Post-Translational Modifications of the DUX4 Protein Impact Toxic Function in FSHD Cell Models

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Objective: Facioscapulohumeral muscular dystrophy (FSHD) is caused by abnormal de-repression of the myotoxic transcription factor DUX4. Although the transcriptional targets of DUX4 are known, the regulation of DUX4 protein and the molecular consequences of this regulation are unclear. Here, we used in vitro models of FSHD to identify and characterize DUX4 post-translational modifications (PTMs) and their impact on the toxic function of DUX4.

Methods: We immunoprecipitated DUX4 protein and performed mass spectrometry to identify PTMs. We then characterized DUX4 PTMs and potential enzyme modifiers using mutagenesis, proteomics, and biochemical assays in HEK293 and human myoblast cell lines.

Results: We identified 17 DUX4 amino acids with PTMs, and generated 55 DUX4 mutants designed to prevent or mimic PTMs. Five mutants protected cells against DUX4-mediated toxicity and reduced the ability of DUX4 to transactivate FSHD biomarkers. These mutagenesis results suggested that DUX4 toxicity could be counteracted by serine/threonine phosphorylation and/or inhibition of arginine methylation. We therefore sought to identify modifying enzymes that could play a role in regulating DUX4 PTMs. We found several enzymes capable of modifying DUX4 protein in vitro, and confirmed that protein kinase A (PKA) and protein arginine methyltransferase (PRMT1) interact with DUX4.

Interpretation: These results support that DUX4 is regulated by PTMs and set a foundation for developing FSHD drug screens based mechanistically on DUX4 PTMs and modifying enzymes.

Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is among the most common forms of muscular dystrophy, affecting an estimated 870,000 people worldwide. FSHD is typically characterized by slowly progressive, asymmetric weakness affecting the muscles of the face, shoulders, trunk, and extremities. FSHD is associated with significant disability that may lead to wheelchair dependence.1 Most patients present...
in late adolescence and early adulthood; however, rare early-onset forms show greater disease severity and systemic manifestations. Unfortunately, there are no approved disease-modifying treatments and care remains supportive.

In the simplest terms, FSHD is caused by abnormal de-repression of the DUX4 gene in the skeletal muscle. DUX4 produces a double homeodomain transcription factor (DUX4) that normally operates in early embryogenesis and is repressed in adult tissues except the thymus, testes, and possibly skin. When expressed in the skeletal muscle, DUX4 activates numerous pathways that are incompatible with normal muscle function, including oxidative stress and apoptosis, among others. The DUX4 open reading frame (ORF) is embedded within D4Z4 macrosatellite repeats located on the human chromosome 4q35. In non-FSHD muscle, this region is typically hypermethylated and embedded in heterochromatin, and DUX4 is not expressed. In FSHD, shorter D4Z4 arrays (FSHD1) or mutations in chromatin modifier genes (FSHD2) lead to 4q DNA hypermethylation, enabling transcription of DUX4. If this occurs on a permissive allele (4q4), containing a polyA signal for DUX4, the DUX4 mRNA arising from the most distal telomeric D4Z4 repeat is polyadenylated, stabilized, and translated into toxic DUX4 protein.

Since the discovery that DUX4 is the causative gene in FSHD, there has been great interest in developing DUX4 inhibition therapies. Because DUX4 protein activates toxic pathways, arguably the most effective DUX4 inhibition strategies will silence DUX4 at the DNA or RNA levels, thereby avoiding protein production. Indeed, several approaches to accomplish DUX4 DNA or mRNA silencing have been previously described, including by our laboratory. These strategies, although promising, offer no remedy for inhibiting DUX4 after protein has already been produced. To date, no one has yet described an approach to directly interfere with the ability of DUX4 protein to transactivate downstream genes. This was the overall goal of the current study.

To develop DUX4 protein inhibitors, it is critical to understand how DUX4 protein functions and is regulated. Previous studies have defined domains within the DUX4 protein that are required for DUX4 transactivation activity and toxicity, including 2 N-terminal DNA binding homeodomains, nuclear localization signals, and a C-terminal transactivation domain that recruits histone acetyltransferases (p300/CBP) to target genes. Once incorporated into a protein, primary amino acids can be modified at the post-translational level to regulate and alter protein function. These post-translational modifications (PTMs) link gene regulation machinery with signal transduction pathways and are known to impact the function, stability, and subcellular localization of transcription factors. We hypothesized that the DUX4 transcription factor was regulated by PTMs and that prospective modifications and the enzymes that add or remove them could be targets for DUX4 inhibition strategies.

Materials and Methods
Mutagenesis
DUX4 modification mutant plasmids were constructed using a recombinant polymerase chain reaction (PCR) method. Mutants were constructed using PCR to amplify the DUX4 ORF, with primers containing the mutated site, using CMV-driven wildtype DUX4 plasmid (AAV.DUX4.V5) as the PCR template. The entire mutant DUX4 ORF was amplified, gel purified, and cloned into PCR-blunt II-Topo, prior to sequence verification. DUX4 mutant ORFs were then cloned into an AAV proviral plasmid. Phospho-null, Phospho-mimic, Methyl-null, basic, and Methyl-mimic mutants were synthesized by Genscript with N-terminal Nhel and C-terminal Acc65I restriction enzyme sites flanking the DUX4 ORF, and then subcloned into AAV proviral plasmid.

Cell Culture
Human embryonic kidney cells (HEK293) and human immortalized myoblasts (WS236, 15 V, biceps, unaffected control cells, ix DUX4 cells) were maintained as previously described.

Briefly, HEK293 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin at 37°C in 5% CO2. Human immortalized myoblasts were cultured in LHCN media containing DMEM supplemented with 16% Medium 199, 15% fetal bovine serum, 30 ng/ml zinc sulfate, 1.4 μg/ml vitamin B12, 55 ng/ml dexamethasone, 2.5 μg/ml human growth factor, 10 ng/ml fibroblast growth factor, 20 mM HEPES, and penicillin/streptomycin. The iDUX4 human immortalized myoblasts were cultured in F10 medium supplemented with 20% fetal bovine serum, 10 ng bFGF, 1 μM dexamethasone, and penicillin/streptomycin.

Protein Immunoprecipitation
HEK293 cells were transfected with a total of 4 μg AAV.CMV, DUX4.V5, PRMT1-GFP, or PRKACA-Flag (1 x 10^6 cells/well) using Lipofectamine 2000 and harvested 16 hours later using cold 1 x phosphate-buffered saline (PBS). Cells were pelleted and lysed in buffer A containing 137 mM NaCl, 50 mM Tris, pH 7.5, 1% NP-40, protease inhibitor cocktail (Sigma), and phosphatase inhibitor (Roche). All steps were performed at 4°C or on ice. Lysate was incubated with protein agarose G for 1 hour while rotating. The supernatant was subsequently incubated with anti-V5 antibody conjugated to agarose resin overnight while rotating. The resin was washed 5 times with buffer A, then resuspended in buffer A supplemented with 1 mM DTT and 1 x LDS-PAGE sample buffer (Invitrogen). DUX4 complexes were eluted by boiling at 95°C for 10 minutes.
Liquid Chromatography and Mass Spectrometry
Immunoprecipitated samples were loaded into a TGX 4 to 15% precast gel (Bio-Rad), resolved and stained with Bio-Safe Coomassie (Bio-Rad). The DUX4 band was excised and disulfide bonds reduced and alkylated. Bands were digested overnight at 37°C with 800 ng of trypsin (Promega) and/or chymotrypsin (Promega) in 100 mM ammonium bicarbonate (Sigma). Peptides were extracted from the gel, dried, and resuspended in loading buffer (2% acetonitrile and 0.1% formic acid). Peptides were separated on a Thermo Dionex UltiMate 3,000 RSLC high-performance liquid chromatography (HPLC) coupled to a Thermo Orbitrap Fusion Tribrid mass spectrometer. Peptides were separated on a PepMap100 C18 microcolumn (5 μm, 100 Å, 0.3 × 50 mm) using a linear gradient 5% to 30% of acetonitrile in water with 0.1% formic acid. The tandem mass spectrometry (MS/MS) data were collected using data dependent acquisition mode.

Mass Spectrometry Data Analysis
Raw data were converted to the mzML format using the MSConvert tool in ProteoWizard (version 3.0.4624) and searched against a database containing the DUX4 sequence (UniProt accession Q9UBX2) with a C-terminal V5 epitope tag (GKPIPNPLLGLDST) and common contaminant proteins (downloaded June 22, 2015; 234 total entries) using the MassMatrix search engine version 2.4.2 and MASCOT (version 2.6.0; Matrix Science). Peptide mass tolerance was set at 20 ppm with a fragment mass tolerance of 0.02 Da. against UniProt human database containing the DUX4 sequence (UniProt accession Q9UBX2) with a C-terminal V5 epitope tag (GKPIPNPLLGLDST). Peptide mass tolerance was set at 10 ppm with a fragment mass tolerance of 0.05 Da. Variable modifications included acetylation of K; mono-, di-, or tri-methylation of K; mono- or di-methylation of R; oxidation of M; and phosphorylation of S,T,Y. Carbamidomethylation of C was included as a fixed modification. Enzyme specificity was set for trypsin and chymotrypsin with up to 4 missed cleavages.

Quantitative PCR
HEK293 cells and human myoblasts (5 × 10^5 cells/well) were transfected with 2 μg of AAV.DUX4.V5 (wild type or mutant construct) using Lipofectamine 2000 (Invitrogen) and the
Human Dermal Fibroblast Nucleofector kit (Amaxa), respectively, according to the manufacturer’s instructions. Cells were harvested 24 hours post-transfection in TRIzol RNA isolation reagent (Life Technologies). RNA was isolated, DNase treated, and reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan gene expression assays (Applied Biosystems) were used to quantify human RPL13A, human ZSCAN4, human TRIM43, and human PRAMEF12. Efficiencies were comparable among all probes. RPL13A was used as the reference gene for normalization. The normalized expression ($\Delta\Delta C_q$) was calculated relative to control pClneo transfected cells.

**Immunofluorescence**

HEK293 cells (187,500 cells/well) were transfected in suspension with 800 ng AAV.DUX4.V5, AAV.DUX4 mutant constructs, or pClneo (transfection control plasmid) using Lipofectamine 2000 reagent (Thermo Scientific). Cells were fixed using 4% paraformaldehyde (PFA) 20 hours after transfection, then permeabilized with 0.25% Triton X-100 in 1× PBS. Cells were then blocked using 3% bovine serum albumin (BSA) for 1 hour and incubated with anti-V5 primary antibody (1:2000; Abcam) in 1× PBS and 10% normal goat serum overnight at 4°C. Next, the cells were incubated with Alexa-594 coupled goat anti-rabbit immunoglobulin G (IgG) secondary antibodies (1:5000; Molecular Probes) in 1× PBS and 10% normal goat serum overnight at 4°C. The slides were mounted in Vectashield (Vector Laboratories) with DAPI.

**Western Blot Protein Visualization**

HEK293 cells (65,000 cells/well) were transfected in suspension with 100 ng AAV.DUX4.V5, AAV.DUX4 mutant constructs.V5 or pClneo (transfection control plasmid) using Lipofectamine 2000 reagent (Invitrogen). Human myoblasts (500,000 cells/well) were transfected with 2.5 μg of the same constructs using Lipofectamine 3,000 reagent (Invitrogen). All cells were harvested 24 hours post-transfection in RIPA buffer containing 10 mM Tris-Ci (pH 8.0), 1% Triton X-100, 0.1% sodium deoxycholate, 1% SDS, 140 mM NaCl, and 1 mM DTT. Total protein was quantified using the Lowry Protein Assay (Bio-Rad) and analyzed by 12% SDS-PAGE. Protein was visualized by Western blot using an anti-V5-HRP antibody (Invitrogen).

**In Vitro Kinase Screen**

A radiometric protein kinase filter-binding assay was used for measuring the kinase activity of the 245 protein kinases with recombinant DUX4 protein (Reaction Biology). The reaction cocktails containing kinase solution and buffer/ATP/test sample mixture, were pipetted into 96-well, V-shaped polypropylene microtiter plates. The reaction cocktails contained 60 mM HEPS-NaOH, pH 7.5, 3 mM MnCl$_2$, 3 μM Naorthovanadate, 1.2 mM DTT, 50 μg/ml PEG$_{2000}$, 1 μM ATP/[γ-32P]-ATP, protein kinase (1–400 ng/50 μl), and DUX4 protein (5 μg/50 μl) with some reactions supplemented with CaCl$_2$, EDTA, phosphatidylserine, 1,2-Dioleyl-glycerol, cGMP, or calmodulin as needed. The assays were incubated at 30°C for 60 minutes. The reaction cocktails were stopped with 10% (v/v) H$_3$PO$_4$ transferred into 96-well glass-filter plates.

### TABLE 1. DUX4 Peptides Containing PTMs

<table>
<thead>
<tr>
<th>AA</th>
<th>Peptide</th>
<th>Site</th>
<th>PTM</th>
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<tbody>
<tr>
<td>24–35</td>
<td>LVWTPSQSEALR</td>
<td>S29</td>
<td>Phosphorylation</td>
</tr>
<tr>
<td>24–35</td>
<td>LVWTPSQSEALR</td>
<td>R35</td>
<td>Monomethylation</td>
</tr>
<tr>
<td>24–35</td>
<td>LVWTPSQSEALR</td>
<td>R35</td>
<td>Dimethylation</td>
</tr>
<tr>
<td>52–62</td>
<td>LAQAIGIPEPR</td>
<td>R62</td>
<td>Monomethylation</td>
</tr>
<tr>
<td>52–62</td>
<td>LAQAIGIPEPR</td>
<td>R62</td>
<td>Dimethylation</td>
</tr>
<tr>
<td>63–71</td>
<td>VQIWFQNER</td>
<td>R71</td>
<td>Monomethylation</td>
</tr>
<tr>
<td>63–71</td>
<td>VQIWFQNER</td>
<td>R71</td>
<td>Dimethylation</td>
</tr>
<tr>
<td>99–111</td>
<td>TAVTGSQTALLLR</td>
<td>S104</td>
<td>Phosphorylation</td>
</tr>
<tr>
<td>130–137</td>
<td>ETGLPESR</td>
<td>R137</td>
<td>Monomethylation</td>
</tr>
<tr>
<td>214–236</td>
<td>AAPALQPSQAAPAEGISQPAPAR</td>
<td>R236</td>
<td>Monomethylation</td>
</tr>
<tr>
<td>259–267</td>
<td>WPPHPGKSR</td>
<td>K265</td>
<td>Acetylation</td>
</tr>
</tbody>
</table>

*Note: The sequences identified by mass spectrometry are listed with the amino acid residues, peptide sequence, site, and type of PTM. Amino acids in boldface and underline represent PTM sites.*

*Abbreviation: PTM = post-translational modification.*
DUX4-Activated Reporter in Cells

HEK293 cells (65,000 cells/well) were transfected with 100 ng plasmid DNA (AAV.CMV.DUX4.V5 or mutants) and 100 ng pLenti.DUX4-activated GFP, using Lipofectamine 2000 (ThermoFisher), and plated simultaneously on 96-well plates. GFP expression was quantified 24 and 48 hours post-transfection using the SPECTRAmax M2 instrument (Molecular Devices). GFP expression was visually monitored with a fluorescent stereomicroscope (Leica M165 FC microscope; Leica Microsystems).

Rapid Immunoprecipitation Mass Spectrometry of Endogenous Proteins

The Rapid Immunoprecipitation Mass Spectrometry of Endogenous (RIPE) assay was performed as previously described. Briefly, cells were fixed with 1% formaldehyde for 8 minutes and quenched with 0.125 M glycine. Chromatin was isolated by the addition of lysis buffer, followed by disruption with a Dounce homogenizer. Lysates were sonicated and the DNA sheared to an average length of 300 to 500 base-pairs. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a NanoDrop spectrophotometer. An aliquot of chromatin (100 μg) was pre-cleared with protein G agarose beads (Invitrogen). Proteins of interest were immunoprecipitated using 10 μg of anti-V5 (Abcam) and protein G magnetic beads. Protein complexes were washed, then trypsin was used to remove the immunoprecipitate from the beads, and the peptides were extracted with 1% formic acid and processed for mass spectrometry.

Table 3. Mutagenesis Strategy for DUX4 Amino Acids

<table>
<thead>
<tr>
<th>Modified amino acid</th>
<th>Null substitution</th>
<th>Mimic substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl-Arg</td>
<td>2 nulls per residue</td>
<td>1 methyl mimic per residue</td>
</tr>
<tr>
<td>Arg to Ala</td>
<td>Arg to Leu</td>
<td></td>
</tr>
<tr>
<td>Arg to Lys</td>
<td>(maintains + charge)</td>
<td></td>
</tr>
<tr>
<td>Phospho-Ser/Thr</td>
<td>1 null per residue</td>
<td>1 phospho mimic per residue</td>
</tr>
<tr>
<td>Ser/Thr to Ala</td>
<td>Ser/Thr to Glu or Asp</td>
<td></td>
</tr>
<tr>
<td>Acetyl-Lys</td>
<td>1 null per residue</td>
<td>1 acetyl mimic per residue</td>
</tr>
<tr>
<td>Lys to Ala</td>
<td>Lys to Gln</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Ala = alanine; Arg = arginine; Asp = aspartic acid; Gln = glutamine; Glu = glutamic acid; Leu = leucine; Lys = lysine; Ser = serine; Thr = threonine.
FIGURE 2: Identification of DUX4 PTM mutants that protect against apoptotic cell death. HEK293 cells were transfected with plasmids expressing wild type DUX4 or the indicated DUX4 PTM mutants, and caspase 3/7 assay performed 48 hours later to assess cell death. Representative data for (A) arginine methylation mutants, (B, C) phosphorylation mutants and (D) acetylation mutants (D). Asterisk (*) indicates significant differences compared to DUX4-WT. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. One-way analysis of variance (ANOVA) test. Representative data for 3 to 4 independent experiments performed in triplicate. Dashed line indicates 50% of DUX4-WT caspase-3/7 activity. RFU = relative fluorescence units.
digested the protein sample. Protein digests were separated from the beads and purified using a C18 spin column (Harvard Apparatus). The peptides were vacuum dried using a speedvac. Digested peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC–MS/MS) on a Thermo Scientific Q Exactive Orbitrap Mass spectrometer in conjunction with a Proxeon Easy-nLC II HPLC (Thermo Scientific) and Proxeon nanospray source. All MS/MS samples were analyzed using X! Tandem (The GPM, thegpm.org; version CYCLONE 2013.02.01.1). Scaffold (version Scaffold_4.6.1, Proteome Software) was used to validate MS/MS based peptide and protein identifications.

**Statistical Analysis**

All statistical analyses (Apo-ONE Homogeneous Caspase-3/7 Assay, Q-PCR, DUX4-activated GFP Reporter Assay) were performed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA) using the indicated tests.

**Results**

To detect DUX4 PTMs, we transfected a V5-epitope tagged DUX4 expression plasmid in HEK293 cells, immunoprecipitated DUX4 with a V5 antibody and performed mass spectrometry (MS). We detected the DUX4 protein migrating near the expected molecular weight of 52 kDa by SDS-Page (Fig 1A). Following in-gel digestion, we used high resolution MS to detect modified DUX4 peptides. We used a trypsin or trypsin/chymotrypsin digestion strategy in two biological replicates and obtained 85% and 43% sequence coverage, respectively. We
identified 17 prospectively modified amino acids, including novel DUX4 serine/threonine phosphorylation sites, arginine monomethylation and dimethylation sites, and a lysine acetylation site (Fig 1B, C and Table 1). We were unable to localize a subset of phosphorylation sites given their close proximity, however, the spectral information indicated that these peptides were phosphorylated (Table 2). Although MS is the most sensitive approach to detect protein modifications, we performed pull-downs and Western blots to confirm that DUX4 was modified on phospho-serine/threonine and methylated arginine residues (Fig 1D). We found evidence that DUX4 protein had arginine monomethylation as well as symmetric dimethylation. Unfortunately, we were unable to use pull-down and Western blot to visualize DUX4 protein phosphorylation due to nonspecific background using phospho-serine antibodies (data not shown).

To determine the functional consequence of prospective DUX4 PTMs, we generated 55 mutants designed to mimic or prevent the MS-identified phosphorylation, methylation, and acetylation sites (Table S1). This total included DUX4 PTM mutants containing phosphorylation sites with ambiguous fragmentation. We generated 3 mutants for every methylated arginine residue and 2 for phosphorylated serine/threonine and acetylated lysine (Table 3). We also generated controls containing mutations in every putatively modified arginine or serine/threonine residue (DUX4-Methyl null and mimic; DUX4-Phospho null and mimic, respectively; see Table S1).

FIGURE 4: DUX4-R71 mutants have a gradient of decreased transactivation. HEK293 cells were co-transfected with plasmids expression the DUX4-activated fluorescence reporter (DRE) and wild type DUX4, DUX4 R71 mutants, or empty vector (pClNeo). GFP expression was visualized 24 hours later. (A) Representative fluorescence photomicrograph of HEK293 cells transfected with the indicated plasmids and DUX4-responsive GFP reporter. (B) Quantification of DUX4-activated fluorescence reporter normalized to DUX4-WT. n = 3 independent experiments. (C) The localization of R71 in the crystal structure of DUX4 with DNA. Asterisk (*) indicates significant differences compared to DUX4-WT. **** p < 0.0001. One-way analysis of variance (ANOVA) test. RFU = relative fluorescence units.
Because DUX4 expression causes apoptosis in numerous cell types, we used a cell death assay as a primary screen to assess the functional impacts of DUX4 PTMs on DUX4-induced cell death. 7,9 To do this, we transfected plasmids expressing wild-type DUX4 or DUX4 PTM mutants into HEK293 cells and performed caspase-3/7 apoptosis

![Graph showing Myoblasts Viability (Trypan Blue) with Live cells/mL values for Empty Vector, DUX4-WT, DUX4-S29D/S31D/T106D, and DUX4-R71A](image.png)

**FIGURE 5:** DUX4 PTM mutants protect against cell death in human myoblasts without affecting DUX4 expression or nuclear localization. (A) Human myoblasts were transfected with plasmids expressing wild type DUX4 or the indicated DUX4 PTM mutants, and viability assessed 48 hours later with trypan blue counting. Asterisks indicates significant differences compared to DUX4-WT. ***p < 0.001, ****p < 0.0001. One-way ANOVA test. (B) Western blot demonstrating V5 epitope-tagged DUX4 protein in transfected HEK293 cells and myoblasts. (C) DUX4 protein was detected using fluorescent anti-V5 antibody (red) 24 hours post-transfection in HEK293 cells. Nuclei were DAPI stained (purple). Representative data from n = 3 independent experiments.
FIGURE 6: Decreased DUX4 target gene activation in DUX4 PTM mutants. (A) HEK293 cells or (B) myoblasts were transfected with plasmids expressing wild type DUX4, empty vector (pClNeo), DUX4 R71A, DUX4-Mutant 2 and DUX4-Mutant 5. Quantitative RT-PCR was performed 24 hours later for DUX4 target genes PRAMEF12, ZSCAN4, TRIM43 and LEUTX. Gene expression was normalized to the reference gene, RPL13A. n = 3 independent experiments performed in triplicate.
assays. We identified 5 mutants that significantly reduced DUX4-associated caspase-3/7 activation (Fig 2A–D). Among these 5 mutants, 3 contained different single mutations in the same methylated arginine residue (R71), such that methylation null and mimic mutations at R71 produced the same protective effect. The other 2 protective DUX4 mutants we identified in the caspase-3/7 screen contained phospho-mimic changes in 3 or 4 serine/threonine residues, respectively (DUX4-S29D/S31D/T106D; aka Mutant 2) and (DUX4-T99E/T102E/S104E/T106E; aka Mutant 5; see Fig 2C). Interestingly, single mutations of prospective phosphorylated serine or threonine residues had no impact on DUX4 toxicity. Notably, the amino acids changed in the 5 protective DUX4 mutants were all located within the DUX4 homeobox DNA binding domains (see Fig 1C).

DUX4 is a transcription factor that activates downstream target genes, and we therefore next sought to determine the impacts of DUX4 PTM mutations on its transactivation function. For this experiment, we specifically focused on the 5 nontoxic mutants identified in

**FIGURE 7:** **DUX4 is a PKA substrate.** A radiometric in vitro kinase assay was performed with (A) purified recombinant DUX4 protein. (B) Activity ratios from a subset of serine/threonine kinases with high activity to DUX4 protein. (C, D) HEK293 cells were transfected with plasmids expressing wild type DUX4 and the indicated serine/threonine kinase, and caspase-3/7 assay performed 48 hrs later. (E) HEK293 cells were transfected with plasmids expressing wild type DUX4 and/or the catalytic subunit of PKA, PRKACA, and caspase 3/7 assay performed 48 hours later. Representative data from n = 3 independent experiments performed in (C) duplicate and (D, E) triplicate. Asterisks indicates significant differences compared to DUX4-WT. ***p < 0.001, ****p < 0.0001. One-way ANOVA test. RFU = relative fluorescence units.
Although none of the 5 mutants were S2 and our structural modeling suggests 7B and Table 7A S2 3 4C 4 5A S2 3 to con
Because FSHD primarily affects muscle, we next soughtments in human embryonic kidney cells (HEK293).
However, crystallization ex periments would be neces-
For the R71 mutants, we quanti-
protein structural data,28 our structural modeling suggests that the DUX4 R71 residue could be involved in mak-
To this point in the study, we performed all experi-
the caspase-3/7 assay. We first tested the ability of wild-
type DUX4 or DUX4 PTM mutants to transactivate a previously described DUX4-responsive GFP reporter construct.6 Although none of the 5 mutants were completely devoid of transactivation activity in this reporter assay, all failed to achieve GFP expression at the level produced by wild-type DUX4 (Figs 3 and 4). For the R71 mutants, we quantified a gradient of GFP expression (R71A < R71L < R71K; 26%, 32%, and 59% of wild-type GFP levels; see Fig 4A, B). Thus, maintaining a basic residue at this location (R71K) produced the most transactivation in this assay among the 3 R71 mutants. Using previously published DUX4 protein structural data,28 our structural modeling suggests that the DUX4 R71 residue could be involved in making electrostatic interactions with DNA (see Fig 4C). However, crystallization experiments would be neces-
sary to definitively demonstrate this.

To this point in the study, we performed all experi-
ments in human embryonic kidney cells (HEK293).
Because FSHD primarily affects muscle, we next sought to confirm the nontoxic effects of DUX4 mutants in human muscle cells. Although we identified 5 nontoxic DUX4 mutants with reduced ability to transactivate a DUX4-responsive reporter construct, 3 contained mutations in the same R71 amino acid, and we therefore used one (R71A) to represent the group in all subsequent experiments. We transfected human myoblasts with plasmids expressing these 3 PTM mutants and measured cell viability using a Trypan Blue assay. Although wild-type DUX4 caused significant myoblast cell death, the 3 DUX4 PTM mutants had no deleterious effects on myoblast viability (Fig 5A). Importantly, these PTM mutants were not associated with decreased DUX4 protein expression in HEK293 cells and myoblasts or altered nuclear localization when expressed in HEK293 cells (Fig 5B, C and Fig S1).

We next assessed the ability of DUX4-R71A, DUX4-Mutant 2, and DUX4-Mutant 5 to activate 4 DUX4 target genes, PRAMEF12, ZSCAN4, TRIM43, and LEUTX. We expressed wild type or DUX4 R71A, DUX4-S29D/S31D/T106D (Mutant 2) and DUX4-T99E/T102E/S104E/T106E (Mutant 5) in HEK293 and human myoblasts, and examined DUX4 target gene expression by quantitative real-time PCR (RT-PCR). Although wild-type DUX4 activated all 4 biomarkers in HEK293s and myoblasts, cells transfected with the 3 DUX4 mutants showed significantly decreased PRAMEF12, ZSCAN4, TRIM43, and LEUTX expression (Fig 6A, B).

Our mutagenesis data to this point suggested that DUX4-associated cell death and target gene activation could be influenced by arginine methylation inhibition and enhanced serine/threonine phosphorylation on the DUX4 protein. Thus, we next sought to identify kinases and arginine methyltransferases that were capable of modifying the DUX4 protein. First, we conducted a compre-
hesive screen to identify serine/threonine kinases that could directly phosphorylate DUX4. To do this, we per-
formed an in vitro radiometric filter binding assay using purified recombinant DUX4 protein and 245 kinases (Fig 7A and Table S2). We identified 92 kinases with significant DUX4 phosphorylation activity (activity ratio value greater than 3; see Table S2). We focused subsequent experiments on a subset of the kinases with a high phosphorylation activity (activity ratio of 9 or greater; Fig 7B).

Our finding that DUX4 phosphorylation mimic mutants protected against DUX4-mediated cell death suggested that overexpression of serine/threonine kinases with high DUX4 phosphorylation activity could also rescue the cell death phenotype. We overexpressed DUX4 with a subset of kinases with high activity to DUX4 (Fig 7C, D) and found the catalytic subunit of protein kinase A, PRKACA, was associated with decreased caspase-3/7 activity (Fig 7D, E). Overexpression of 4 other protein kinase A subunits had no inhibitory effect on caspase-3/7 activity (see Fig 7D). Another kinase, TBK1,

### TABLE 4. Selected List of DUX4 Interacting Proteins

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Gene name</th>
<th>Enzyme function</th>
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<tr>
<td>Protein arginine N-methyltransferase 1</td>
<td>PRMT1</td>
<td>Methylation</td>
</tr>
<tr>
<td>rRNA 2'-O-methyltransferase fibrillarin</td>
<td>FBL</td>
<td>Methylation</td>
</tr>
<tr>
<td>DNA-dependent protein kinase catalytic subunit</td>
<td>PRKDC</td>
<td>Phosphorylation</td>
</tr>
<tr>
<td>Histone deacetylase 1</td>
<td>HDAC1</td>
<td>Acetylation</td>
</tr>
<tr>
<td>Histone deacetylase 2</td>
<td>HDAC2</td>
<td>Acetylation</td>
</tr>
<tr>
<td>Ubiquitin-like modifier-activating enzyme 1</td>
<td>UBA1</td>
<td>Ubiquitination</td>
</tr>
<tr>
<td>Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1</td>
<td>RPN1</td>
<td>Glycosylation</td>
</tr>
</tbody>
</table>

Note: Modifying enzymes identified by RIME are listed with enzymatic function. Abbreviation: RIME = Rapid Immunoprecipitation Mass Spectrometry of Endogenous Proteins assay.
We performed co-immunoprecipitation (co-IP) of DUX4 and two PRMT1 splice variants (v1 and v2) in HEK293 cells. Representative data from n = 3 independent experiments. (B) Human myoblasts were transfected with a WT-DUX4 expression plasmid, or no DNA, in the presence or absence of increasing concentrations of SAA. Caspase 3/7 assay was performed 48 hours later. Representative data from n = 3 independent experiments performed in duplicate or singlet. (C) iDUX4 human myoblasts were treated with increasing concentrations of SAA and caspase-3/7 assay was performed 48 hours after DUX4 induction. n = 4 independent experiments performed in triplicate. Asterisks indicates significant differences compared to DUX4-WT. *p < 0.05, **p < 0.01, ****p < 0.0001. One-way ANOVA test. RFU = relative fluorescence units.

also showed weaker ability to reduce DUX4-associated caspase-3/7 activation in vitro (see Fig 7D). Importantly, DUX4 and PRKACA form a complex when overexpressed in HEK293 cells (Fig 7F). In a similar experiment, we found no evidence for association of DUX4 with Tbk1 (data not shown).

To isolate arginine methyltransferases that associate with the DUX4 complex, we used RIME, which combines crosslinking, immunoprecipitation, and MS to identify transient or distant interactions.27 We performed RIME in human myoblasts expressing V5 epitope-tagged WT DUX4 and identified protein arginine methyltransferase 1 (PRMT1) as a component of the DUX4 complex (Tables 4 and S3). Using cotransfection and pull-down assays, we found that DUX4 associates with the two predominant and ubiquitous PRMT1 isoforms (splice variants 1 and 2)29 upon overexpression in HEK293 cells (Fig 8A).

These results supported that DUX4 is a substrate of protein kinase A (PKA) and PRMT1, and suggested that these interactions could offer novel targets for small molecule treatments of FSHD. Specifically, we hypothesized that drugs that activate the PKA pathway, or inhibit PRMT1, could protect against DUX4-mediated toxicity. We identified 5 published candidate compounds with prospectively desired properties (PKA activator 8-Br-cAMP; PKA-targeting phosphatase inhibitor LB-100; PRMT inhibitors Adox, AMI-1 and salvianolic acid [SAA]).30 To determine if small molecule perturbation of PKA and PRMT1 could protect from DUX4-induced death, we treated human DUX4-expressing cells with increasing doses of each drug and measured caspase-3/7 activity (Fig 8B, C, Figs S2 and S3). Only one compound, salvianolic acid (SAA), showed promising results and yet data with SAA were inconsistent and inconclusive using 5 different human cell lines. Specifically, high doses of SAA (100, 150, and 200 μM) reduced caspase-3/7 activity in normal human myoblasts electroporated with a CMV.DUX4 expression plasmid (see Fig 8B). We observed a similar trend in iDUX4 myoblasts, which are human myoblasts containing a doxycycline-inducible DUX4 transgene (see Fig 8C).25 However, SAA caused toxicity in 2 different lines of differentiated primary myotubes from patients with FSHD, and reduced proliferation of HEK293 cells at 100 μM (see Fig. S3 and data not shown).
Taken together, our results support that the DUX4 protein contains multiple post-translational modifications that may impact its toxic function; and that DUX4 protein associates with and is a target of PKA and PRMT1 enzyme complexes. Although the limited drug screen we performed was inconclusive, this study defined prospective new targets for more extensive future drug screens.

Discussion
This is the first study to identify methylation and phosphorylation as critical regulators of DUX4-mediated toxicity. We characterized arginine methylation null mutants and 2 phosphorylation mimic mutants that prevented DUX4-associated apoptosis and decreased DUX4 target gene activation. We also found that DUX4 forms a complex with the arginine methyltransferase PRMT1 and a catalytic subunit of the serine/threonine kinase PKA, thereby identifying new prospective targets for FSHD drug development.

Arginine methylation plays an important role in the regulation of transcription factors by affecting DNA binding and target gene activation. Consistent with this, we found that DUX4 R71 is critical for DUX4 transactivation activity. We demonstrated that the arginine methyltransferase PRMT1 is a component of the DUX4 complex. PRMT1 is the predominant type I arginine methyltransferase accounting for 85% of arginine methyltransferase activity in mammalian cells. In the skeletal muscle, PRMT1 plays a central role in differentiation and regeneration. In particular, PRMT1 methylation of the transcription factor MyoD affects DNA binding and target gene activation in C2C12 cells and PRMT1 inhibition impacts the recruitment of the transcription factor Myc to specific target promoters. Further studies are required to determine whether PRMT1 is directly responsible for recruiting DUX4 to target gene promoters and the molecular consequences of the DUX4-PRMT1 interaction.

Our data demonstrate that serine/threonine phosphorylation of DUX4 and overexpression of DUX4 with the catalytic subunit of serine/threonine kinase PKA protect against DUX4-mediated toxicity. These results reveal that serine/threonine phosphorylation of the DUX4 protein is protective. The beneficial effects of DUX4 phosphorylation could result from diminished activation of target genes or by affecting protein recruitment to the DUX4 transcriptional complex resulting in impaired transactivation. There is evidence for such mechanisms in other systems. For example, in the silkworm, PKA phosphorylation of the transcription factor BR-C impairs transcriptional activity, whereas a phosphorylation mimic mutant of EZH2, a PKA substrate, has enhanced interactions with STAT3, resulting in decreased oncogenic activity. Interestingly, activation of the PKA pathway represses DUX4 mRNA expression. These results suggest a dual mechanism by which the PKA pathway negatively regulates DUX4 by repressing DUX4 expression at the mRNA level and abrogating DUX4-mediated toxicity at the protein level.

Related to the mutagenesis screen we performed, it is possible that the various mutations we inserted into DUX4 could disrupt DUX4 protein function by simply altering the native structure of the homeodomains, and could be unrelated to PTMs. Understanding this caveat, we used results from the DUX4 PTM mutagenesis screen to explore potential DUX4 modifying enzymes and were able to identify 2 PTM enzymes as DUX4 interactors. Our results suggested that enhancing DUX4 serine/threonine phosphorylation by activating the PKA pathway or inhibiting serine/threonine phosphatases could serve as novel therapeutic approaches for FSHD. Additionally, a prior study found that PKA pathway activation with beta-adrenergic receptor agonists, cAMP analogs, and a constitutively active PKA mutant led to decreased DUX4 mRNA expression and decreased DUX4 target gene activation in the myotubes of patients with FSHD. However, our data indicate that cAMP activator 8-Br-cAMP or phosphatase inhibitor LB-100 did not ameliorate DUX4-mediated caspase cleavage in proliferating cells. Further studies are necessary to investigate the therapeutic potential of additional phosphatase inhibitors or novel methods of PKA pathway activation.

Although PRMT inhibitors have been studied extensively in cancer with several compounds advancing to clinical trials, this is the first report to explore a therapeutic role for PRMT inhibitors in muscle disease. Our screen of arginine methylation inhibitors in human myoblasts initially identified a promising compound, SAA, which improved DUX4-mediated toxicity in human myoblasts. However, overall, our results with SAA were inconclusive, as the compound was toxic to differentiated myotubes and slowed proliferation of HEK293 cells at higher doses. SAA is derived from the root of *Salvia miltiorrhiza* and has been used in traditional medicine in Asian countries for a wide range of ailments, including heart disease, dementia, and cancer. Additionally, oral administration of SAA is safe in rodents. Although SAA has been shown to inhibit PRMT1 activity in vitro, SAA has diverse cellular roles and impacts several signal transduction pathways. It has been shown to induce or inhibit apoptosis in different disease models. These results point to the importance of testing the safety and efficacy of SAA and other potential therapeutics in multiple FSHD disease models, using a battery of different outcome measures.

In conclusion, in this first study to explore DUX4 post-translational modification, we identified and
functionally characterized several modified amino acids in the DUX4 protein. We found several DUX4 residues that are essential for DUX4 target gene activation and DUX4-mediated cell death. In addition, we identified potential modifiers of the DUX4 transcriptional complex including PRMT1 and PKA. In future studies, we will continue to explore the therapeutic potential of targeting DUX4 PTMs in FSHD disease models, with a goal of identifying novel FSHD-modifying drugs.

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Author Contributions

Potential Conflicts of Interest

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