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RESEARCH

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SIX transcription factors are necessary for the activation of DUX4 expression in facioscapulohumeral muscular dystrophy

Amelia Fox[1], Jonathan Oliva[1], Rajanikanth Vangipurapu[1] and Francis M. Sverdrup[1*]

Abstract

Background Facioscapulohumeral muscular dystrophy (FSHD) is a common and progressive muscle wasting disease that is characterized by muscle weakness often first noticed in the face, the shoulder girdle and upper arms before progressing to the lower limb muscles. FSHD is caused by the misexpression of the Double Homeobox 4 (DUX4) transcription factor in skeletal muscle. While epigenetic derepression of D4Z4 macrosatellite repeats underlies DUX4 misexpression, our understanding of the complex transcriptional activation of *DUX4* is incomplete.

Methods To identify potential *DUX4*-regulatory factors, we used small interfering RNAs (siRNAs) to knockdown SIX family transcription factors (SIX1, 2, 4, 5) in patient-derived FSHD1 and FSHD2 myoblasts that were differentiated to form multinucleated myotubes. Quantitative real-time polymerase chain reaction was used to measure changes in *DUX4* mRNA, DUX4 target gene expression and myogenic markers. Staining for SIX1 and SIX2 with specific antibodies was performed in FSHD myoblasts and myotubes. To assess reciprocal effects of DUX4 on *SIX1*, *2*, and *4* expression, we utilized a doxycycline-inducible DUX4 myoblast cell line.

Result We show that SIX1, 2 and 4 transcription factors, regulators of embryonic development, muscle differentiation, regeneration and homeostasis, are necessary for myogenic differentiation-dependent *DUX4* expression in FSHD muscle cells. Using siRNA, we demonstrate SIX1, SIX2, and SIX4 to be critical factors involved in the induction of *DUX4* transcription in differentiating FSHD myotubes in vitro. siRNA dual knockdown of SIX1 and SIX2 resulted in a ~ 98% decrease of *DUX4* and DUX4 target genes, suggesting that SIX1 and SIX2 are the most critical in promoting *DUX4* expression. Importantly, we show that DUX4 downregulates *SIX* RNA levels, suggesting negative feedback regulation.

Conclusions In this study, we identified a family of developmental regulators that promote aberrant *DUX4* expression in FSHD1 and FSHD2 differentiating muscle cells. Our findings highlight the critical involvement of SIX transcription factors (SIX1, 2, 4) in the pathogenesis of FSHD by serving as necessary factors that function in the promotion of *DUX4* expression following epigenetic derepression of the D4Z4 repeats.

Keywords FSHD Facioscapulohumeral muscular dystrophy, DUX4 Double homeobox 4, SIX transcription factors, D4Z4 macrosatellite repeats, siRNA Small interfering RNA

*Correspondence:
Francis M. Sverdrup
fran.sverdrup@health.slu.edu
[1] Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, Saint Louis, MO, USA

Background

Facioscapulohumeral muscular dystrophy (FSHD) is a genetically inherited disease with a clinical phenotype characterized by progressive muscle weakness and wasting, often first noticed in the face, shoulders, and upper arms, eventually affecting the lower limb muscles [1, 2]. Most FSHD patients exhibit noticeable symptoms

within their second decade of life; however, the disease onset and severity are highly variable [3–6]. There are two main classifications of FSHD (FSHD1 and FSHD2), with clinically identical symptoms, that are distinguished by genetic differences [7, 8]. FSHD1, affecting 95% of patients, results from a pathogenic contraction of the D4Z4 macrosatellite repeat array at the subtelomeric region of chromosome 4q35, resulting in 1–10 residual repeats compared to 11–100 repeats in unaffected persons [3, 9–11]. FSHD2, however, develops from loss-of-function mutations in one or more chromatin modifiers (e.g., SMCHD1, DNMT3B, LRIF1) [3, 10, 12, 13]. Despite the genetic differences, there is a consensus behind the cause of FSHD resulting from loss of epigenetic repression at D4Z4 repeats, alterations of the chromatin structure (e.g., loss of DNA methylation and repressive histone marks) and the inappropriate expression of transcription factor, Double Homeobox 4 (DUX4) in skeletal muscle [3, 14, 15].

DUX4 plays a critical role during early embryonic development and, when ectopically expressed, in the pathogenesis of several diseases [1, 16–19]. During normal development, DUX4 is expressed in two- and four-cell stage embryos and activates the zygotic genome transcriptional program by inducing the transcription of hundreds of genes [16, 20]. Subsequently, *DUX4* expression is downregulated at the 8-cell stage and silenced in most somatic tissues by repeat-mediated epigenetic repression [15, 21]. However, loss of repression and transcriptional activation of DUX4 by a complex and incompletely described mechanism are responsible for one of the most common muscular dystrophies, FSHD [16, 19, 22]. Bursts of *DUX4* expression in the diseased state might lead to progressive muscle degeneration, inflammation, fat infiltration and inadequate muscle regeneration [16, 23, 24]. Importantly, aberrant DUX4 expression has also been implicated in several cancers, where its expression leads to MHC class I antigen suppression, immune evasion and loss of checkpoint blockades, resulting in cancer progression and immunotherapy failure [16, 19, 25, 26]. Due to DUX4 being the common link between FSHD1 and FSHD2, targeting *DUX4* expression has been recognized as an attractive therapeutic strategy and understanding DUX4 regulation is crucial for identifying new potentially druggable targets [27–29]. Although there have been factors previously identified to promote DUX4 activation [30–37], the mechanism remains poorly described and potentially many transcriptional and epigenetic regulators remain to be identified.

Here, we investigated SIX (*sine oculis*) transcription factors, which are part of an established and evolutionarily conserved network of transcription factors known as the PSED (PAX-SIX-EYA-DACH) network [38–40]. This network has critical regulatory roles in the development and regeneration of many tissues including most sensory organs, the kidney, and skeletal muscle [38–42]. There are three distinct subclasses of SIX transcription factors that are grouped by similarities in their amino acid sequences: SIX1/2 (*sine oculis*), SIX3/6 (*optix*), and SIX4/5 (*DSIX4*) [38, 40, 43]. However, only SIX1, SIX2, SIX4, and SIX5 are expressed during embryonic myogenesis, in proliferating myogenic stem cells and in adult myofibers [38, 44]. During muscle development, these transcription factors function by controlling myogenic regulatory factors (MRFs) to direct myogenic cell fate decisions, muscle differentiation and muscle regeneration through stem cell renewal [38, 45]. During these processes, SIX transcription factors can function as transcriptional activators or repressors independently and/or in association with a cofactor, such as EYA (transcriptional activator) or DACH (transcriptional repressor) [38]. Importantly, when absent or ectopically expressed, SIX transcription factors and their related cofactors are responsible for several congenital disorders (e.g., BOR syndrome, hearing loss and craniofacial abnormalities) and cancers by promoting increased invasion and metastasis [46–48]. Although the roles of SIX transcription factors in development and cancer have been extensively studied, their potential contributions in muscular dystrophies, like FSHD, have yet to be defined. In this study, we elucidate the necessary contribution of SIX transcription factors in the regulation of *DUX4* transcription in FSHD.

Methods

Experimental design

We utilized immortalized patient-derived FSHD1 and FSHD2 cells to study the effects on *DUX4* transcription by siRNA knockdown of SIX transcription factors (SIX1, 2, 4, 5). Early and late differentiation markers were analyzed by qPCR analysis to monitor any differential changes following siRNA knockdown. Immunofluorescence was used to look at the expression of necessary transcription factors to determine if they could be responsible for the restricted expression of DUX4 in rare myonuclei. Non-FSHD myoblasts with a doxycycline-inducible *DUX4* transgene were utilized to study the regulation of DUX4 expression on *SIX* gene transcription. Our data provides strong evidence of the necessary involvement of SIX genes in *DUX4* regulation.

Cell culture system

FSHD1 (54–2; 3 D4Z4 repeat units), FSHD1 (16-ABIC; 7 D4Z4 repeat units), and FSHD2 (MB200) patient-derived immortalized myoblasts were grown in Ham's F-10 Nutrient Mix (Gibco, Waltham, MA) with 20% FBS (Corning, Corning, NY), 100 U/100 µg penicillin–streptomycin

(Gibco), 10 ng/mL of recombinant human FGF (Promega Corporation, Madison, WI), and 1 μM dexamethasone (Sigma-Aldrich, Saint Louis, MO). To induce differentiation to form multi-nucleated myotubes, we used Dulbecco's modified Eagle's medium/F-12 mix (Gibco) with 100 U/100 μg penicillin–streptomycin, and 10 μg/mL knockout serum (Gibco) and differentiated our cells for 40–48 h. For our inducible DUX4 cell system (iDUX4), we used codon-altered MB135 cells with a doxycycline-inducible DUX4 promoter gifted by Dr. Stephen Tapscott. MB135 iDUX4 cells were supplemented with 3 ug/mL puromycin (Thermo Fischer Scientific, Waltham, MA) for selection. DUX4 was induced with doxycycline (Thermo Fischer Scientific) for 8 and 24 h. To differentiate MB135 iDUX4 cells, the media was switched to standard differentiation media supplemented with 3ug/mL puromycin. Doxycycline was added for the last 24 h of differentiation prior to harvesting.

Transfections of small interfering RNA (siRNA)

Silencer Select siRNAs for human SIX1, SIX2, and SIX4 were purchased from Thermo Fischer Scientific (Waltham, MA). Myoblasts were plated at 1×10^5 in 12-well plates and transfected using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA) two hours later according to manufacturer instructions. Per well, 2 μl of Lipofectamine RNAiMAX and 1 μL (10 pmol) siRNA were combined in 100 μl Opti-MEM Reduced Serum Medium (Gibco, Waltham, MA). Three biological replicates were used for each condition. 72 h after transfection, myoblasts were harvested, and the RNA was isolated using the E.Z.N.A. Total RNA Kit 1 (Omega BioTek, Norcross, GA). To observe effects in multinucleated myotubes, growth media was replaced with differentiation media 48 h after the initial transfection to ensure depletion of the target gene. RNA was isolated for qPCR analysis with two technical replicates per biologic replicate. All experiments were repeated at least three times to ensure reproducibility.

siRNA sequences

The siRNAs were all purchased from Thermo Fisher Scientific (Waltham, MA). Silencer Select Negative Control No.1 (cat. 4390843) was used throughout all experiments. SIX1 (s12874, sense: AGAACGAGAGCGUAC UCAAtt, antisense: UUGAGUACGCUCUCGUUCUtg); SIX2 (s21094, sense: GGGAAUAAAUUAUACACC Att, antisense: UGGUGUAUAAUUUAUUCCCtt); SIX4 (s224246, sense: GGUUGAUACUGUCUGUGAAtt, antisense: UUCACAGACAGUAUCAACCat); SIX5 (s45075, sense: GAAAUGCGGUUGCUGAAGAtt, antisense: UCUUCAGCAACCGCAUUUCtg); EYA1 (s4904, sense: GACUGAAGGUGGAUUGUCAtt, antisense: UGACAA UCCACCUUCAGUCtt); EYA2 (s4908, sense: GCGAUU GUCUGGAUAAACUtt, antisense: AGUUUAUCCAGA CAAUCGCtg); EYA2 (s4909, sense: GCCUUAUGAUGG AAGAGAUtt, antisense: AUCUCUUCCAUCAUAAGG Cca); EYA3 (s4912, sense: GCUUUGGAGUCACAG GUCAtt, antisense: UGACCUGUGACUCCAAAGCtg); EYA4 (s4794, sense: GGACUUAAGUACCUACAGUtt, antisense: ACUGUAGGUACUUAAGUCCtg).

Total RNA extraction and qPCR

Total RNA was extracted using spin-columns from the E.Z.N.A Total RNA Kit purchased from Omega BioTek according to the kit instructions (Norcross, GA). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to analyze to relative expression levels using Quant Studio 5 from Applied Biosystems by Thermo Fischer Scientific (Foster City, CA).

TaqMan assay for qPCR

All TaqMan primer probe sets were purchased from Thermo Fisher Scientific (Waltham, MA) and compared to an endogenous control, RPL30 (Hs00265497_m1). Human DUX4 and DUX4 targets were used: DUX4 (Hs07287098_g1); MBD3L2 (Hs00544743_m1); LEUTX (Hs01028718_m1); and ZSCAN4 (Hs00537549_m1). Human SIX genes were used: SIX1 (Hs00195590_m1); SIX2 (Hs00232731_m1); SIX4 (Hs00213614_m1); and SIX5 (Hs05053086_s1). To look at markers of differentiation, MYOG (Hs01072232_m1); MYOD (Hs00159528_m1); MYH1 (Hs00428600_m1); MYH2 (Hs00430042_m1); MYH3 (Hs01074230_m1); MYH4 (Hs00757977_m1); MYH7 (Hs01110632_m1); MYH8 (Hs00267293_m1); CKM (Hs00176490_m1) were used. SIX1 and SIX2 target genes and cofactors were used: PGK1 (Hs00943178_g1); SLC4A7 (Hs00186192_m1); EYA1 (Hs00166804_m1); EYA2 (Hs00193347_m1); EYA3 (Hs00544914_m1); EYA4 (Hs01012399_m1). For each assay, TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific, Walham, MA) or TaqMan Fast Advanced Virus Master Mix (Thermo Fisher Scientific) were used to carry out qPCR.

DUX4 assay

To determine relative *DUX4* expression, a two-step process was used in which cDNA was first synthesized with oligo dT priming using the ProtoScript II First-Strand cDNA Synthesis kit (New England BioLabs, Ipswich, MA). To denature the template RNA, 6 μL of total RNA of interest with 2 μL of Oligo dT was incubated for 5 min at 65 °C. Then 10 μL of the Reaction Mix (2X) and 2 μL of the Enzyme Mix (2X) was mixed with the RNA. Samples were incubated for 1 h at 42 °C and then the enzyme

was inactivated for 5 min at 80 °C. cDNA was used in the standard qPCR protocol.

Western blot

Cells treated by siRNA knockdown were collected by adding direct SDS lysis buffer to each well. Following collection, the samples were boiled for 10 min, passed through a Qiashredder (Omega Bio-Tek, Norcross, GA), and then run on a NuPAGE 4–12% Bis–Tris polyacrylamide gel (Thermo Fisher Scientific, Waltham, MA) with SDS Running buffer (Thermo Fisher Scientific). The gel was then transferred to an Immobilon-FL polyvinylidene difluoride transfer membrane (Millipore, Burlington, MA) using the NuPAGE transfer buffer (Life Technologies, Carlsbad, CA) with 10% methanol. After the membrane transfer, the membrane was left to dry overnight. The next day, the membrane was activated by soaking in methanol and 1X TBS before blocking in Odyssey TBS Blocking buffer (Li-Cor, Lincoln, NE) for 1 h. After blocking, the primary antibody of interest and an appropriate α/β-Tubulin (loading control) were added to the membrane at a concentration of 1:1000 in Odyssey TBS Blocking buffer (Li-Cor) and 0.2% Tween 20 overnight at 4 °C. The next day the membrane was washed (4X for 5 min) using 1X-TBST (0.1% Tween 20) before adding a near-infrared fluorescent secondary antibody (Li-Cor) diluted at 1:15,000 in the Odyssey TBS Blocking buffer (Li-Cor) with 0.2% Tween 20 and 0.01% SDS. The membrane was incubated in the secondary antibody for 1 h at room temperature. After a series of washes with 1X TBST (0.1% Tween 20) and a rinse of 1X TBS to remove all the Tween 20, the Li-Cor Odyssey CLx was used to image the blot. The Empiria Studio software was used to determine the percentage of selective protein knockdown.

Primary and secondary antibodies

The primary antibodies that were used for western blotting were α-Tubulin Mouse (Li-Cor; 926–42,213), β-Tubulin Rabbit (Li-Cor; 926–42,211), SIX1 Rabbit mAb (Cell Signaling Technologies, Danvers, MA; 12891S), SIX2 Rabbit pAb (Novus, Centennial, CO; NBP2-54917), SIX4 Rabbit pAb (Abcam, Cambridge, UK; ab176713), and anti-Myosin hc (clone MF20; R&D Systems, Minneapolis, MN; MAB4470). The secondary antibodies that were used were Goat anti-Rabbit IRDye 680RD (Li-Cor; 926–68071), Goat anti-Mouse IRDye 680RD (Li-Cor; 926–68670), Goat anti-Mouse IRDye 800CW (Li-Cor; 926–32210), and Goat anti-Rabbit IRDye 800CW (Li-Cor; 926–32211).

Immunofluorescence

Cells were fixed using 4% paraformaldehyde (Thermo Fischer Scientific) for 20 min at room temperature, permeabilized with 2% Triton for 15 min, washed with 1X PBS (Corning), blocked with 5% donkey/or goat serum (Millipore) and 1% BSA (Thermo Fischer Scientific). Primary antibodies were incubated at 1:100 and/or 1:300 at 4 °C overnight. The primary antibodies used were SIX1 Rabbit mAb (Cell Signaling Technologies, Danvers, MA; 12891S), SIX2 Rabbit pAb (Novus, Centennial, CO; NBP2-54917) and MF20 (R&D Systems, Minneapolis, MN; MAB4470). The next day the cells were washed with 1X PBS and incubated with a secondary antibody (1:300) at room temperature for 1 h. The secondary antibodies were Alexa Fluor 488 donkey anti-rabbit (Abcam; A21206), Alexa Fluor 488 goat anti-rabbit (Abcam; AB150077), Alexa Fluor 594 goat anti-mouse (Thermo Fischer Scientific; A11005) and DAPI (Thermo Fischer Scientific; 62248). Cells were then mounted and imaged using a Keyence Imaging Microscope under 40X magnification. For fusion index, the total nuclei and nuclei detected in myotubes were manually calculated (three fields captured). Clusters of nuclei (> 2) per myotube was the standard in calculating the fusion index.

Statistical analysis

All figures and statistical analysis were generated using GraphPad Prism 9. All samples tested were normalized to the non-targeting control group. An unpaired two-tailed *t-test* and unpaired one-tailed *t-test* were used to determine statistical significance. Samples were considered significant with a p value < 0.05.

Results

Knockdown of SIX1, SIX2 and SIX4 suppresses DUX4 expression in differentiating FSHD muscle cells

DUX4 is a pioneer transcription factor that can bind heterochromatic genomic regions to transcriptionally activate hundreds of downstream targets, leading to the FSHD phenotype [3, 49, 50]. *DUX4* is induced during early myogenic differentiation of FSHD myoblasts, in vitro [13, 51]; however, the transcriptional components that activate *DUX4* expression in skeletal muscle and other non-muscle tissues associated with FSHD are incompletely understood. To identify transcription factors that drive bursts of *DUX4* expression in FSHD myotubes, we screened factors critical during muscle development and myogenic differentiation by using small interfering RNAs (siRNAs) to knockdown each intended target in immortalized patient-derived FSHD1 (54–2 and 16-ABIC) and FSHD2 (MB200) myoblast lines. We transfected myoblasts with siRNAs selectively targeting *SIX* genes (*SIX*1, 2, 4, 5) 48 h prior to inducing differentiation for two days to generate multinucleated myotubes. For each transfection, there were three biological replicates.

Quantitative real-time PCR (qPCR) was used to quantify the relative *DUX4* and DUX4 target mRNA levels.

We found that selective knockdown of SIX1, SIX2 or SIX4, individually, led to significant decreases in *DUX4* mRNA levels in FSHD1 (54–2) myotubes, with *SIX1* and *SIX2* knockdown having the most pronounced effects on *DUX4* levels (Fig. 1A). Additional FSHD2 (MB200) and FSHD1 (16-ABIC) cell lines were used for further validation and were found to largely replicate the effects seen in the 54–2 FSHD1 cell line (fig. S1A and fig. S2A, respectively). However, in FSHD2 (MB200) cells, *DUX4* mRNA levels were not significantly reduced with SIX4 individual knockdown, as seen in the FSHD1 line (fig. S1A). DUX4 targets (*MBD3L2*, *LEUTX*, and *ZSCAN4)* were also significantly reduced, largely mirroring the decreases in *DUX4* mRNA across all tested cell lines (Fig. 1A, fig. S1A and fig. S2A). In the second FSHD1 (16-ABIC) cell line, *DUX4* is more weakly expressed, leading to an increase in variability between each replicate; however, although not all DUX4 targets were significantly reduced, all three targets trended down (fig. S2A). Based on the relative expression levels from qPCR, we determined *SIX5* mRNA was nearly undetectable in our cell system (table S1). These results suggest the individual involvement of SIX1, SIX2 and SIX4 in the transcriptional activation of *DUX4* gene expression in FSHD1 and FSHD2 myotubes.

Next, we knocked down various combinations of *SIX1*, *SIX2*, and *SIX4* to identify potential non-redundant roles among these paralogs and to determine if we could further suppress *DUX4* mRNA levels. Dual combinations containing *SIX2* siRNA had the most significant reduction of *DUX4* and DUX4 target transcripts with the combined knockdown of *SIX1* and *SIX2* eliciting the greatest decrease in *DUX4* and target gene mRNA levels (~98% decrease of *DUX4* mRNA) (Fig. 1B**,** fig. S1B, and fig. S2B). Across all tested cell lines, the combined knockdown of all three SIX genes resulted in a nearly complete extinction of *DUX4* and DUX4 target gene expression (~90–98% decrease) in FSHD1 (54–2 and 16-ABICs) and FSHD2 (MB200) myotubes (Fig. 1C, fig. S1C, and fig. S2C). siRNAs selectively targeting *SIX1*, *SIX2*, and *SIX4* effectively knockdown their respective target mRNA levels in both FSHD1 and FSHD2 myotubes (Fig. 1D and fig. S1D). Western blotting analysis and quantification confirmed selective protein depletion of SIX1, SIX2 and SIX4 by their respective siRNAs (Fig. 1E-G). Together, these data suggest that expression of SIX transcription factors (SIX1, SIX2, and SIX4) is necessary to promote *DUX4* transcription during FSHD myogenic differentiation, with SIX1 and SIX2 playing the most prominent roles.

Combined knockdown of SIX1, SIX2, and SIX4 does not inhibit myogenic differentiation

SIX proteins are well documented to participate in muscle differentiation and regeneration through their control of myogenic regulatory factors (MRFs) [38]. To rule out the possibility that knockdown of SIX proteins indirectly affected *DUX4* expression by inhibiting myogenic differentiation, we monitored the myotube fusion index and measured myogenic markers after the combined knockdown of SIX1, SIX2, and SIX4 in differentiating FSHD1 (54–2) and FSHD2 (MB200) cells. Consistent between the tested cell lines, we observed no delays in myotube formation (48 h) or changes in the fusion index of the generated multinucleated myotubes in comparison to cells treated with negative control siRNA (Fig. 2A-C, fig. S1E-G, and fig. S2D). We also measured gene expression of several myogenic markers. We screened markers of myogenic commitment (myoblasts determination protein 1, *MYOD1*), early differentiation (myogenin, *MYOG*), and late differentiation (creatine kinase M-Type, *CKM*).

Additionally, myosin heavy chains involved in embryonic development and regeneration (myosin heavy chain, *MYH3* and *MYH8*), slow fiber types (myosin heavy chain 7, *MYH7*), and fast fiber types (myosin heavy chains, *MYH1*, *MYH2*, and *MYH4*) were analyzed. qPCR analysis revealed that the combined knockdown of *SIX1*, *SIX2*, and *SIX4* had no differential effects on *MYOD*, *MYOG*, *MYH1* and *MYH4* mRNA levels, suggesting that knockdown of SIX proteins does not generally inhibit early myotube differentiation (Fig. 2D and E). However, there were differential effects on *CKM* (decreased), *MYH2* (increased), *MYH7* (decreased), *MYH3* (decreased), and *MYH8* (decreased) with the combined knockdown of all

(See figure on next page.)

Fig. 1 siRNA knockdown of SIX1, SIX2 and SIX4 suppresses DUX4 mRNA in FSHD1 myotubes. FSHD1 (54–2) myoblasts were transfected with siRNA targeting SIX1, SIX2, and SIX4 (**A**) individually and (**B-C**) combinatorially (two days prior to differentiating into myotubes for 48 h). Shown are the relative mRNA levels for (**A-C**) *DUX4*, (**A-C**) DUX4 targets (*MBD3L2*, *LEUTX,* and *ZSCAN4*) and **D** each *SIX* target. **E-G** Western blots show individual protein depletion of SIX1 (MW: ~ 36 kDa), SIX2 (MW: ~ 36 kDa) and SIX4 (MW: ~ 83 kDa). Alpha-tubulin (MW: 52 kDa) is shown as the loading control. Each experimental group had three biological replicates. Each experimental group was normalized to a negative si-Control with the mean and standard deviation depicted. Asterisks demonstrate statistical significance between the control group using an unpaired two tailed *t*-test (ns = not significant; *$p < 0.05$)

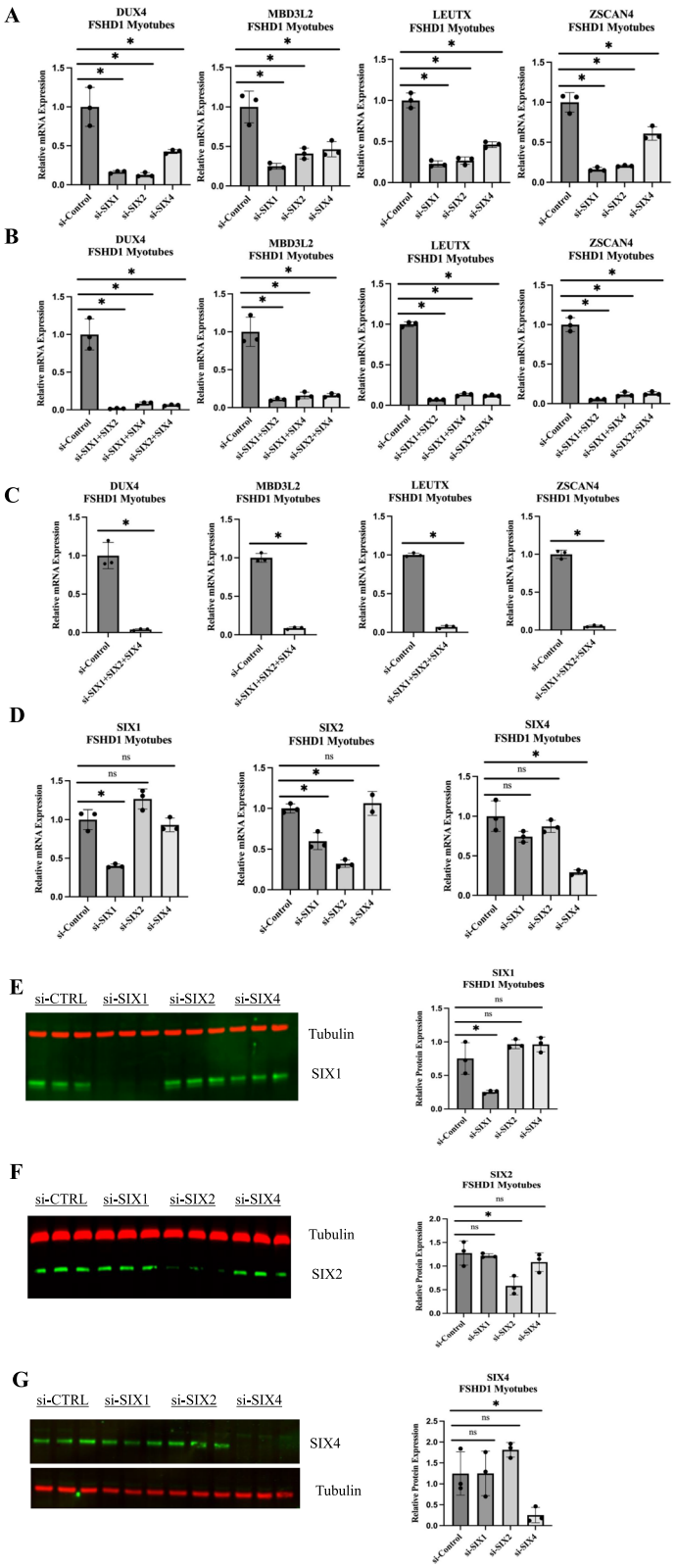

Fig. 1 (See legend on previous page.)

three SIX genes (Fig. 2D-G). Overall, these results suggest that the effects of SIX1/2/4 knockdown on *DUX4* expression are not due to impairment of myogenic differentiation, but rather establishes their role as a necessary component for DUX4 activation.

EYA coactivators participate in activating *DUX4* transcription

Since SIX1 (in contrast to SIX2 and SIX4) does not possess an intrinsic transcriptional activation domain and requires coactivators to positively affect transcription [52], we knocked down EYA genes (*EYA1*, *EYA2, EYA3*, and *EYA4*) in our FSHD myoblast lines to assess their potential involvement in *DUX4* regulation. We found individual knockdown of EYA1 did not significantly affect *DUX4*, but knockdown of EYA3 or EYA4 resulted in a 43–49% decrease of *DUX4* mRNA (Fig. 3A). We found that the combined knockdown of EYA1, EYA3 and EYA4 resulted in a 54% decrease of *DUX4* and a 40–70% decrease of DUX4 target mRNA levels upon myogenic differentiation of FSHD1 (54–2) myoblasts, with combinations containing EYA3 having the most pronounced effect on DUX4 target genes (Fig. 3B and fig. S3A). EYA2 was found to be lowly expressed and individual knockdown of *EYA2* had no effects on DUX4 target genes (fig. S4A). siRNAs selectively targeting *EYA1*, *EYA2*, *EYA3* and *EYA4* successfully knocked down each of their intended targets (Fig. 3C and fig. S4B). These data are consistent with EYA coactivators playing some role in promoting DUX4 expression through their interaction with SIX transcription factors.

Knockdown of SIX proteins suppresses DUX4 expression in a differentiation-dependent manner

Having established the requirement of SIX transcription factors to promote a differentiation-mediated increase in *DUX4* expression, we tested whether siRNA knockdown of SIX transcription factors would also suppress the relatively low levels of DUX4 expression in undifferentiated myoblasts [51, 53–55]. We transfected FSHD1 (54–2) and FSHD2 (MB200) myoblasts with *SIX* siRNAs and maintained the cells for 72 h prior to qPCR analysis to allow for sufficient protein turnover. We found individual and combined knockdown of *SIX1*, *SIX2*, and *SIX4* showed no significant effects on the low levels of *DUX4* and target mRNA in FSHD1 and FSHD2 myoblasts (Fig. 4A-C and fig. S5A). siRNAs targeting SIX1, SIX2, and SIX4 in myoblasts selectively knocked down each intended target gene (Fig. 4D). To determine if SIX transcription factors were otherwise active in myoblasts, we looked at the expression of known targets of SIX1 (*PGK1, SLC4A7*) and SIX2 (*EYA1*, *SLC4A7*) [56–58]. Knockdown of *SIX1* resulted in a 26% decrease in *PGK1* RNA levels and a 46% decrease in *SLC4A7* RNA levels (Fig. 4E), indicating that SIX1 does exhibit transcriptional activity towards known targets in myoblasts. Conversely, knockdown of *SIX2* in myoblasts resulted in a modest 25% decrease in *EYA1* RNA levels but failed to decrease *SLC4A7* RNA (Fig. 4E). In differentiating myotubes, however, knockdown of *SIX2* resulted in a more substantial decrease in EYA1 levels (54–59% decrease) and significantly decreased *SLC4A7* RNA levels (42–64% decrease), suggesting a dependence on the induction of differentiation to increase SIX1 and SIX2 transcriptional activity (Fig. 4F). Taken together, these data indicate that the regulation of *DUX4* expression by SIX1, SIX2, and SIX4 occurs primarily during the differentiation-induced increases of *DUX4*, and that while SIX1 and SIX2 have demonstrable transcriptional activity in myoblasts, their ability to regulate DUX4 requires myogenic differentiation signaling.

SIX1 and SIX2 do not govern restriction of DUX4 expression to a subset of nuclei

DUX4 transcription is restricted to a rare subset of nuclei in differentiating FSHD myotubes, resulting in clusters of adjacent nuclei importing DUX4 protein due to the syncytial nature of multinucleated myotubes, yet the reason for this restriction is not understood [50, 59, 60]. One possibility is the restricted expression of necessary transcription factors that drive *DUX4* transcription. To determine if the restricted transcription of *DUX4* in a subset of nuclei was due to restricted expression of SIX proteins in the same nuclei, we stained for SIX1 and SIX2, due to their prominent role in regulating *DUX4* transcription. Immunofluorescence staining in FSHD1 cells revealed SIX1 and SIX2 protein to be present in every nucleus

(See figure on next page.)

Fig. 2 Combined knockdown of SIX1, SIX2, and SIX4 does not inhibit myogenic differentiation. FSHD1 (54–2) myoblasts were transfected with siRNAs targeting SIX1, SIX2, and SIX4, and differentiated to generate multinucleated myotubes. **A** Multinucleated myotubes with clusters of nuclei for the individual and combined knockdown of SIX1/2/4. **B** Immunofluorescent staining for nuclei (blue) and MF20 (red) for si-Control and si-SIX1/2/4 conditions. **C** Fusion index was calculated for the conditions shown in **B**. **D-G** Myogenic markers for (**D**) myogenic commitment, **D** early and late differentiation, **E** fast fiber-types, **F** slow fiber-types and **G** regenerative were assessed. For fusion index, there were three fields captured with three biological replicates. Each experimental siRNA group ($n = 3$) was normalized to a negative si-Control with the mean and standard deviation depicted. Asterisks demonstrate statistical significance between the control vs experimental group using an unpaired two tailed *t*-test (ns = not significant; *$p < 0.05$)

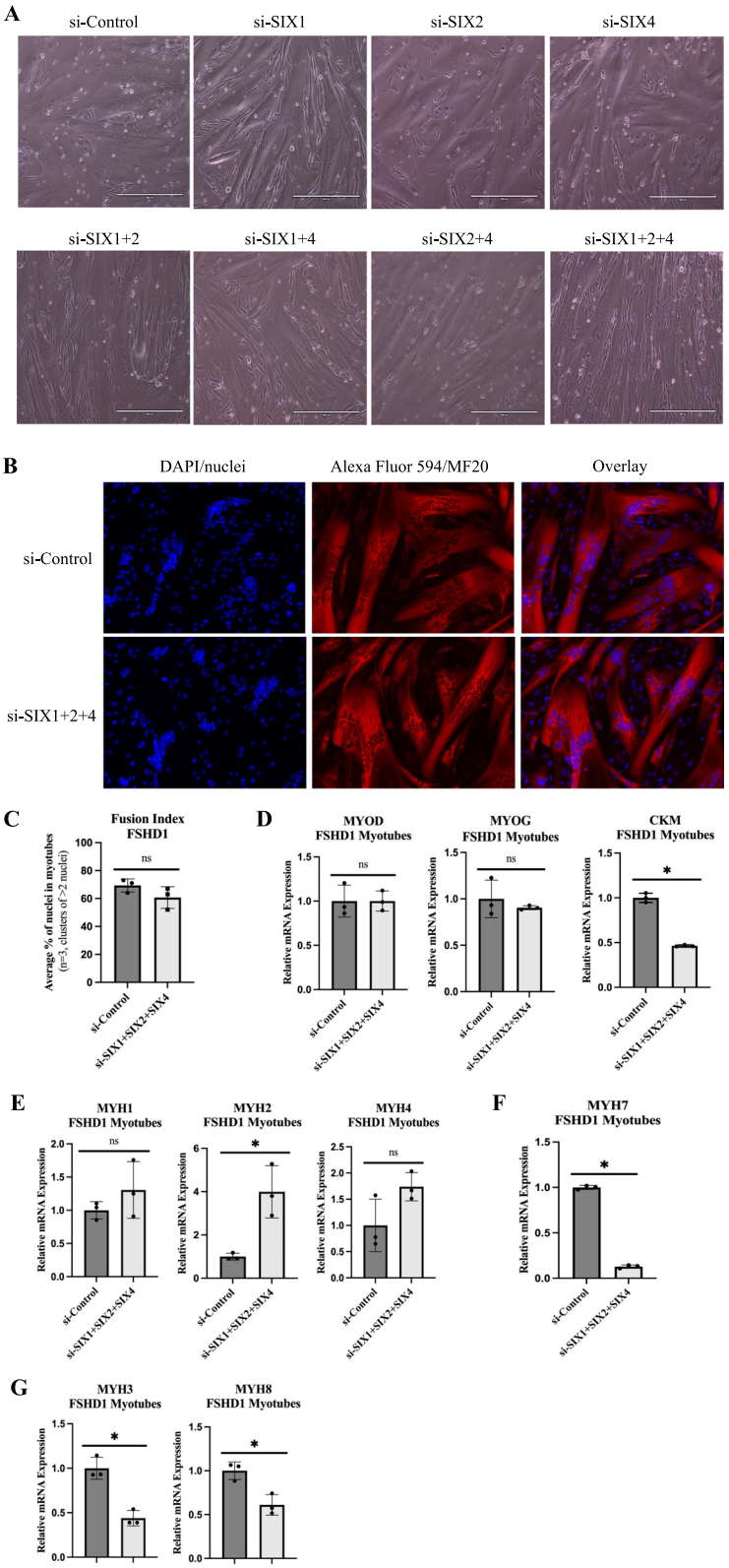

Fig. 2 (See legend on previous page.)

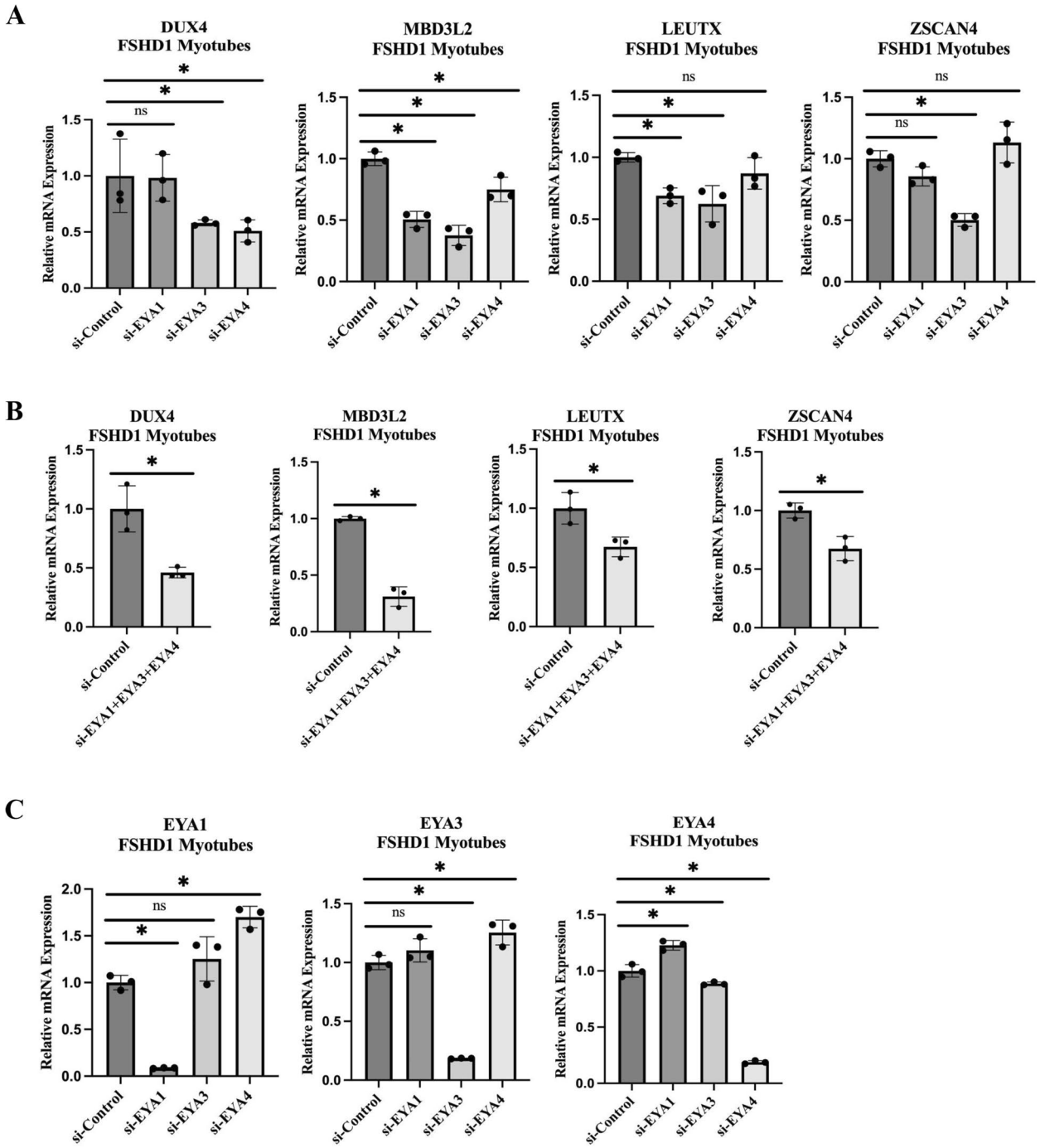

Fig. 3 Involvement of SIX coactivator, EYA, in DUX4 regulation. FSHD1 (54–2) myoblasts were transfected with EYA1, EYA3 and EYA4 and differentiated to form multinucleated myotubes. Shown are the relative mRNA levels from qPCR for (**A-B**) *DUX4*, **A-B** DUX4 targets (*MBD3L2*, *LEUTX* and *ZSCAN4*) and **C** each EYA target. Each experimental group ($n=3$) was normalized to a negative si-Control with the mean and standard deviation depicted. Asterisks demonstrate statistical significance between the control vs experimental group using an unpaired two tailed *t*-test (ns = not significant; $*p<0.05$)

in proliferating myoblasts and along multinucleated myotubes, albeit with some variation in staining intensity within myotubes (Fig. 5A-D). Staining intensity was significantly decreased with siRNA knockdown of SIX1 and SIX2 and increased with overexpression of SIX1 and SIX2, confirming specificity of staining (Fig. 5A-D).

Overall, these results indicate that SIX1 and SIX2 expression does not explain the restricted expression of DUX4 to a subset of muscle cell nuclei in FSHD.

DUX4 suppresses *SIX* gene expression

Previous RNA-seq studies demonstrated that *SIX1*, *SIX2* and *SIX4* expression was decreased after forced DUX4 expression [51, 61]. In light of the important role these factors play in driving *DUX4* expression, we hypothesized the existence of a negative feedback loop in which DUX4, induced in differentiating myotubes in a SIX transcription factor-dependent manner, would suppress SIX expression to limit its own transcription. Since DUX4 is induced in only a subset of nuclei during FSHD myogenic differentiation, this suppression would not be accurately assessed in analysis of RNA derived from the myocyte population. To overcome this limitation, we utilized immortalized non-FSHD human myoblasts (MB135) engineered with a doxycycline-inducible promoter driving *DUX4* so that the effects of DUX4 could be monitored in the population [61]. Induction of *DUX4* by treatment of MB135-iDUX myoblasts with doxycycline increased DUX4 target genes (*MBD3L2*, *LEUTX* and *ZSCAN4*) in a concentration and time-dependent manner with saturation of DUX4 target gene expression at 0.5–1.0 µg/mL doxycycline at 8 h and 0.25–0.5 µg/mL at 24 h (Fig. 6A-B). To determine the regulation of *SIX1*, *SIX2* and *SIX4* individually under high induction of DUX4, we treated the cells for 24 h with a near saturating concentration of doxycycline and found *SIX1*, *SIX2*, and *SIX4* expression were each significantly decreased at a doxycycline concentration that models an induction of DUX4 expression, consistent with previous profiling studies (Fig. 6C-E) [51, 61]. These results were replicated in differentiated MB135-iDUX4 cells, where we induced DUX4 with a near-saturating concentration of doxycycline for 24 h. We found that the induction of doxycycline increased DUX4 targets, largely replicating the effects seen in the treated myoblasts (Fig. 6F). Additionally, under the high induction of DUX4, we found *SIX1*, *SIX2*, and *SIX4* to be significantly reduced, as seen in the induced myoblasts (Fig. 6G) Together, these results suggest that high expression of DUX4 suppresses *SIX1*, *SIX2*, and *SIX4* in a negative feedback loop.

Discussion

The transcription factor DUX4 plays a critical role in early embryonic development, where it is involved in zygotic genome activation to orchestrate developmental gene expression before being silenced in most somatic tissues [4, 7, 16, 50]. Inappropriate DUX4 expression in adult skeletal muscle causes the progressive myopathic disease FSHD [8]. Additionally, DUX4 expression is activated in a subset of cancers, where it promotes immune evasion, resistance to checkpoint blockade and immunotherapy failure in metastatic disease [26, 62]. Consequently, there is immense interest in DUX4 as a therapeutic target and understanding the factors that promote its expression is critical to therapeutics development [63–65].

The hallmark of FSHD patient-derived muscle cells in tissue culture is myogenic differentiation-dependent transcriptional bursts of *DUX4* in rare nuclei [21, 54, 55, 66]. In FSHD, loss of repeat-mediated epigenetic repression at D4Z4 macrosatellite repeats is observed with specific decreases in repressive epigenetic marks including DNA methylation, histone 3 lysine 9 trimethylation (H3K9me3) and H3K27me3 [9]; however, there is little detail on the complex transcriptional activation events that ultimately produce *DUX4* transcripts. Previous work has identified the long non-coding RNA *DBE-T* that may initiate *DUX4* transcription though recruitment of ASH1L, and WDR5 has also been shown to be required [32, 35]. Additionally, several epigenetic regulators were found to promote *DUX4* expression including ASH1L, BRD2, KDM4C, SMARCA5 and BAZ1A [33]. While the restriction of transcriptional bursts to rare nuclei remains to be understood, it is presumed that myogenic differentiation signals drive *DUX4* expression in those nuclei that have escaped epigenetic silencing, and indeed myogenic enhancers upstream of D4Z4 repeats have been described, although no specific transcription factors were demonstrated to bind this region [9, 64, 65, 67]. A well-established myogenic differentiation signal is p38 MAPK activation, which we and others have shown positively regulates transcription of *DUX4*, both in vivo and in vitro, supporting p38 as a drug target in FSHD [28, 68]. Yet, the dynamic interactions between p38 MAPK and the factors required for *DUX4* regulation remain to be

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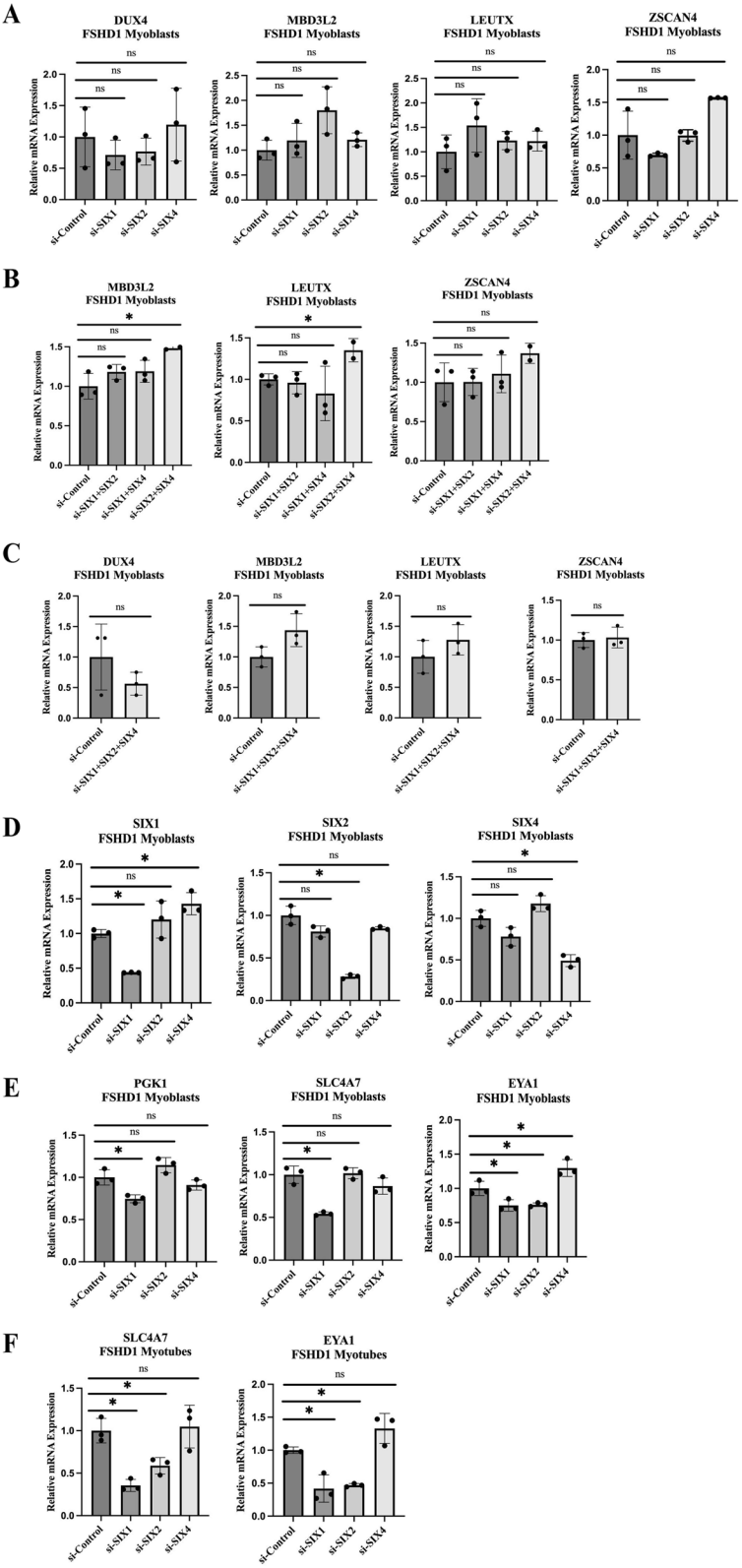
Fig. 4 Low level of DUX4 expression in myoblasts are unaffected by SIX1/2/4 knockdown. FSHD1 (54–2) were transfected with SIX1, SIX2, and SIX4 siRNAs in proliferating myoblasts 72 h before harvesting. qPCR results show relative mRNA expression for **A** and **C** *DUX4*, **A-C** DUX4 targets and **D** siRNAs targeting each respective SIX target. **E-F** Effects on SIX1 and SIX2 targets (*PGK1*, *SLC4A7* and *EYA1*) were analyzed in **E** myoblasts and **F** myotubes. All conditions were carried out in triplicates with the mean and standard deviation depicted. Each experimental group ($n = 3$) was normalized to a negative si-Control with the mean and standard deviation depicted. Asterisks demonstrate statistical significance between the control vs experimental group using an unpaired two tailed *t*-test (ns = not significant; $*p < 0.05$)



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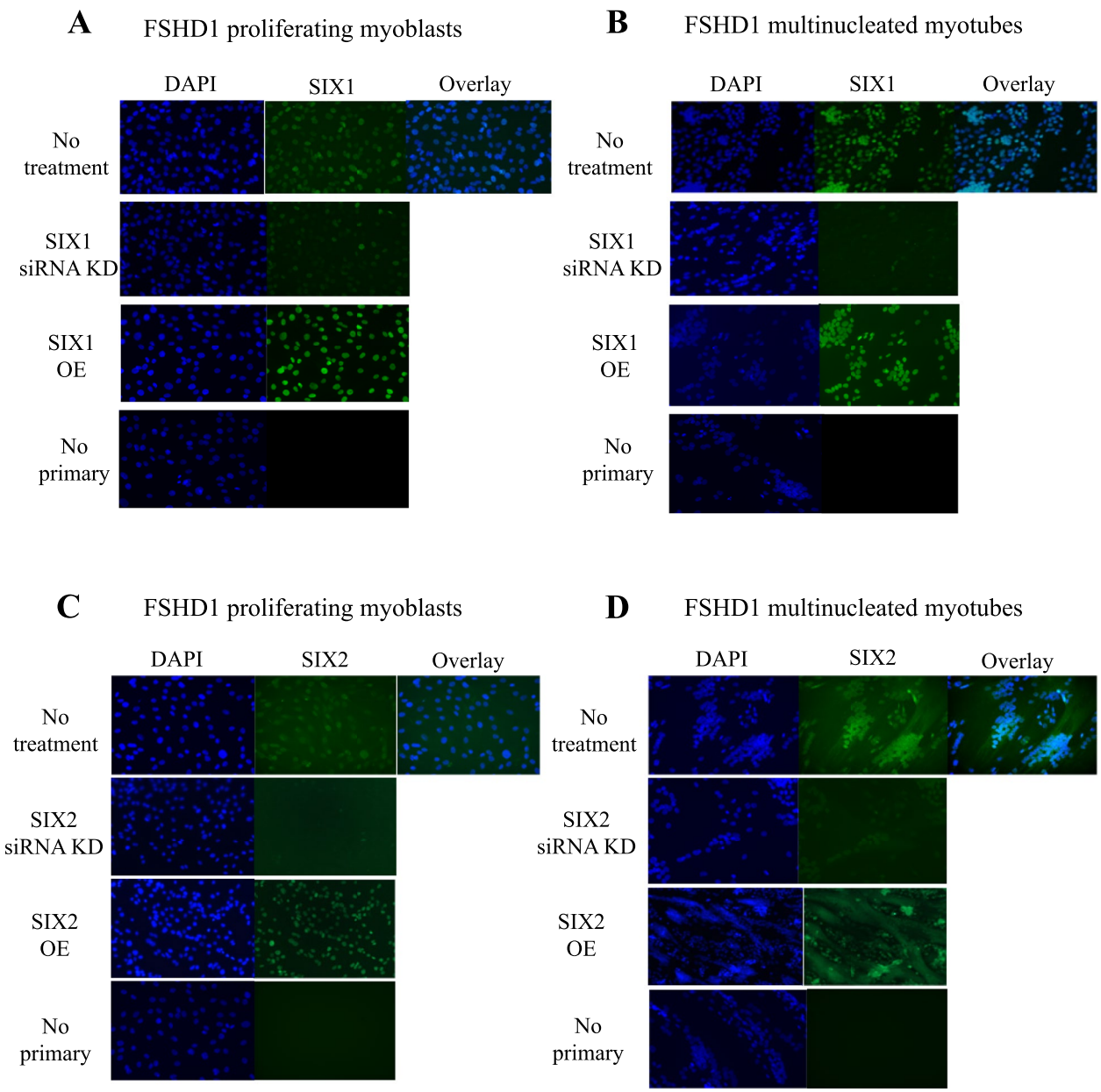

Fig. 5 SIX1 and SIX2 protein expression in FSHD myoblasts and myotubes. FSHD1 (54–2) proliferating myoblasts (left) and myotubes (right) were stained with (**A-B**) anti-SIX1 and (**C-D**) anti-SIX2 antibodies (green) and nuclei counterstained with DAPI (blue)

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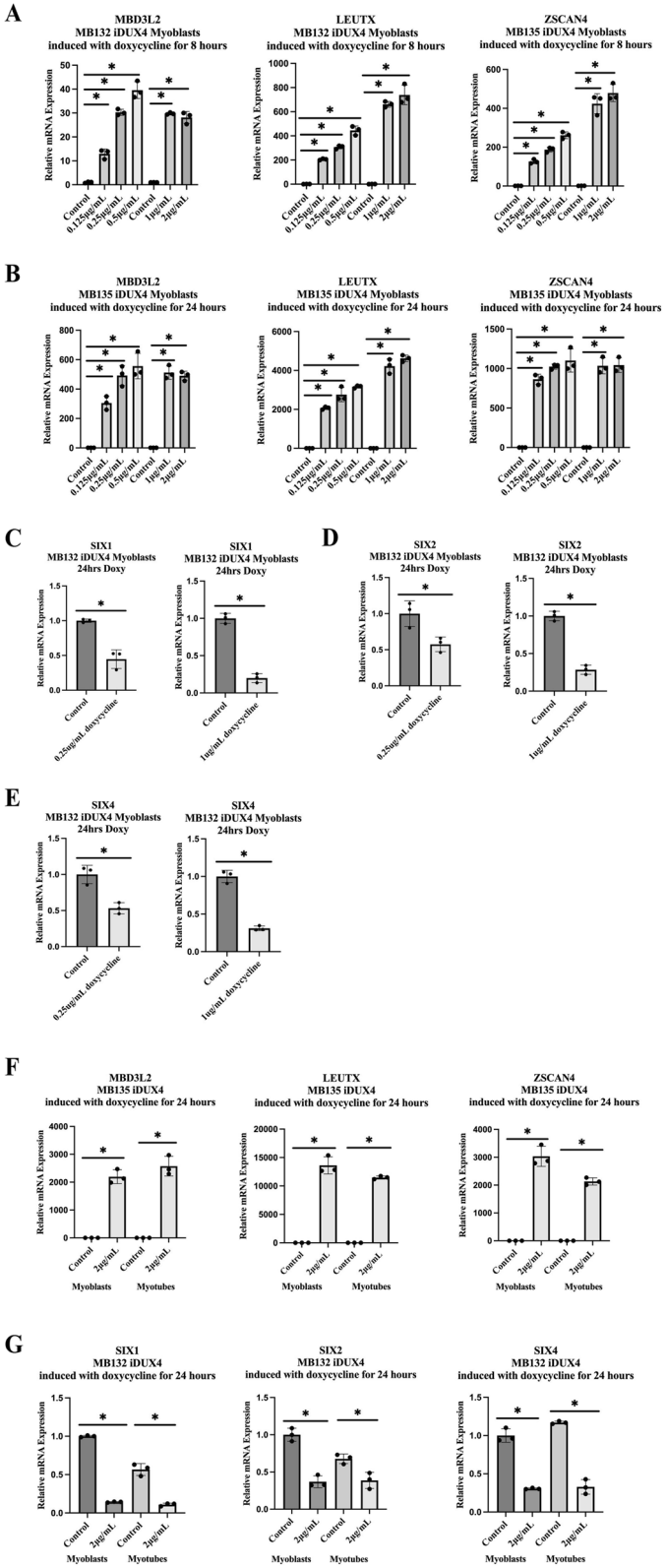
Fig. 6 Forced DUX4 expression downregulates SIX1, SIX2 and SIX4. Stable non-FSHD (MB135) doxycycline inducible DUX4 (iDUX4) myoblasts were induced with various concentrations of doxycycline (0.125, 0.25, 0.5, 1, and 2 µg/mL) for (**A**) 8 h and (**B**) 24 h. Shown are the relative mRNA levels from qPCR for (**A** and **B**) DUX4 targets *MBD3L2*, *LEUTX* and *ZSCAN4*, (**C**) *SIX1*, (**D**) *SIX2* and (**E**) SIX4 at 0.25 µg/mL and 1 µg/mL of doxycycline for 24 h. iDUX4 myoblasts were then differentiated with the addition of 2 µg/mL doxycycline for the last 24 h of differentiation. Shown are the relative mRNA levels for (**F**) DUX4 targets and (**G**) SIX1, SIX2, and SIX4. Each experimental group ($n = 3$) was normalized to a negative control (DMSO) with the mean and standard deviation depicted. Asterisks demonstrate statistical significance between the control vs experimental group using an unpaired two tailed *t*-test (*$p < 0.05$)



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elucidated. Modulation of other signaling pathways and effectors also regulates *DUX4*, including Wnt/β-catenin activation, β2-adrenergic agonism/protein kinase A activation, bromodomain and extra-terminal domain (BET) inhibition, casein kinase 1 δ/ϵ inhibition and other kinase pathways, yet the intersection of these pathways with *DUX4* transcription has yet to be defined [66, 69–72].

In this study, we demonstrate that SIX transcription factors are necessary for myogenic differentiation-dependent bursts of DUX4 in patient-derived muscle cells. We found using combined siRNA knockdown of SIX1, SIX2 and SIX4, that *DUX4* expression was nearly completely suppressed (~98%) in FSHD1 (54–2 and 16-ABIC) and FSHD2 (MB200) myotubes. Combinations containing *SIX2* siRNA were the most effective at reducing *DUX4* mRNA levels, highlighting the particular importance of SIX2 in driving *DUX4* transcription. Suppression of *DUX4* was context-dependent, occurring only in differentiating cells in which *DUX4* expression bursts are induced. Knockdown of SIX1, SIX2 and SIX4 failed to affect the low-level expression of *DUX4* in undifferentiated FSHD myoblasts. Direct targets of SIX1 (*PGK1, SLC4A7*) and SIX2 (*EYA1*) are reduced by SIX1 and SIX2 knockdown, respectively, suggesting they are transcriptionally active in myoblasts. Importantly, since SIX protein levels are not induced during differentiation, these data indicate that SIX protein activity in promoting *DUX4* expression is likely dependent on myogenic differentiation signaling. As mentioned above, one such aspect of myogenic signaling is p38 MAPK activation, which orchestrates genome-wide transcriptional changes [73, 74]. Although SIX factors are not known to be regulated by p38-mediated phosphorylation, other kinases have been described to regulate phosphorylation levels of SIX transcription factors, such as PKC, CK2, Cdc2 kinase and GSK3β [75–78]. Therefore, it is possible that p38 modulates SIX factors, directly or indirectly, during muscle differentiation. Another aspect of myogenic signaling may be the availability of other transcription factors that could act synergistically with SIX proteins, a common theme for SIX transcription factor activities in driving different stages of muscle development [38]. An alternative interpretation is that the chromatin environment is changed during differentiation to allow SIX transcription factors access to DUX4 regulatory regions. The potential regulation of SIX transcription factors by myogenic signaling pathways such as p38 warrants further investigation.

We investigated the potential regulation of *DUX4* transcription by the established and widely explored SIX coactivator, EYA [38, 52, 79, 80]. Knockdown of EYA1, EYA3, and EYA4 partially suppressed *DUX4* expression in FSHD1 myotubes, demonstrating an involvement in DUX4 regulation; however, EYA suppression was moderate compared to knockdown of SIX1, 2, or 4. Interestingly, EYA has been found to interact directly with chromatin remodeling complex ATPases to allow for the SIX-EYA complex to promote DNA accessibility and initiate transcriptional activation [38, 81]. Given the involvement and importance of p38 MAPK's recruitment of chromatin remodeling complexes and the proposed activation of EYA by p38 phosphorylation, this potential mechanism needs to be further explored [82, 83]. Alternatively, SIX2 has been shown to directly recruit the SWI/SNF chromatin remodeling complex independent of EYA and is known to activate transcription in the absence of EYA cofactors [84]. Our data suggests that SIX proteins utilize both EYA-dependent and EYA-independent mechanisms to drive DUX4 expression.

Important for making inferences about *DUX4* regulation, we demonstrated that knockdown of SIX genes does not inhibit myogenic differentiation. Multinucleated myotubes formed similarly between control cultures and combined SIX1, SIX2, and SIX4 knockdown cultures in two FSHD1 (54–2 and 16-ABIC) and one FSHD2 (MB200) patient-derived cell lines. Markers of differentiation such as *MYOD1*, *MYOG* and *MYH1* were unaffected. One late differentiation marker, creatine kinase, M-type (*CKM*), was significantly decreased by combined SIX knockdown; however, *CKM* is known to be directly regulated by SIX transcription factors [85]. RNA levels for *MYH3* and *MYH8*, genes encoding developmental and regenerative myosin heavy chains, were both decreased by combined knockdown. Interestingly, we found differential effects on genes involved in myofiber type specification. While the slow myofiber type gene, *MYH7*, was dramatically decreased, the fast fiber type gene, *MYH2,* was increased by combined SIX1/2/4 knockdown. These changes are surprising given the well-characterized role of SIX factors in promoting fast fiber gene expression in mice [38, 86]. SIX transcription factors accumulate preferentially in the nuclei of Type 2 fast-fiber skeletal muscles, the fibers preferentially affected with FSHD [38, 87]. Therefore, it will be important to investigate the potential connection between muscle fiber types expressing SIX family members and those that are selectively lost in degenerating FSHD muscle. For example, FSHD affects fast-twitch oxidative (MYH2; Type 2A) and glycolytic muscle fibers (MYH1, Type 2X), reducing maximum force capacity in select muscle groups that are affected [88, 89]. Nonetheless, since combined SIX knockdowns did not prevent myogenic differentiation, we were able to demonstrate their critical role in *DUX4* induction during differentiation.

A poorly understood phenomenon of FSHD is the restricted and sporadic nature of DUX4 expression in only a subset of nuclei. In vitro, DUX4 is expressed at

relatively low levels in undifferentiated myoblasts; however, following differentiation, there is a burst of DUX4 in rare sentinel nuclei [55]. In this study, we demonstrated the requirement of SIX1/2/4 to induce *DUX4* transcriptional activation. Because DUX4 could be regulated by several factors, we wanted to determine if its restricted expression pattern was due to the absence or presence of necessary transcription factors, like SIX1 and SIX2. We found that although SIX1 and SIX2 are necessary for DUX4 expression, they are present in all myonuclei in our FSHD cultures and thus their expression pattern does not explain the restricted expression of DUX4 to a subset of nuclei. Together, these data support the idea that DUX4 requires, in addition to SIX transcription factors, some other rare myogenic signaling event or cumulative epigenetic injury to escape silencing. Additionally, we investigated the potential for feedback regulation between DUX4 and SIX1, SIX2 and SIX4 using a doxycycline-inducible DUX4 cell model that allows DUX4 expression in every cell nucleus. Overall, we found *SIX1*, *SIX2* and *SIX4* were downregulated following DUX4 induction, suggesting negative feedback regulation. Previously, it was demonstrated that ectopic DUX4 expression can lead to several feedback and feedforward mechanisms contributing to its toxic persistent expression in the disease state [90]. Conversely, in embryonic development, DUX4 is expressed in a discreet window, inducing zygotic genome activation (ZGA) before being silenced as development proceeds [20, 91]. Therefore, it will be important to identify drivers of *DUX4* during ZGA, the mechanisms of suppression, and similarities and differences with FSHD muscle cells.

Where do SIX factors bind to exert their effect on *DUX4* transcription and do they act directly or indirectly? Himeda et al. described myogenic enhancers upstream of the 4q D4Z4 repeats that activate *DUX4* expression in FSHD skeletal myocytes [67]. In particular, *DUX4* myogenic enhancer 2 (DME2) exhibits a strong muscle-specific interaction with the *DUX4* promoter and in silico analysis of the sequence revealed the presence of a consensus binding motif for SIX1/4 (MEF3) as well as other regulators of muscle gene expression [67], suggesting that SIX proteins directly activate *DUX4* transcription through DME2. Another possibility is that they bind to a DUX4 regulatory region more proximal to *DUX4* (e.g. within D4Z4 repeats). Alternatively, SIX factors may act indirectly through activating other factors that then directly promote *DUX4* transcription. It is also not clear if SIX1/2/4 are functionally acting at the same site or if they participate in separate steps of *DUX4* transcription by binding at multiple locations and/or during sequential steps within a transcription cascade. It will be important to experimentally determine genomic binding regions for each SIX family member to understand their individual roles in promoting *DUX4* activation using chromatin binding assays.

SIX transcription factors are known master regulators during the development of the head, sensory organs, kidney and skeletal muscle through their control of progenitor cell populations and differentiation mechanisms [38, 41, 46, 92]. Interestingly, *SIX* genes are expressed in the pre-placodal ectoderm and later in sensory cranial placodes, precursors of sensory organs of the head [93, 94]. Recently, it has been shown that mutations in SMCHD1, most commonly associated with FSHD2, are also responsible for the congenital defect arrhinia, complete lack of the external nose [95, 96]. Inoue et al. demonstrated a potential connection with DUX4 expression by showing that defects in SMCHD1 can allow DUX4 expression during cranial placode differentiation, causing DUX4 toxicity and cell death, providing a plausible link between SMCHD1 mutations and developmental defects such as arrhinia [41]. It is tempting to speculate that SIX transcription factors are a common link to cells capable of expressing DUX4 and that other co-morbidities in FSHD, such as high frequency hearing loss, may be due to a similar toxicity in which DUX4 is expressed during development of otic placodes. Understanding the nature of *DUX4* regulation by SIX transcription factors will be essential for understanding the pathogenesis of FSHD, comorbidities potentially linked to disease severity in FSHD, and cancer. Further investigation will be needed to identify other transcriptional and epigenetic factors regulating DUX4 expression to develop a mechanistic understanding of pathogenic DUX4 expression.

Conclusions

Our study identified novel *DUX4*-regulatory transcription factors that are necessary for aberrant DUX4 expression in FSHD. Knockdown of SIX1, SIX2 and SIX4 results in a nearly complete suppression of *DUX4* mRNA, in vitro. Our findings highlight a negative feedback loop between SIX1, SIX2 and SIX4, under high induction of DUX4. Importantly, further investigation will be necessary to identify genomic regions bound by each protein to develop a mechanistic understanding.

Abbreviations

FSHD	Facioscapulohumeral muscular dystrophy
FSHD1	Facioscapulohumeral muscular dystrophy type 1
FSHD2	Facioscapulohumeral muscular dystrophy type 2
DUX4	Double Homeobox 4
SIX	*sine oculis*
siRNA	Small interfering RNA
qRT-PCR	Quantitative real-time polymerase chain reaction

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
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Declarations

Ethics approval and consent to participate
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Competing interests
The authors declare no competing interests.

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