet et al., 2014; Dumont and Rudnicki, 2016). Notably, when we treated xenograft mice for 14 days with the p38 inhibitor losmapimod, the total number of both human and mouse cells increased compared to non-treated animals (Fig. 5 and Suppl. Fig. 5), consistent with p38 inhibition promoting myoblast proliferation even as the expression of a late differentiation marker (*MYH2*) was induced and maintained.

The molecular mechanisms that tie *DUX4* expression to p38 activity remain to be elucidated. A surprise finding from these studies is that DUX4 expression is dependent on both the alpha and beta p38 isoforms (Figs. 3, 4). The result that simultaneously knocking down both isoforms does not suppress DUX4 more than solely knocking down p38β implies that each kinase plays a distinct mechanistic role such that when p38β is knocked down, DUX4 is suppressed even when p38α levels are increased. p38β is generally expressed at lower levels than the other isoforms (Cuadrado and Nebreda, 2010) and appears to be dispensable for muscle differentiation (Perdiguero et al., 2007; Ruiz-Bonilla et al., 2008). This offers an opportunity to explore the utility of p38 isoform-selective inhibitors to balance efficacy and safety. Due to its role in inflammation (Allen et al., 2000), previous efforts have focused on developing p38α inhibitors for various inflammatory diseases (Kumar et al., 2003). Most of these inhibitors also inhibit p38β due to the high sequence similarity between the closely related proteins (Kondoh et al., 2016), and no p38β-selective inhibitors have been described. Given the roles of p38α in muscle differentiation as well as its role in inflammation (Page et al., 2010), it is worth pursuing the development of p38β-selective inhibitors. Interestingly, p38β inhibition has independently been suggested as therapeutic strategy for muscle cachexia (Ding et al., 2017).

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Since DUX4 is a transcription factor that is likely regulated by post-translational modifications, it was important to determine if p38 inhibition had any effect on the transcriptional activation function of DUX4. We used the p300 acetyltransferase activity inhibitor A-485 as a control since DUX4 has been reported to interact with p300 to activate target genes (Choi et al., 2016). Indeed, A-485 inhibited induction of DUX4 targets *MBD3L2* and *LEUTX*. Interestingly, *ZSCAN4* RNA levels were not decreased by A-485, suggesting that this DUX4 target is induced independent of p300 acetyltransferase activity even though DUX4 has been shown to induce p300-dependent acetylation of histones in vicinity of the *ZSCAN4* DUX4 binding sites (Choi et al., 2016). The significance of this finding remains to be explored. In the case of p38 inhibitors, there are minor and inconsistent decreases in some DUX4 targets by p38 inhibitors. It is difficult to conclude from these data if p38 plays any role in DUX4 activity or if the small decreases are assay artifacts due to potential interference of cytomegalovirus promoter by p38 inhibitors (Bruening et al., 1998). Nonetheless, high concentrations of p38 inhibitors do not block DUX4 activity and the primary effect of p38 inhibition in FSHD cells is to suppress the synthesis of *DUX4* mRNA.

p38 inhibitors suppress the expression of DUX4 and its targets at clinically relevant doses. It is clear from in vivo testing of two clinically advanced inhibitors that p38 inhibition effectively reduces the peak levels of *DUX4* expression in a dose dependent manner (Fig. 5). The doses of 5 mg/kg b.i.d. for PH-797804 and 6 mg/kg b.i.d. for losmapimod reduce *DUX4* and DUX4 targets RNA levels approximately 80 percent. These dosing levels have been used in translational animal models (Willette et al., 2009; Xing et al., 2012). Losmapimod has been extensively characterized in humans. Efficacy and safety studies have used repeated dosing with losmapimod at 7.5 mg, twice daily (O'Donoghue et al., 2016; Fisk et al., 2018). A detailed study of drug exposure demonstrated a stable plasma trough concentration of around 16 ng/ml (39 nM) (Ino et al., 2015). Losmapimod was efficacious in our xenograft mouse model with a terminal drug concentration in the plasma of 16 nM. These data suggest there is sufficient muscle exposure by dosing losmapimod at 7.5 mg b.i.d. to substantially reduce DUX4 in FSHD patient muscle. A more

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detailed analysis of pharmacokinetic and pharmacodynamic relationships, specifically in muscle tissue, will enable accurate prediction of human dose requirements.

To conclude, data presented here support the repurposing of existing p38α or dual p38α/β inhibitors as potential therapeutics to suppress *DUX4* expression in FSHD and suggests the development of p38β-selective inhibitors to perhaps more specifically target *DUX4*. These studies uncover the exciting possibility of a disease-modifying treatment for FSHD by targeting the root cause of disease through p38 MAPK inhibition.

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Authorship Contributions

Participated in research design: Oliva, Galasinski, Campbell, Meyers, Tapscott, Sverdrup

Conducted experiments: Oliva, Richie, Campbell, Modi, Zhong, Sverdrup

Contributed new reagents or analytic tools: Tawil

Performed data analysis: Oliva, Richie, Campbell, Sverdrup

Wrote or contributed to writing of the manuscript: Oliva, Galasinski, Campbell, Tapscott, Sverdrup

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Figure Legends

Figure 1. p38 inhibitor PH-797804 reduces *DUX4* and DUX4 target gene expression in FSHD patient-derived proliferating myoblasts and differentiating myotubes. A. PH-797804 concentration-response curve. Differentiating MB200 (FSHD2) cells were treated with PH-797804 in an 11-point dilution series for 40 hours. RNA levels for DUX4 target *MBD3L2* and differentiation markers *MYOG* and *MYH2* were determined in cell lysates by qRT-PCR. B. Ph-797804 in FSHD myoblasts. Proliferating cultures of 54-2 (FSHD1) and MB200 (FSHD2) myoblasts were treated with 100 nM PH-797804 for 72 hours. RNA was isolated and analyzed for DUX4 targets *MBD3L2*, *ZSCAN4* and *LEUTX*. C and D. Differentiating cultures of 54-2 (FSHD1) and MB200 (FSHD2) muscle cells were treated with varying concentrations of PH-797804, as indicated, for 40 hours and the cultures analyzed for *DUX4*, DUX4 target (*MBD3L2*, *ZSCAN4* and *LEUTX*) and differentiation marker (*MYOG* and *MYH2*) RNA levels by qRT-PCR. Data are expressed as relative expression (means with standard deviations) with the expression in absence of inhibitor set to one. *, $P < 0.01$ versus Control (unpaired, two-tailed t test). **, $P < 0.01$ versus Control (one-way ANOVA with Dunnett's post test).

Figure 2. DUX4 activity: p38 inhibitors do not block DUX4 transcriptional activation function. Non-DUX4 expressing 54-6 (normal) myoblasts were transfected with a DUX4 expression plasmid and treated with varying concentrations of control p300 inhibitor A-485 (A), p38 inhibitor PH-797804 (B) or p38 inhibitor pamapimod (C), as indicated, for 20 hours. Cultures were analyzed for DUX4 targets *MBD3L2*, *ZSCAN4*, and *LEUTX* RNA levels by qRT-PCR. Data are expressed as relative expression with the expression in absence of inhibitor set to 100.

Figure 3. p38 siRNAs suppress *DUX4* in FSHD myoblasts. 54-2 (FSHD) (A.) or MB200 (FSHD2) (B.) myoblasts were transfected with siRNAs targeting p38α (si-p38α), p38β (si-p38β) or control siRNAs (si-CTRL) twice starting 72 hours before harvest and again 24 hours before harvest to ensure efficient knockdown. Myoblasts were maintained in growth media before RNA was isolated and analyzed for p38

(left panels) and DUX4 targets *MBD3L2, ZSCAN4 and LEUTX* (right panels) expression levels. Data are expressed as relative expression (means with standard deviations) with the expression in the presence of si-CTRL set to one. *, $P < 0.01$ versus si-CTRL (one-way ANOVA with Dunnett's post test).

Figure 4. p38 siRNAs suppress *DUX4* in FSHD myotubes. A. 54-2 (FSHD1) myoblasts were transfected with siRNA targeting p38α (si-p38α), p38β (si-p38β) or control (si-CTRL) siRNAs, as indicated, 24 hours prior to induction of differentiation. Cells were induced to differentiate and harvested 40 h later. RNA was isolated and analyzed for p38 (left panel) and *DUX4, MBD3L2, ZSCAN4 and LEUTX* (right panel) expression levels. B. MB200 (FSHD2) myoblasts were transfected with the indicated siRNAs on two consecutive days prior to inducing differentiation. Twenty-four hours after the second transfection, cells were induced to differentiate and RNA was isolated 40 hours later and analyzed as in A. Data are expressed as relative expression (means with standard deviations) with the expression in the presence of si-CTRL set to one. *, $P < 0.01$ versus si-CTRL (one-way ANOVA with Dunnett's post test).

Figure 5. p38 inhibitors suppress DUX4 expression in a mouse xenograft pharmacology model of FSHD gene regulation. A. PH-797804 was administered to xenograft mice twice daily by subcutaneous injections at the indicated doses for four days. RNA was isolated from excised TA muscles and analyze for *DUX4*, *MBD3L2*, *ZSCAN4* and *LEUTX* RNA levels. B. Losmapimod was administered orally to xenograft mice twice daily at the indicated doses for four days. RNA was isolated and analyzed as in A. C. Losmapimod was administered orally to xenograft mice twice daily at 6 mg/kg for 14 days. RNA and DNA were isolated from excised TA muscles and analyzed for differentiation marker *MYH2* RNA levels and 2-copy genes h*TERT* and mTfrc DNA levels. Data are expressed as relative expression (means with standard errors) with the expression in the Vehicle groups set to one For *MYH2* analysis, the 14 day levels were compared to levels in 4 day animals (Day 4) not treated with drugs, which was set to one. *, $P < 0.01$ versus Vehicle (one-way ANOVA with Dunnett's post test). #, $P = 0.030$ versus Vehicle (unpaired two-tailed t test).

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Tables

Table I. Potency of Inhibitors in differentiating cultures of FSHD1 and FHD2 muscle cells.

Inhibitor	Mechanism/Selectivity	FSHD1 EC_{50} (nanomolar)	FSHD2 EC_{50} (nanomolar)	Clinical Advancement
Acumapimod (BCT197)	p38α/β	13	6.3	Phs II, Active
ARRY-614 (Pexmetinib)	p38/Tie2	0.33	0.18	Phs I, Discontinued
BMS-582949	p38α, 5X selective over p38β	17	4.2	Phs II, Discontinued
Doramapimod (BIRB796)	p38α/β/γ/δ	0.83	4.6	Phs II, Discontinued
Losmapimod (GW856553)	p38α/β	14	2.4	Phs III, Discontinued
Neflamapimod (VX-745)	p38α, 22X selective over p38β	12	13	Phs II, Active
Ralimetinib (LY2228820)	p38α/β JNK2, JNK3 > JNK1	4.0	1.6	Phs II, Discontinued
Pamapimod (RO4402257)	P38α, 34X selective over p38β	5.5	2.2	Phs II, Discontinued
PH-797804	p38α, 4X selective over p38β	0.15	0.41	Phs II, Discontinued
SB202190	p38α/β	6.0	5.2	Tool Compound
SB203580	p38α/β	17	9.5	Tool Compound
TAK-715	p38α, 28X selective over p38β	4.4	12	Phs II, Discontinued
Talmapimod (SCIO 469)	p38α, 10X selective over p38β	1.6	5.0	Phs II, Discontinued
VX-702	p38α, 14X selective over p38β	4.1	14	Phs II, Discontinued
Tomivosertib (eFT-508)	MKNK1/2 (MNK1/2)	>10,000	>10,000	Phs II, Active
PF-3644022	MAPKAPK2/3 (MK2/3)	>10,000	>10,000	Tool Compound
MK2-IN-1	MAPKAPK2 (MK2)	>10,000	>10,000	Tool Compound

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FIGURES

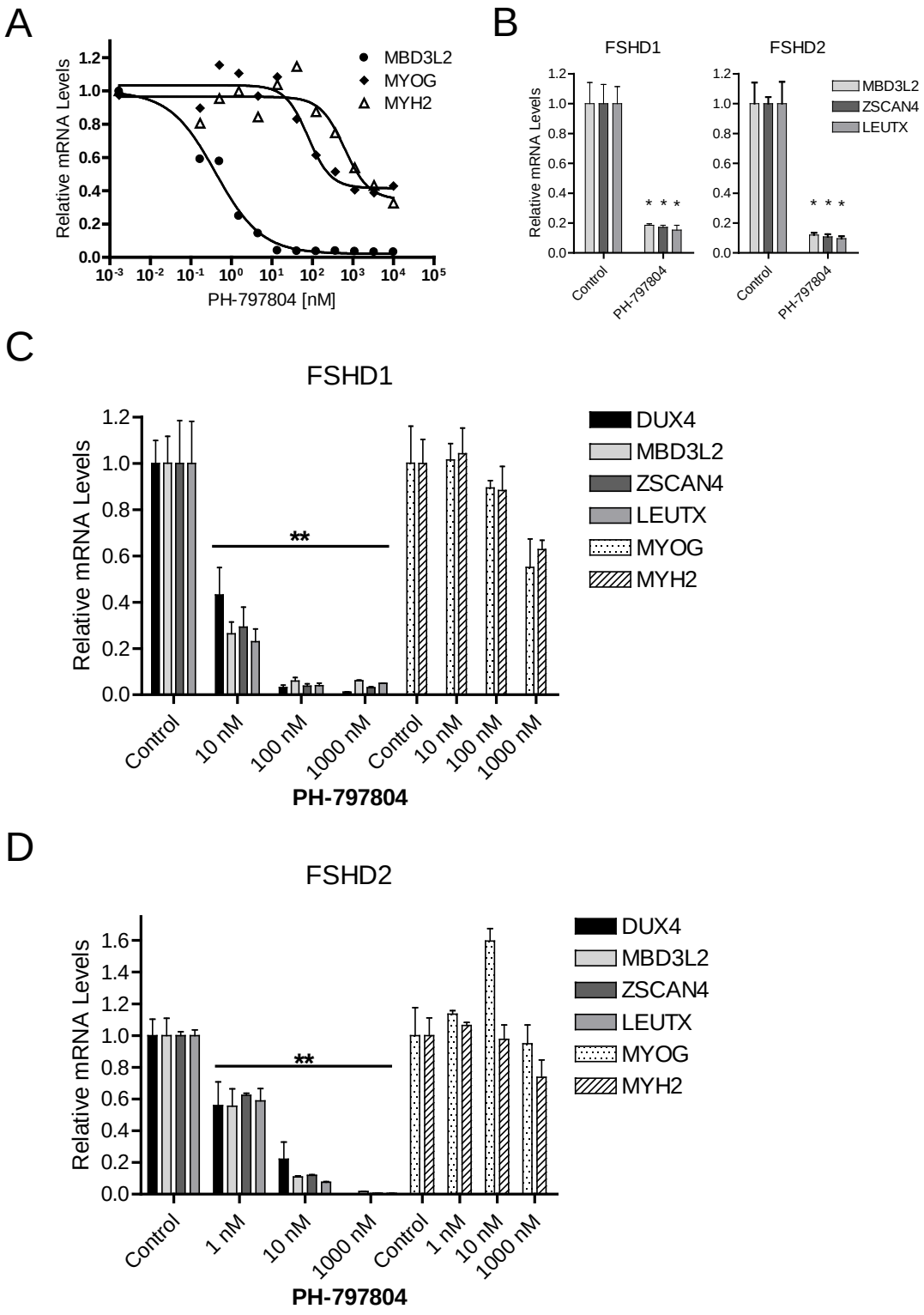

Figure 1.

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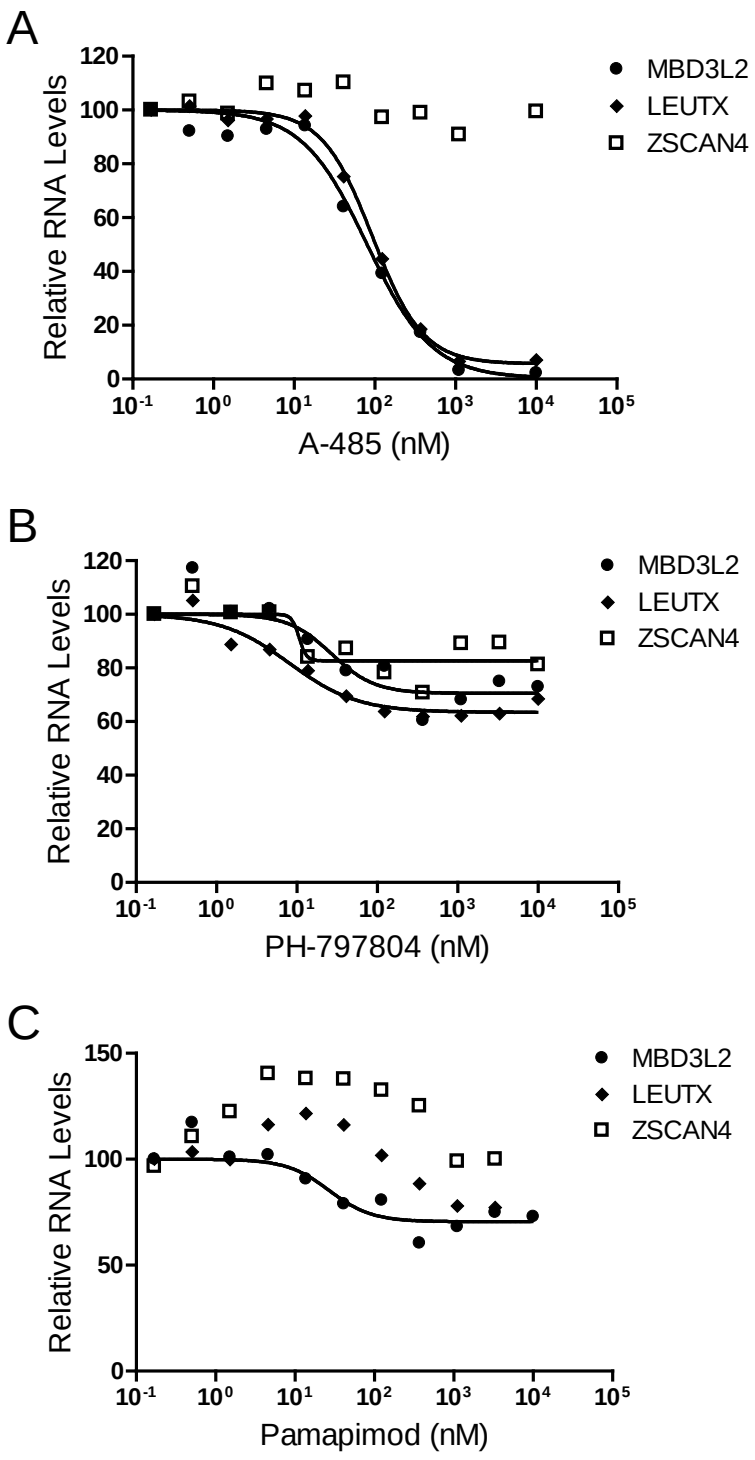

Figure 2.

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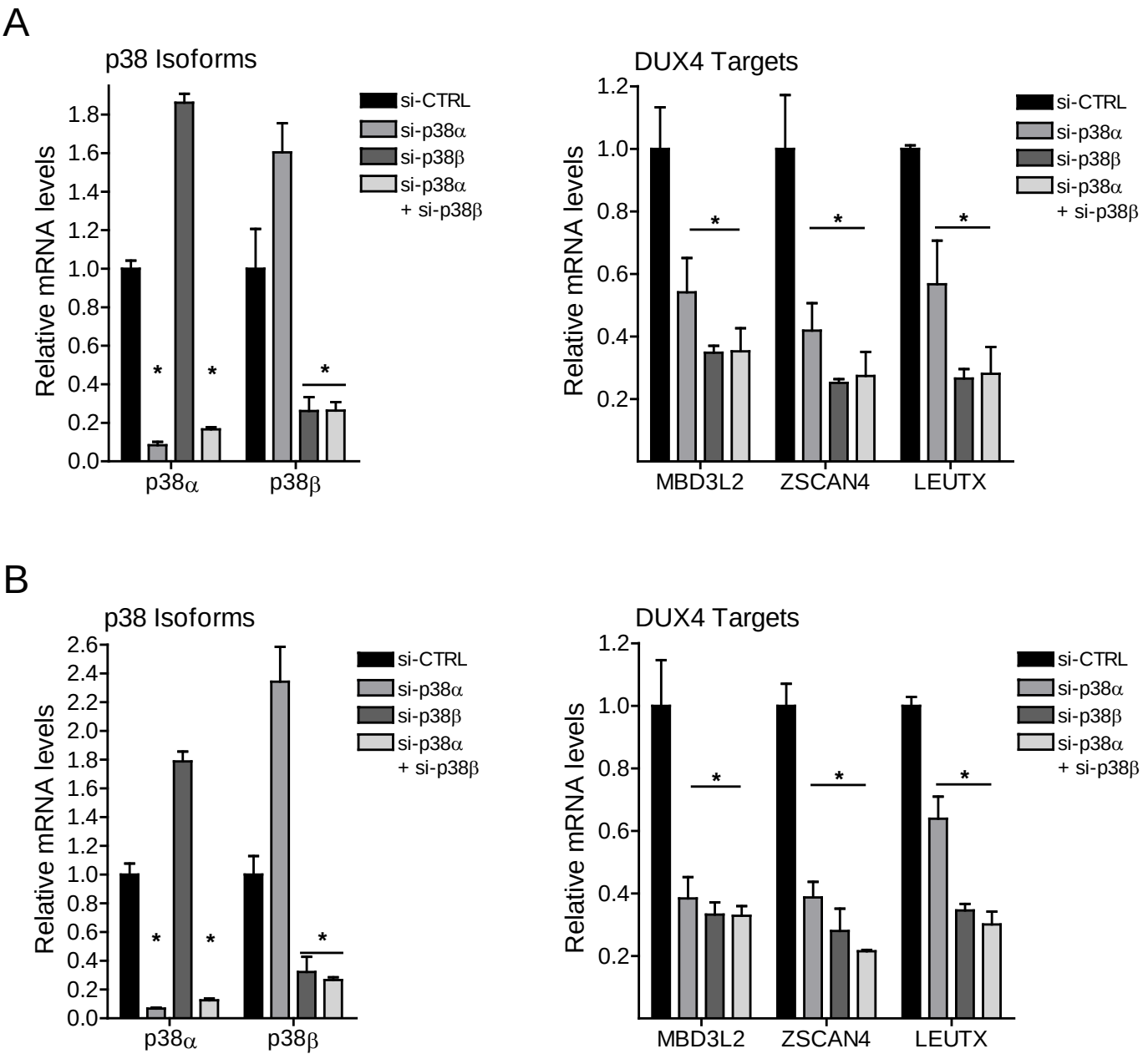

Figure 3.

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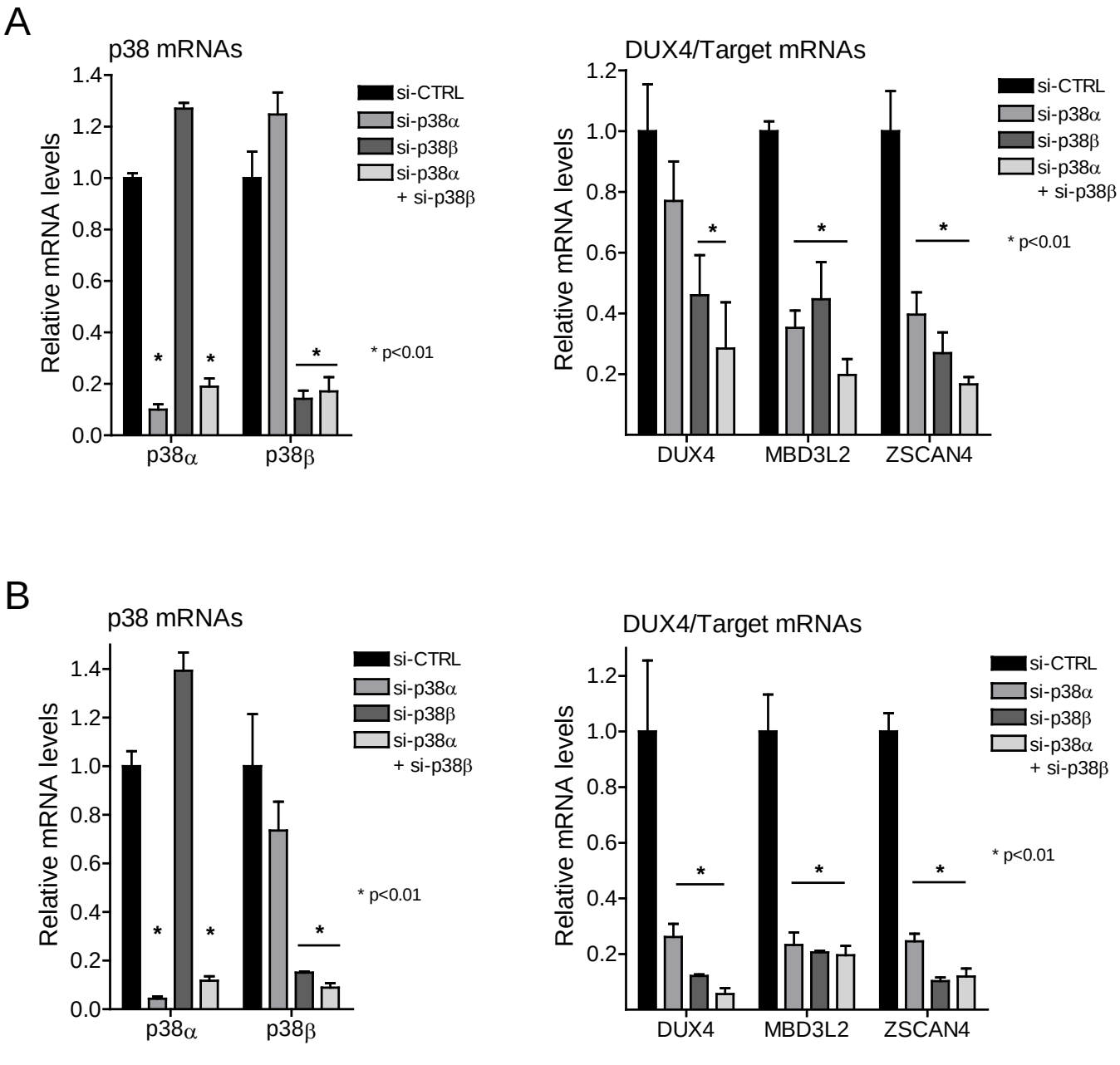

Figure 4.

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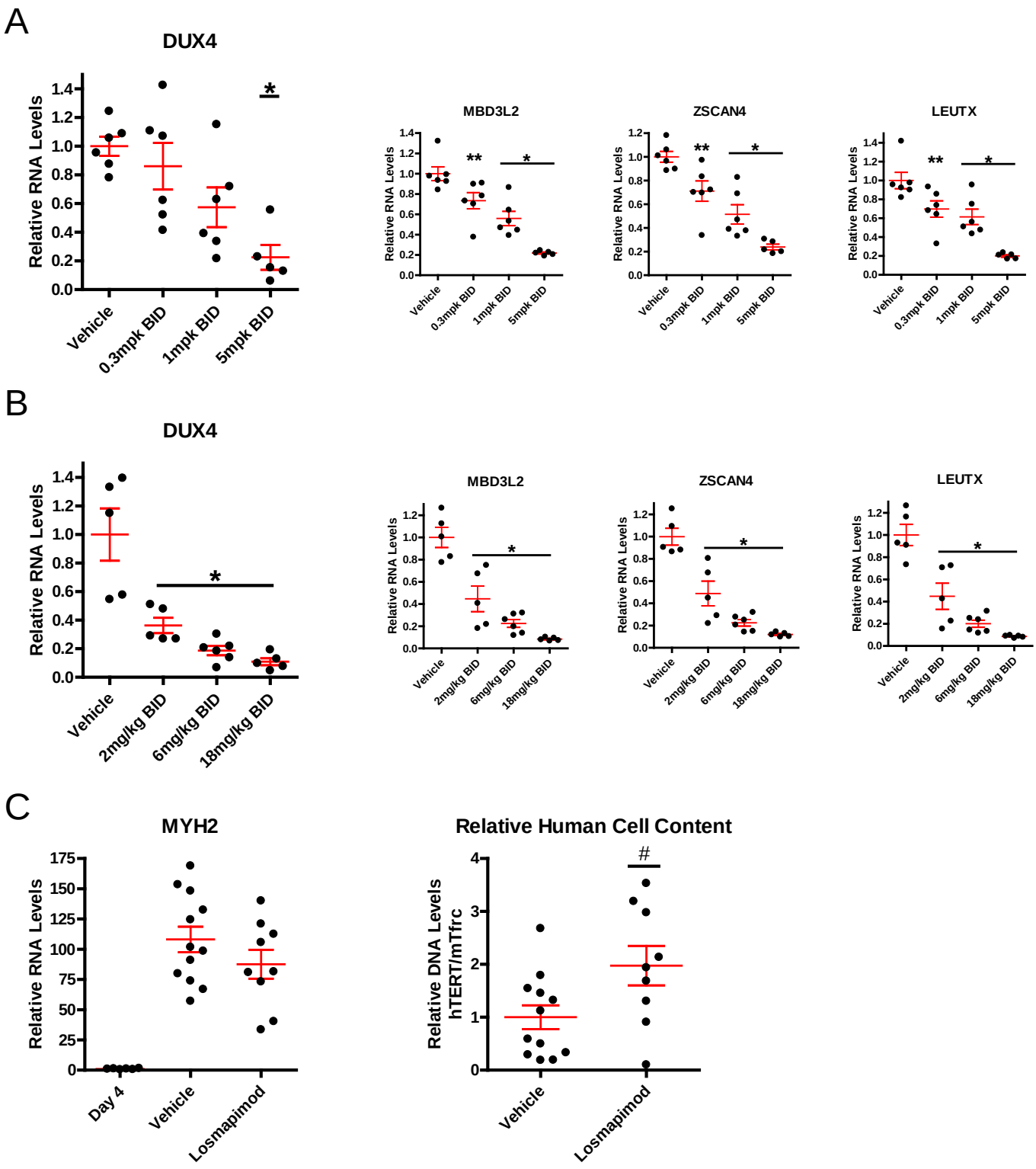

Figure 5.