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A cre-inducible DUX4 transgenic mouse model for investigating facioscapulohumeral muscular dystrophy

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Abstract

The Double homeobox 4 (DUX4) gene is an important regulator of early human development and its aberrant expression is causal for facioscapulohumeral muscular dystrophy (FSHD). The DUX4-full length (DUX4-fl) mRNA splice isoform encodes a transcriptional activator; however, DUX4 and its unique DNA binding preferences are specific to old-world primates. Regardless, the somatic cytotoxicity caused by DUX4 expression is conserved when expressed in cells and animals ranging from fly to mouse. Thus, viable animal models based on DUX4-fl expression have been difficult to generate due in large part to overt developmental toxicity of low DUX4-fl expression from leaky transgenes. We have overcome this obstacle and here we report the generation and initial characterization of a line of conditional floxed DUX4-fl transgenic mice, FLExDUX4, that is viable and fertile. In the absence of cre, these mice express a very low level of DUX4-fl mRNA from the transgene, resulting in mild phenotypes. However, when crossed with appropriate cre-driver lines of mice, the double transgenic offspring readily express DUX4-fl mRNA, protein, and target genes with the spatiotemporal pattern of nuclear cre expression dictated by the chosen system. When cre is expressed from the ACTA1 skeletal muscle-specific promoter, the double transgenic animals exhibit a developmental myopathy. When crossed with tamoxifen-inducible cre lines, DUX4-mediated pathology can be induced in adult animals. Thus, the appearance and progression of pathology can be controlled to provide readily screenable phenotypes useful for assessing therapeutic approaches targeting DUX4-fl mRNA and protein. Overall, the FLEx-DUX4 line of mice is quite versatile and will allow new investigations into mechanisms of DUX4-mediated pathophysiology as well as much-needed pre-clinical testing of DUX4-targeted FSHD interventions in vivo.

Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is caused by epigenetic dysregulation of the 4q35 D4Z4 macrosatellite repeat, which leads to aberrant myogenic expression of the Double
homeobox 4 (DUX4) gene. DUX4 encodes a paired homeobox domain transcriptional activator and regulator of cleavage-stage genes and therefore is not typically expressed in healthy somatic cells [1–10]. In FSHD, however, DUX4 misexpression in differentiated skeletal muscle ultimately initiates numerous potentially detrimental events including the induction of apoptosis [11–14], and activation of the inflammatory immune response [15, 16]. While several DUX4 alternative mRNA isoforms are generated by alternate 5' splice site usage in the first exon, only the DUX4-full length (DUX4-fl) mRNA is pathogenic [4, 5, 17]. DUX4-fl expression is highly regulated and apparently restricted to testis and cleavage-stage embryos where the DUX4-mediated transcriptional program is key for human zygotic genome activation, supporting a normal function for DUX4-FL in germ cells and during early human development [1, 3, 5, 18, 19]. However, individuals meeting the genetic, epigenetic, and clinical criteria for FSHD express stable DUX4-fl mRNA and DUX4-FL target genes in their skeletal muscles, initiating a detrimental cascade of events which culminate in muscle pathology and disease [4–6, 9, 10, 20, 21]. Somatic expression of DUX4-fl mRNA per se is not necessarily pathogenic since rare expression can be detected in some cultures of healthy myogenic cells and muscle biopsies, albeit at levels significantly lower than those found in similar tissues from FSHD-affected subjects, suggesting that the levels and/or timing of somatic DUX4 expression dictate disease [6–8]. Thus, aberrantly increased DUX4 expression is the primary mediator of FSHD pathophysiology, the DUX4-fl mRNA and protein are prime FSHD therapeutic targets, and animal models for FSHD should be based on expression of DUX4.

FSHD is an autosomal dominant, gain-of-function disease and thus should be amenable to modeling in mice by overexpression of DUX4-fl mRNA. However, animal models of FSHD have suffered from serious issues with respect to pathogenic expression levels, tissue distribution, and cellular toxicity [12, 22–26]. Early attempts to model the disease in mice using viral over-expression produced massive pathology and physiological effects not characteristic of FSHD [13], resulting in conclusions based on artifacts [27]. Conversely, the D4Z4-2.5 transgenic mouse model, in which a pathogenic FSHD1-sized D4Z4 repeat array consisting of 2.5 D4Z4 repeat units [28, 29], including the distal DUX4 polyadenylation site rendering the distal repeat unit capable of producing the stable DUX4-fl mRNA [4], suffered from too little DUX4 expression in muscle and failed to show an FSHD-like phenotype [23]. When levels of the FSHD modifier gene, Smchd1, were decreased, the levels of DUX4-fl were increased and the phenotypes were exacerbated, however, the model still failed to recapitulate FSHD-like muscle pathology [26]. Interestingly, a tetracycline regulated transgenic knock-in DUX4 mouse model that suffered from severe pathology and early lethality was recently rescued by inserting an inefficient DUX4 mRNA polyadenylation signal to decrease leaky DUX4-fl transgene expression allowing for animals to survive and develop FSHD-like phenotypes in muscles [24, 30]. Together, these cases highlight both the importance and difficulty of mimicking the pathogenic mechanisms of FSHD in mouse models [31].

In FSHD, the DUX4 gene is typically expressed in only a small fraction (<1%) of myogenic cells, ultimately leading to debilitating muscle pathology over time [5, 6, 32]. This may account, in part, for the generally late onset of clinical symptoms in FSHD patients. In addition, DUX4-fl is expressed in sporadic bursts in differentiated FSHD muscle cells, an event that is epigenetically suppressed in healthy and asymptomatic subjects [7, 14, 33]. However, DUX4-fl expression in affected FSHD muscle, even when bursting, is still extremely rare, highly variable, and difficult to detect [5, 6, 14]. Thus, based on our current understanding of the FSHD pathogenic mechanism, adult-onset, mosaic transgene expression in skeletal muscle is necessary for modeling an FSHD-relevant DUX4 expression profile and is key to achieving FSHD-like pathophysiology. Yet nothing is known about potential developmental or extra-muscular contributions of DUX4-fl expression to FSHD. Ideally, a mouse model for investigating this
disease should incorporate flexibility in dictating DUX4-fl expression, and thus allow some of these critical questions to be addressed.

Here we report the generation and initial characterization of a conditional DUX4-fl transgenic mouse model, termed FLexDUX4. Similar to the situation in FSHD, DUX4-fl is expressed at extremely low levels in adolescents and adult FLexDUX4 mice. Mating with cre-expressing lines of mice leads to induction of DUX4-fl in the spatiotemporal manner of the cre driver, thereby providing a highly flexible model for investigating the effects of DUX4-fl expression. When crossed with a skeletal muscle-specific, tamoxifen inducible cre-expressing mouse strain, skeletal muscles of the double transgenic offspring can be induced to produce mosaic expression patterns of DUX4-fl expression in a fraction of skeletal myonuclei, similar to the spontaneous mosaic bursting of DUX4-fl expression found in FSHD skeletal myocytes [14], ultimately resulting in an FSHD-like myopathy. Thus, FLexDUX4 mice are a useful model for both developmental and disease-relevant studies on DUX4-fl, and provide a suitable model for FSHD therapeutic interventions targeting DUX4-fl mRNA, protein, and certain downstream pathways.

Materials and methods

Ethics statement

All animal procedures were approved by the local IACUC committees at the University of Massachusetts Medical School and the University of Nevada, Reno. The work performed at the University of Nevada, Reno was approved under protocol #0701. The work performed at the University of Massachusetts Medical School was approved under protocols #A-2447-13 and #A-2535-16. Anesthesia was performed using isoflurane inhalation. Euthanasia was performed using isoflurane inhalation followed by exsanguination.

Transgenic mouse generation and crosses

Transgenic mice were generated by genOway SA (France) using the FLex directional switch system to bypass the embryonic lethality from leaky embryonic DUX4 transgene expression [34, 35]. The DUX4 genomic sequence (S1 Sequence), containing all three exons and both introns, was synthesized in vitro with silent mutations made into the two known 5' splicing donor sites for the DUX4-s isoforms. This sequence was cloned into the proprietary genOway Rosa26 targeting vector, and completely sequenced. Murine C57BL/6 ES cells were transfected and clones were selected and screened for homologous recombination into the Rosa26 locus, first by PCR, then confirmed by Southern blotting. Properly targeted ES cells were injected into blastocysts and implanted to generate male chimeras. The chimeras were mated to C57BL/6 females to confirm germline transmission. Chimeras were mated with the genOway proprietary ubiquitous Flp recombinase expressing mice for in vivo removal of the Neomycin selection cassette. The F1 hemizygous FLexDUX4 mice devoid of the Neomycin resistance gene were delivered to the Jones lab.

C57BL/6 mice and the following Cre driver lines were purchased from Jackson Labs (Bangor, Maine): ACTA1-MCM refers to B6.Cg-Tg(ACTA1-cre/Esr1<tm1(cre/ERT2)]Ejb/J (JAX 008085), Sox2-cre refers to B6.Cg-Tg(Sox2-cre)1Amc/J (JAX 008454), ACTA1-cre refers to B6.Cg-Tg(ACTA1-cre)79Jme/J (JAX 006149), UBC-cre/ERT2 refers to B6.Cg-Tg(UBC-cre/ERT2)1Ejb/J (JAX 008085), and Pax7-cre/ERT2 refers to B6.Cg-Pax7<tm1(cre/ERT2)Gaka>/J (JAX 017763).

Transgenic mouse genotyping

Genomic DNA was isolated from 2mm tail snips from <10-day-old mice. PCRs were performed with ~100ng genomic DNA, 1X GoTaq Buffer, 200μM dNTPs, 0.5U GoTaq DNA
polymerase (Promega), 600nM primer TJ76F (5’ -CAATACCTTTCTGGAGTTCTCTGCTGC),
400nM primer TJ77R (5’ - CTCGTGTAGACAGACCTAGACAATTTGTTG), 200nM primer
TJ78R (5’ - TGCAGGACAACGGCCACACACC). Reactions were incubated at 94˚C for 3min,
then cycled 30 times (94˚C for 20sec, 62˚C for 20sec, 72˚C for 35sec), followed by a final 2min
extension at 72˚C. Reactions were separated on a 1.5% agarose gel and visualized by ethidium
bromide staining. Homozygous pups produce a 409bp product, hemizygous pups produce
409bp and 175bp products, and wild type pups produce a 175bp product.

Transgene recombination
Genomic DNA was isolated from the organic phase of TRIzol RNA tissue extractions as per
manufacturer’s protocol. Two PCRs were performed on genomic DNA (50ng/reaction) using
0.1μl GoTaq (Promega) in 1X GoTaq buffer, 200μM dNTPs, 400nM primer TJ76F (5’–CAAT
TACCTTTCTGGAGTTCTCTGCTGC), and either 400nM reverse primer TJ77R (5’–CTCGTG
TAGACAGACCTAGACAATTTGTTG) for detecting the non-recombined transgene (406bp
product) or 400nM reverse primer 14A2-Rev (5’–AGGCTCGCAAGGGCCCTGCTT) for detect-
ing the recombined transgene (440bp product). Reactions were incubated at 94˚C for 3min,
then cycled 32 times (94˚C for 20sec, 62˚C for 20sec, 72˚C for 35sec), followed by a final 2min
extension at 72˚C. Reactions were electrophoresed in separate wells of the same 1.5% agarose
gel and visualized by ethidium bromide staining. Band intensities were quantified and the
recombination rate was calculated as % recombination = TJ76F;14A2-Rev band / (TJ76F;
TJ77R band + 76F;14A2-Rev band) x 100.

Tamoxifen (TMX) administration
For intraperitoneal (IP) injections of TMX, tamoxifen free base (Sigma #T5648) was dissolved
in 100% ethanol (200mg/ml) at 55˚C and added to warm sterile corn oil (ThermoFisher
S25271) to make a 20mg/ml stock. Stock TMX aliquots were warmed to 37˚C, briefly, and
diluted further with warm corn oil 10-fold just prior to use. Twelve-week-old mice were
weighed and injected IP on two consecutive days with the appropriate volume (100μl for a 20g
mouse) of TMX for a final concentration of 10mg/kg. Alternatively, when specifically indi-
cated, TMX-laced chow (400mg/kg tamoxifen citrate) purchased from Envigo (TD.130860)
was provided ad libitum for 5 days providing an average daily dose of ~40mg/kg for 20-25g
body weight assuming a 3-4g daily food intake.

DNA methylation analysis
Genomic DNA was isolated from dissected muscle samples using TRIzol (ThermoFisher), as
per manufacturer’s instructions, bisulfite converted, and analyzed using the bisulfite sequenc-
ing 4qA (BSSA) protocol, as described [7, 36].

Gene expression analysis by RT-PCR
Total RNA was extracted from dissected mouse muscles homogenized in 10 volumes TRIzol
(ThermoFisher) using the TissueLyser LT (Qiagen), as per manufacturer’s instructions, fol-
lowed by clean-up using the RNeasy mini kit (Qiagen) to remove all remaining genomic DNA.
Quantitative expression of the 1.4kb DUX4-fl RT-PCR product was performed as described [4].
Control reactions skipping the first-strand synthesis step were performed to ensure purity of
the RNA samples and assuring the results were not from contaminating genomic DNA. Quan-
titative DUX4-fl mRNA expression was analyzed using nested qRT-PCR, as described [37].
Expression of DUX4 target genes was analyzed by qPCR using 5-50ng cDNA, generated with
iScript Supermix (Bio-Rad) per manufacturer’s instructions. Expression of DUX4-fl was normalized to 18S rRNA or Rpl37 RNA as indicated, expression of target genes was normalized to Rpl37 RNA. Oligonucleotide primer sequences for DUX4-fl, Rpl37, and 18S rRNA are as previously reported [33]. Oligonucleotide primer sequences for Trim36, Wfdc3, and Cxcr4 are as previously reported [23].

**Alcian blue staining**

The E16.5 embryos were obtained from crossing male ACTA1-cre/+ with female FLExDUX4/+, and the extraembryonic membrane of each embryo was collected and used for genotyping. Alcian blue staining of the fetal E16.5 cartilaginous skeleton was performed as described [38]. Briefly, embryos were dissected at stage 16.5 d.p.c. in PBS and the extraembryonic membrane was removed and used for genotyping. Embryos were fixed in Bouin’s fixative for 2h, washed in ammonium hydroxide:ethanol solution for 24h with 6 changes of solution and equilibrated in 5% acetic acid twice for 1hr each. Embryos were soaked in alcian blue stain for 2h, washed twice in 5% acetic acid for 1h each, then cleared in methanol (2X for 1h each) and then finally in BAB (benzyl alcohol:benzyl benzoate) solution. The stained embryos were imaged using the Leica MZ7.5 and Zeiss Axioacam.

**Histology**

Freshly dissected muscles were kept moist, coated with O.C.T. compound, frozen in liquid nitrogen-cooled isopentane and stored at -80°C until sectioning. The 10–12μm cryosections were mounted on slides, air-dried for 30 min before staining or storage. Sections were fixed with cold acetone for 5 min for Hematoxylin and Eosin staining or picrosirius red staining.

**Picrosirius red staining**

Staining was performed as described [39]. Cryosections (12μm) cut mid belly of the Tibialis anterior muscle or heart were mounted and fixed in 4% paraformaldehyde/PBS for 10 min, rinsed with dH₂O, and then dehydrated with a series of 30 sec ethanol washes (70%, 95%, 100%) and air dried. Sections were stained for 1 hr in fresh picrosirius red solution (0.1% direct red 80, 1.3% saturated picric acid), washed twice with 0.5% acetic acid and three times with dH₂O. Stained sections were dehydrated with a series of ethanol washes (70% for 30 sec, 95% for 30 sec, and 100% for 1 min), cleared with xylene for 5 min, and mounted with Cytoseal 60. A series of micrographs from each muscle section were captured using a 10X objective on a Leica DM2000 and reconstituted to form an entire muscle cross-section. A custom script was written in MATLAB (Mathworks) to determine the number of pixels stained red and the total number of pixels stained. Muscles from 3 mice, 5 sections per muscle, for each treatment were analyzed.

**DUX4 Immunohistochemistry**

Tissue sections were fixed with 4% paraformaldehyde/PBS on ice for 20 min, permeabilized with 0.25% TritonX-100/PBS for 10 min, then incubated with blocking solution (5% normal goat serum (NGS), 2% BSA, 0.01% TritonX-100/PBS) for 30 min. Sections were incubated with DUX4 E5-5 antibody (1:200, abcam) at 4°C overnight, then subsequently incubated with Alexa 594 Goat anti-rabbit IgG (1:500, ThermoFisher) at room temperature for 40 min. Stained sections were mounted in ProLong Gold with DAPI for nuclear staining.
Four-limb hanging test

A 6x6-inch wire mesh made of 2-mm thick stainless steel wire was hand-held ~35-cm above a layer of soft bedding material. Mice were allowed to grasp the middle of the mesh in an upright position and acclimated. The mesh was slowly rotated until inverted, the timer was started, and time until the mouse fell to the bedding was recorded. Each mouse was given three trials per session and the longest hang time was recorded.

Results

Generation and Characterization of the FLExDUX4 mouse line

Our goal was to generate a viable and fertile transgenic mouse model containing an intact human DUX4 transgene that, upon controlled induction, produces mosaic expression and a myopathic phenotype [4, 10, 18]. While the DUX family of transcription factors is functionally conserved in mammals, the intact human DUX4 gene is specific to old-world primates and not found in mice [2, 3, 19, 40]. Therefore, investigating the potential pathogenic effects of DUX4-fl expression in mice requires the human DUX4 gene to be incorporated into the mouse genome. To accomplish this, we synthesized a DUX4 transgene that retained the intact human DUX4-fl gene structure, including the 5’ untranslated region (UTR), all three exons and both introns, the endogenous PAS, and the distal auxiliary elements that enhance DUX4 mRNA cleavage and polyadenylation events (Fig 1A, S1 Sequence) [1, 5, 17, 41, 42]. Importantly, many of these elements are prime targets for sequence-based therapies [43, 44]. We incorporated four silent mutations in the 5’ splice acceptor sites for the two most prominent DUX4 alternative mRNA isoforms found in myogenic cells. These transcripts encode the non-pathogenic DUX4-short (DUX4-S) isoforms, which have a dominant negative effect on DUX4-FL; thus, it was important to prevent their expression from our DUX4 transgene [5]. We also chose not to add any epitope tag to the DUX4-fl mRNA sequence that might interfere with splicing, downstream functions of the protein, or therapeutic targeting. Following transfection of our DUX4 expression construct into murine C2C12 cells, we confirmed its functionality by RT-PCR, and the lack of detectable expression of DUX4-s mRNA (S1 Fig). Finally, because DUX4-FL is highly cytotoxic during vertebrate development, resulting in past failures or extreme phenotypes of transgenic mice [12, 22–24], the transgene was engineered using the FLEX directional switch system (Fig 1B) to bypass the embryonic lethality from leaky embryonic DUX4 transgene expression [34, 35]. The FLEXDUX4 transgene targeted the Rosa26 locus such that it would recombine in the antisense orientation. The transgene was flanked by heterologous loxP sites that recombine unidirectionally upon exposure to Cre recombinase, resulting in DUX4-fl mRNA expression under transcriptional control of the Rosa26 promoter. This design resulted in the successful generation of the hemizygous FLEXDUX4 line of mice in a C57BL/6 background, formally referred to as B6(Cg)-Gt(ROSA)26Sortm1(DUX4+/Pjj)J, which we have made available to the FSHD research community prior to this initial publication (Jackson Laboratories catalog #028710). The available hemizygous line has been crossed with the background mouse strain (C57BL/6) at least 5 times. DNA methylation analysis of the transgene shows no changes in the pattern of transgene hypomethylation across all five generations (S2 Fig).

Initial observations of the FLEXDUX4/+ hemizygous mice indicated that they are viable, fertile, live a normal life span (~2yrs), and appear generally healthy with the exception of a very mild alopecia (Fig 2A–2D) and slightly reduced size (Fig 2F). Hemizygous males were similar in weight to non-transgenic C57BL/6 littermate controls through the first 8 weeks after birth, at which point the FLEXDUX4/+ males displayed slower growth, resulting in mice ~14% (~5g)
smaller by 20 weeks of age. Female hemizygous mice were comparable in size to controls through 12 weeks before their weight gain slowed, resulting in ~11% (~3g) smaller adult females. Surprisingly, despite their more normal size and weight, alopecia becomes more severe in adult female hemizygous mice than in their male counterparts (S3 Fig).

**FLExDUX4/FLExDUX4** homozygous mice were similarly viable and fertile, but showed a much more severe alopecia compared with hemizygous and non-transgenic littermates, that periodically appears between 3–8 weeks of age and then remains in older animals (Fig 2E and S4 Fig). Most **FLExDUX4** homozygous mice lived a normal life span, although some developed rectal prolapse after 6 months of age and had to be euthanized. In addition, the homozygous **FLExDUX4** mice had generally soft stools, and males developed an inflamed preputial gland.

https://doi.org/10.1371/journal.pone.0192657.g001

**Fig 1. Transgene map and transgenic model generation.** (A) The synthesized FLExDUX4 transgene, which maintains the 3 exon and 2 intron structure of the human DUX4 gene, but lacks the DUX4 promoter, was inserted in the antisense orientation to the Rosa26 promoter. The transgene is flanked by heterologous lox sites (loxP and lox511) that unidirectionally recombine in the presence of cre recombinase to invert the FLExDUX4 transgene to the sense orientation. DUX4-fl mRNA is then transcribed from the Rosa26 promoter, processed, and terminated by the DUX4 exon3 PAS. (B) The genome targeting scheme for knocking in to the Rosa26 locus utilized the FLEx system methodology. PGK-NeoR: phosphoglycerate kinase I promoter regulating the neomycin resistance gene. DTA: Diphtheria toxin A gene used for counter selection.
(S4 Fig), suggesting potential inflammation in the gastrointestinal tract. Homozygous \textit{FLExDUX4}/+ mice were healthy, active, and fertile with a very mild alopecia that is readily distinguishable from wildtype littermates starting at two weeks of age. (E) Homozygous \textit{FLExDUX4}/\textit{FLExDUX4} mice are also healthy, active, and fertile but show more prominent alopecia than hemizygous mice. F) These transgenic mice are initially similar in size and weight as their control non-transgenic siblings; however, the hemizygous and homozygous are smaller as adults. The graph shows average weights for \( n = 5+ \) animals for each genotype measured each week for 20 weeks.

Fig 2. The \textit{FLExDUX4} lines of mice. (A-D) Hemizygous (\textit{*}FLExDUX4)+ mice are healthy, active, and fertile with a very mild alopecia that is readily distinguishable from wildtype littermates starting at two weeks of age. (E) Homozygous \textit{FLExDUX4}/\textit{FLExDUX4} mice are also healthy, active, and fertile but show more prominent alopecia than hemizygous mice. F) These transgenic mice are initially similar in size and weight as their control non-transgenic siblings; however, the hemizygous and homozygous are smaller as adults. The graph shows average weights for \( n = 5+ \) animals for each genotype measured each week for 20 weeks.

https://doi.org/10.1371/journal.pone.0192657.g002

Together, these transgene-linked phenotypes with apparent transgene dosage effects led us to suspect that the \textit{DUX4} transgene was being expressed at very low levels in the absence of cre-mediated recombination, despite the gene being in the antisense orientation to the \textit{Rosa26} promoter.

To assess potential leaky expression of the \textit{DUX4} transgene, we performed sense strand-specific quantitative reverse transcriptase PCR (qRT-PCR) for polyadenylated \textit{DUX4-fl} mRNA
using RNA extracted from several tissues of FLExDUX4 hemi- and homozygous mice. Sense DUX4-fl mRNA was detected in skeletal muscle, liver, and skin of all mice (Fig 3A). Homozygous mice, on average, showed higher DUX4-fl expression as a group in all tissues except skin (Fig 3A), but there was mouse-to-mouse and tissue variation, and the dosage effect was not absolute or significant between groups for any tissue, despite the more severe phenotypes (S4 Fig). Regardless, the overall DUX4-fl mRNA levels detected were still very low, comparable to those found in differentiated human FSHD myocytes (17Abic MT [6]) (Fig 3A). To assess the scope of RNA transcript isoforms generated from the DUX4 transgene, qualitative RT-PCR was performed using PCR primers spanning DUX4 exons 1–3 (Fig 3B), which amplify all known DUX4 mRNA isoforms [5]. The predominant DUX4 transcript found in gastrocnemius and tibialis anterior muscles was the predicted 1.4 kb product for DUX4-fl that retains intron 1, with a minor 1.2 kb product in which intron 1 was spliced out, as is the case in FSHD muscle cells [5, 6, 45]. DNA sequencing confirmed that the 1.4 kb product is derived from the transgene, contains the conserved splicing site changes, and is expected to encode the full open reading frame for the pathogenic DUX4-fl mRNA (S2 Sequence). Thus, skeletal muscle preferentially generates the pathogenic DUX4-fl transcript, however, strikingly, both liver and skin predominantly expressed many shorter RNAs distinct from the previously described DUX4-s 0.35 kb and 0.45 kb isoforms [5, 6], and little to none of the 1.4 kb DUX4-fl transcript (Fig 3B). The nature of these aberrant transcripts is not clear, however, since the first strand cDNA synthesis was performed with an oligo-dT primer, the negative controls resulted in no amplified products (Fig 3B), and similarly the non-transgenic controls showed no products of any size, all of these isoforms most likely represent polyadenylated isoforms or truncations of DUX4 transgene transcripts. DNA sequence analysis of these short products confirmed they are in fact derived from the transgene suggesting that FLExDUX4 mice express novel short DUX4 transgene transcripts in non-muscle tissues (Fig 3B). Although our approach for the efficient expression of the DUX4-fl isoform was successful in skeletal muscle, there may be tissue-specific RNA processing of the DUX4 transgene in non-muscle tissues, which utilize variable 5’splicing donor sites and likely affects the specificity of our DUX4-fl qRT-PCR assay. It should be noted that the DUX4 qRT-PCR assay was designed and validated as specific for the DUX4-fl mRNA in human muscle tissue and myogenic cells, not in skin or liver, since DUX4-fl is not known to be expressed in these tissues [7, 46]. Thus, one should interpret results with caution when using this assay alone as a measure for DUX4-fl mRNA levels in non-myogenic tissues of this mouse model.

To determine if this unexpected transgene expression was due to aberrant recombination, genomic PCRs for the sense and antisense transgene were performed and no indication of any genomic recombination was found (S5 Fig), thus confirming that the mRNAs are produced from antisense transcription at the Rosa26 locus. We conclude that a sense polyadenylated DUX4-fl mRNA is being produced in skeletal muscle from the inverted transgene (Fig 1A) by antisense transcription at the Rosa26 locus. Thus, in the absence of induction, FLExDUX4 hemi- and homozygous mice have a very low level of DUX4-fl mRNA in their skeletal muscles throughout their lifetime, yet exhibit no overt muscle phenotype. This low chronic DUX4-fl expression without any induction may represent a useful model for testing therapies targeting DUX4-fl mRNA.

Since hemi- and homozygous FLExDUX4 mice express low levels of DUX4-fl mRNA in skeletal muscles, we analyzed their muscles for DUX4-FL-mediated expression profiles and signs of histopathology. As anticipated, since there was no transgene recombination, DUX4-FL immunostaining of FLExDUX4/+ skeletal muscles did not reveal DUX4-FL positive myonuclei (S6 Fig), indicating that the DUX4-fl mRNA expression seen in the muscles of these mice is likely due to very low-level expression from many or all myonuclei. DUX4-FL protein
functions as a transcriptional activator in human and mice [23, 47]; to assess if functional levels of DUX4-FL protein were being made, we analyzed expression of WAP four-disulfide core domain 3 (Wfdc3), a well-documented direct murine target of overexpressed DUX4-FL protein [18, 23, 27], in gastrocnemius muscle (S7 Fig). Interestingly, Wfdc3 mRNA levels were not significantly induced in either the hemi- or homozygous FLExDUX4 muscle tissue (S7 Fig), indicating that either no protein was being made or, more likely, that protein levels in the individual myonuclei were too low to affect downstream gene transcription. Histological evaluation of skeletal muscles similarly failed to reveal any increase in centralized nuclei, immune cell infiltration, or fibrosis between hemi- and homozygous FLExDUX4 mice and C57BL/6
controls (Fig 4 and S8 Fig). Together, this data supports a model of FLExDUX4 mice producing very low levels of leaky DUX4-fl mRNA expression in all muscle cells, however, the expression levels are too little on a per cell basis to activate or at least detect induction of downstream DUX4 target genes. This is in contrast to the situation in FSHD, where a small number of myonuclei burst with high and ultimately pathogenic levels of DUX4-fl mRNA and protein expression, leading to downstream target gene activation [5, 14, 48]. This model can be readily scaled and may be useful for assessing therapeutic approaches targeting DUX4-fl mRNA in vivo.

Developmental induction of DUX4-fl expression is lethal.

While FLExDUX4 hemi- and homozygous mice express very low levels of DUX4-fl and display mild phenotypes in fur, digestive tract, and muscle fiber type, they show no signs of a skeletal myopathy and are generally healthy. In order to generate a model suitable for investigating DUX4-induced myopathy, it was necessary to induce DUX4-fl expression levels using cre-mediated recombination. As designed, cre expression in the tissues of double transgenic mice would lead to inversion of the transgene to the sense orientation such that DUX4-fl mRNA expression would be mediated by the ubiquitous and fairly strong Rosa26 promoter [49] (Fig 1A). To assess the functionality of the floxed transgene design and ensure that cre-mediated recombination leads to increased expression of DUX4-fl mRNA, we initially crossed the FLExDUX4 mice with two developmentally regulated cre expression lines of mice (Table 1). Crossing with a Sox2-cre mouse [50], in which cre is expressed from the Sox2 (SRY-box containing 2) promoter in the mouse epiblast, resulted in decreased litter sizes and no double transgenic

**Fig 4. FLExDUX4 mice display no overt myopathy despite low-level DUX4-fl expression.** Skeletal muscles were analyzed for histopathology using hematoxylin and eosin (H&E) staining at 8, 12, 15, and 23 weeks (shown). Representative tibialis anterior sections are shown for (A) control C57BL/6, (B) FLExDUX4/+, and (C) FLExDUX4/FLExDUX4 female mice. Scale bar = 100μm.

Table 1. Results of FLExDUX4 crosses to cre expressing lines of mice.

<table>
<thead>
<tr>
<th>Cre line</th>
<th>Phenotype of double transgenic FLExDUX4;cre offspring</th>
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<tr>
<td>Sox2-cre [50]</td>
<td>Embryonic lethal</td>
</tr>
<tr>
<td>ACTA1-cre [51]</td>
<td>Stillborn E19; skeletal abnormalities</td>
</tr>
<tr>
<td>UBC-creER T2 [52]</td>
<td>Viable; may develop severe ataxia and severe neurological phenotypes requiring euthanasia after 3 weeks in absence of TMX</td>
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<tr>
<td>Pax7-creER T2 [53]</td>
<td>Viable and apparently healthy in absence of TMX</td>
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<tr>
<td>ACTA1-MerCreMer [54]</td>
<td>Viable, very mild muscle phenotype in absence of TMX; TMX-inducible myopathic phenotype</td>
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https://doi.org/10.1371/journal.pone.0192657.t001
mice after multiple matings, supporting previous work that DUX4-fl expression during early vertebrate development is lethal [12].

To circumvent the developmental lethality of early ubiquitous DUX4-fl expression, FLEX-DUX4 mice were crossed with ACTA1-cre mice [51], which express cre in striated muscles from the human Skeletal α-actin (ACTA1) promoter, recapitulating the expression profile from the endogenous murine Acta1 promoter. Male FLEXDUX4/+ mice crossed with female ACTA1-cre mice and, conversely, female FLEXDUX4/+ mice crossed with male ACTA1-cre mice both produced litters of normal size and typical Mendelian inheritance patterns (Fig 5). However, all double transgenic pups (n = 7) were stillborn and smaller after developing nearly to full term (E19.5) compared with single transgenic or WT pups (n = 9) that were uniformly viable and healthy. RT-PCR analysis of mRNA expression confirmed that pathogenic DUX4-fl mRNA was expressed in all pups containing the FLEXDUX4 transgene, and at higher levels in the limbs of FLEXDUX4;ACTA1-cre double transgenic stillborn animals compared with their FLEXDUX4/+ sibling controls (Fig 5D). Thus, while mouse development can withstand the very low level of leaky DUX4-fl expression found in the FLEXDUX4 hemi- and homozygous mice, increased expression levels in striated muscle during development are lethal.

Although FSHD is typically adult-onset, there is an early-onset, very severe infantile form of the disease (IFSHD) that is poorly understood [55, 56], and early, potentially developmental events preceding clinical presentation of FSHD are not known. One hypothesis is that bursts of DUX4-fl expression occur earlier in IFSHD and thus start the detrimental cascade of pathophysiology sooner than in the classical adult FSHD1. An alternative and not mutually exclusive hypothesis is that all forms of FSHD have a developmental origin, establishing a course for FSHD pathology well before clinical symptoms appear. DUX4-fl mRNA has been detected in genetically FSHD1 human embryonic tissues [57], supporting both hypotheses and suggesting that its developmental increase in expression may be detrimental and a contributing factor to increased IFSHD severity. Since the Acta1/ACTA1 promoter is active in the myotomal region of somites as early as 9.5 days post-coitum (d.p.c) [51], we took advantage of this developmental DUX4-fl expression model, and analyzed embryos for DUX4-fl dependent musculoskeletal defects (Fig 6). The 16.5 d.p.c. embryos were isolated from crosses of male ACTA1-cre/+ mice with female FLEXDUX4/+ mice and analyzed with alcian blue staining to reveal the embryonic cartilaginous skeleton [38, 58]. Interestingly, all ACTA1-cre;FLEXDUX4 embryos (n = 12) had overall smaller bodies compared with single transgenic siblings (Fig 6A compared with Fig 6E), aberrantly developed spinal columns (Fig 6B compared with Fig 6F), and underdeveloped rib cages (Fig 6C and 6D compared with Fig 6G and 6H). None of these phenotypes were found in any sibling controls (n = 36). Thus, although these specific developmental phenotypes do not directly translate to known FSHD or IFSHD clinical phenotypes, they clearly demonstrate that small increases in DUX4-fl expression levels in striated muscle during development can have dramatic and deleterious effects.

Inducible crosses generate phenotypic DUX4-fl-expressing mice

Although the FLEXDUX4 mice are viable and relatively healthy despite the presence of low levels of DUX4-fl during development, the fully penetrant stillborn phenotype of the ACTA1-cre; FLEXDUX4 double transgenic animals indicated that an adult mouse model would require postnatal induction of DUX4-fl expression. We therefore crossed FLEXDUX4 mice with inducible cre expressing mouse lines (Table 1). We chose three mouse lines for the crosses, each serving different purposes: induced ubiquitous expression (UBC-cre/ERT2 line [52]), induced limb and craniofacial skeletal muscle expression (ACTA1-MerCreMer line [54]), and induced muscle satellite cell expression (Pax7-cre/ERT2 line [53]). Each line expresses a cre protein
fused to estrogen receptors for strong cytoplasmic retention, and mutated such that it translocates to the nucleus only in the presence of tamoxifen (TMX), and not estrogen [59]. All three crosses with FLExDUX4 mice successfully produced litters of normal size with Mendelian inheritance, and numerous viable double transgenic offspring that survived into adulthood.

Fig 5. Increased expression of DUX4-fl in embryonic muscle is lethal by stage E19. In order to boost DUX4-fl expression levels in skeletal muscle, the FLExDUX4/+ mice were crossed with ACTA1-cre mice. Litters were of normal size (7–10 pups) with both (A) live and (B) stillborn pups. (C) Genotyping showed live births for all three control genotypes; however, all resulting ACTA1-cre/+; FLExDUX4/+ double transgenic animals were stillborn. D and E) RT-PCR shows that the full-length 1.4kb DUX4-fl mRNA transcript is expressed at increased levels in the forelimbs and hindlimbs of (D) stillborn ACTA1-cre/+; FLExDUX4/+ animals compared with (E) live born FLExDUX4/+ sibling pups. This increased expression correlates with expression of cre recombinase. Scale bar = 5mm.

https://doi.org/10.1371/journal.pone.0192657.g005
Fig 6. Embryonic expression of DUX4-fl results in skeletal developmental abnormalities. Stage E16.5 embryos were isolated from crosses between ACTA1-cre/+ male and FLExDUX4/+ female mice and stained with alcian blue to visualize the mouse fetal cartilaginous skeleton. The ACTA1-cre/+; FLExDUX4/+ double transgenic embryos (E-H) were smaller than controls (A-D), and were the only genotype (n = 12) to show skeletal deformations in the spine, limbs, and rib cage compared with control (n = 36) littermates (FLExDUX4/+, ACTA1-cre/+, and WT). Bars = 1mm.

https://doi.org/10.1371/journal.pone.0192657.g006
The UBC-cre/ERT2;FLExDUX4 mice ubiquitously express a TMX-inducible cre from the human ubiquitin C promoter [52], and, in the absence of TMX administration, should not express levels of DUX4-fl mRNA above those caused by antisense transcription of the inverted FLExDUX4 transgene. Therefore, double transgenic animals were not anticipated to have any adverse phenotypes. However, despite apparently normal embryonic development and birth-rates, the UBC-cre/ERT2;FLExDUX4 pups (stage P10) showed delayed fur growth compared with FLExDUX4/+ siblings (Fig 7A), which further manifested as a mild alopecia by 3 weeks of age (Fig 7B). Unlike the FLExDUX4 hemi- and homozygous mice that remained healthy, after 5 weeks of apparently normal behavior (Fig 7C), the health of UBC-cre/ERT2;FLExDUX4 mice began to rapidly deteriorate and they developed additional phenotypes that became much more severe (Fig 7D). Animals developed severe kyphosis and tremors, becoming unsteady and eventually ataxic (S1 Movie). Between 5–12 weeks of age, the mice (n = 5/6) had lost their righting reflex and began having spontaneous seizures characterized by periods (> 30 sec) of wild and uncontrolled running (S2 Movie), and required euthanasia.

These severe phenotypes beyond those seen in the FLExDUX4 hemi- and homozygous mice suggested increased levels of DUX4-fl expression in the double transgenic animals. Analysis of genomic DNA isolated from tissues of UBC-cre/ERT2;FLExDUX4 mice confirmed low levels of cre-dependent transgene recombination specifically in the brain, but not in liver or muscle, and were absent in the FLExDUX4/+ lines (S9 Fig), indicating a low level of functional nuclear cre in the brains of double transgenic mice in the absence of TMX. This leaky transgene recombination in the brain, but not the muscle, likely explains the severe neurological phenotype without an obvious muscle phenotype.

To assess the phenotypic DUX4-fl expression levels, we performed qRT-PCR analysis. However, the DUX4-fl mRNA levels detected in brain tissue from UBC-cre/ERT2;FLExDUX4 double transgenic animals were surprisingly similar to the DUX4-fl expression found in the brains of non-recombined FLExDUX4/+ mice (Fig 8A). In fact, there were no significant differences in DUX4-fl mRNA expression levels assayed by qRT-PCR between FLExDUX4/+ and UBC-cre/ERT2;FLExDUX4 mice for any of the tissues analyzed. Previous analysis of the FLExDUX4 single transgenic mice showed that non-myogenic tissues can produce variable short DUX4 transcripts that can affect the qRT-PCR assay, and the results may not reflect the expression level of the pathogenic DUX4-FL protein (Fig 3). To determine the levels of functional DUX4-FL protein in these tissues, expression of Wfdc3, a direct target of DUX4-FL protein with minimal expression in FLExDUX4/+ mice, was analyzed and showed a markedly distinct expression pattern (Fig 8B). Despite similar DUX4-fl qRT-PCR profiles between mouse strains, Wfdc3 expression was induced > 250-fold in the brains of UBC-cre/ERT2;FLExDUX4 mice compared with FLExDUX4/+ mice, but only mildly, yet significantly, induced in liver or muscle tissues, indicating that only the double transgenic brain had high levels of functional DUX4-FL protein. To further address the discrepancy between DUX4 mRNA expression and DUX4-FL target gene expression in the brain, we again performed a DUX4 RT-PCR to qualitatively analyze the mRNA isoforms generated from the DUX4 transgene in these tissues from both mouse models (Fig 8C). As before (Fig 3), gastrocnemius muscle from the FLExDUX4/+ and UBC-cre/ERT2;FLExDUX4 mice only produced the pathogenic DUX4-fl mRNA isoform, containing intron 1, and did not generate any DUX4-s or other alternative short mRNAs from the transgene. In contrast, brains of FLExDUX4/+ mice and, to a lesser degree, the UBC-cre/ERT2;FLExDUX4 mice expressed a combination of DUX4-fl mRNA and multiple short unique transcripts that, upon DNA sequencing (Fig 8C, *), were confirmed to be novel short DUX4 variants. Thus, alternative splicing in non-muscle tissue again contributed to a high noise for the DUX4-fl qRT-PCR analysis in the brain. Thus, the extreme behavioral and physical phenotypes of the UBC-cre/ERT2;FLExDUX4 double transgenic animals can likely be explained by
brain-specific leakiness of cre, leading to brain-specific transgene recombination and, ultimately increased levels of DUX4-fl mRNA and functional DUX4-FL protein in the brain.

Upon observation of the internal organs, the subiliac lymph nodes (central drainage) and popliteal lymph nodes (hind leg drainage) [60] were strikingly swollen in the double transgenic mice compared with C57BL/6 and FLExDUX4 controls (S10 Fig). After weighing, both types of lymph nodes were found to be significantly enlarged in the DUX4-fl expressing FLExDUX4/+ (4.0 mg SD = 1.641 and 1.7 mg SD = 0.427, respectively) and UBC-creERT2;FLExDUX4 (12.5 mg SD = 8.031 and 4.4 SD = 2.935, respectively) mice compared with control mice (2.5 mg SD = 0.601 and 0.9 mg SD = 0.326, respectively) (S10 Fig). Enlarged lymph nodes can be indicative of acute inflammation, and several lines of evidence suggest inflammatory immune processes may be involved in mediating FSHD pathophysiology [9, 15, 16, 61, 62], supporting that an inflammatory immune response was stimulated by low-level DUX4-fl expression in these mice. Overall, we conclude that UBC-cre/ERT2;FLExDUX4 animals, with or without TMX
Fig 8. Tissue-specific DUX4-fl alternative mRNA isoforms are generated from the FLExDUX4 transgene. Three severely phenotypic 12-week-old UBC-creERT2;FLExDUX4 (dTG) mice and three sibling FLExDUX4/+ mice were analyzed by qRT-PCR for expression of (A) DUX4-fl and (B) Wfdc3, a direct DUX4-FL target gene, in liver, brain, and gastrocnemius muscle. The results of the three individual mice for each condition are summarized in the graphs on the right (U = UBC-creERT2/+; F = FLExDUX4/+; dT = dTG). (C) RT-PCR analysis of FLExDUX4/+ and FLExDUX4;UBC-creERT2 mice for DUX4 mRNA isoforms shows tissue-specific splicing and variation in expression levels. Multiple novel short DUX4 mRNAs (confirmed by sequencing) are detected in the brain and liver, but not gastrocnemius. Significance was calculated using Welch’s t-test; n.s. = not significant (p > 0.05), * = p < 0.05, ** = p < 0.005.

https://doi.org/10.1371/journal.pone.0192657.g008
induction, express elevated levels of DUX4-FL and may be useful for evaluating certain systemic effects of DUX4-fl expression for preclinical testing of molecules targeting DUX4-fl mRNA or protein. However, due to severe neurological effects, this model did not recapitulate an FSHD-like phenotype, and the progressive severity of the phenotype with age requires utilizing young mice and administering euthanasia by 10–12 weeks of age.

**Generation of an adult FSHD-like phenotype using the FLExDUX4 model**

Our current understanding of FSHD is that the aberrantly increased DUX4-fl expression is generally restricted to skeletal muscles [33]. However, unlike other muscle diseases, in which all of the myocytes of an affected individual share the same functional deficit [63], it is estimated that <1% of FSHD myonuclei express the pathogenic DUX4-fl mRNA at any particular time [5, 6], and that sporadic bursts of DUX4-fl expression from an increased number of myonuclei may lead to pathology [14]. Affected muscle has mosaic expression, and DUX4-fl expressing cells are in the minority. To best recapitulate this situation in our mouse model, we crossed FLExDUX4 mice with the ACTA1-MerCreMer (ACTA1-MCM) line [54], which expresses TMX-inducible cre in skeletal and craniofacial muscles, but not in cardiac muscle. Presumably, the resulting ACTA1-MCM;FLExDUX4 mice would express low levels of leaky DUX4-fl, similar to an unaffected FSHD patient, and, upon limited TMX administration, would recombine the transgene in a fraction of myonuclei, dependent upon the amount of TMX, to produce a mosaic DUX4-fl expression pattern in the skeletal muscles. This would result in high levels of DUX4-fl expression from each recombined myonucleus, thus generating a DUX4-mediated FSHD-like myopathy.

Crossing ACTA1-MCM males with FLExDUX4 females produced large litters (8–10 pups) of viable healthy double transgenic offspring at normal Mendelian ratios. Typically, researchers aim for a 100% recombination rate for the transgene and use up to 75mg/kg TMX in successive intraperitoneal (IP) injections [64] to achieve this goal. However, we are interested in a much lower recombination rate to produce mosaic expression of the DUX4 transgene and avoid lethality. To obtain our desired phenotype, we chose two methods: 1) feeding a TMX-laced chow *ad libitum* for 5 days for a predicted administration of 40mg/kg/day, and 2) direct administration of TMX using a dosage of 20mg/kg in corn oil delivered by IP injection on two successive days. All TMX-treated ACTA1-MCM;FLExDUX4 mice and controls were monitored daily for activity and overall health. Both groups of TMX-treated ACTA1-MCM;FLExDUX4 mice rapidly displayed phenotypes consistent with a progressive myopathy (S11 Fig, S3–S10 Movies). The mice fed TMX chow lost weight and became ataxic within 8 days of TMX exposure (S3 Movie); however, the mice did not appear to like eating the chow and food intake was lower than with untreated food. Thus, analyzing the effects of TMX induction of DUX4-fl was compounded by low and variable food intake and resulting weight loss, which translated to a variable and uncontrolled dosage. We conclude that TMX-laced chow is not an appropriate route of administration for this mouse model. In contrast, there was no such variable with the mice administered TMX via IP injections. In addition, concerns about the potential effects of TMX on muscle are mitigated using this IP method since the dose is low and exposure is very short due to TMX clearance [65]. By seven days post-injection, all double transgenic mice showed a noticeable decline in appearance, activity (S4–S6 Movies), and strength (S7–S10 Movies) and by nine days post-injection, they required euthanasia. By contrast, TMX-injected FLExDUX4/+ and ACTA1-MCM/+, as well as non-injected ACTA1-MCM;FLExDUX4 controls, showed no adverse effects. Qualitative movement (compare movies of control animal, S4 and S5 Movies with movie of affected animal, S6 Movie) and strength measurements by a four-limb hanging test (compare movies of control animals, S7...
and S8 Movies with movies of affected animals, S9 and S10 Movies) revealed clear declines specific to the TMX-injected ACTA1-MCM;FLExDUX4 animals (e.g. 2 min maximum assay hanging times for all controls and <2 sec for all TMX-injected ACTA1-MCM;FLExDUX4 affected animals). Thus, TMX-injected ACTA1-MCM;FLExDUX4 mice develop an expected FSHD-like phenotype.

All mice, including age-matched controls, were sacrificed nine days post-injection (13 weeks old) and muscle samples were assayed for DUX4-fl expression and histopathology. Surprisingly, despite the clear phenotypes, significant increases in the steady state levels of DUX4-fl mRNA were not detected by qRT-PCR in the phenotypic ACTA1-MCM;FLExDUX4 (+TMX) mice (Fig 9A). However, immunostaining revealed mosaic DUX4-FL protein expression in the myonuclei (Fig 10I–10L), reminiscent of the sporadic natural bursting of expression seen in FSHD myonuclei, and three downstream target genes, Wfdc3, Trim36, and Cxcr4 (Fig 9B–9D), were greatly induced by TMX in the skeletal muscles of ACTA1-MCM;FLExDUX4 mice. Thus, detection of these DUX4-FL downstream targets is a more accurate method for assessing DUX4-FL levels than direct detection of DUX4-fl mRNA by qRT-PCR. In addition, skeletal muscle tissues that expressed DUX4-FL showed large infiltrates of mononuclear cells (Figs 10I and 11L and S12 Fig) and became mildly fibrotic (Fig 12). Thus, initial analysis of the skeletal muscles of phenotypic ACTA1-MCM;FLExDUX4 mice indicate they share three key pathological characteristics of FSHD muscle: mosaic DUX4-FL expression that leads to DUX4-FL target gene expression, mononuclear cell infiltration, and increased fibrosis [5, 14, 15, 21, 61, 66].

Histological analysis of the tibialis anterior, gastrocnemius, and quadriceps muscles (Fig 11) showed severe pathology in the TMX-induced double transgenic animals, including the highly heterogeneous fiber size distribution characteristic of regeneration, mononuclear cell infiltration, and a staining pattern indicative of necrosis and phagocytosis (Fig 11I–11L). Similarly, the tibialis anterior muscles in the TMX-induced double transgenic animals showed increased fibrosis (Fig 12A–12D), indicative of aberrant deposition of extra-cellular matrix components during tissue healing, resulting in the loss of muscle architecture common in many muscular dystrophies including FSHD [66–69]. These phenotypes were absent from the muscles of the double transgenic mice without TMX (Fig 11E–11H) and ACTA1-MCM (Fig 11A–11D) control mice. Interestingly, while not a dramatic phenotype, the non-induced ACTA1-MCM;FLExDUX4 showed patches of centralized nuclei and several small irregular fibers, indicative of muscle damage and regeneration (Fig 11H) that are not found in muscles from the FLExDUX4/+ mice. This correlated with slightly higher levels of DUX4-fl mRNA and target gene expression than those found in skeletal muscle from single transgenic mice (Fig 9), due to a low level of leaky nuclear MerCreMer specifically in skeletal muscle leading to very low mosaic recombination of the transgene (S5 Fig). Thus, these non-induced double transgenic mice alone may be a useful model of very mild DUX4-mediated pathology.

FSHD, like many myopathies, elicits an inflammatory immune response in affected muscles that, in turn, may contribute to muscle pathophysiology [15, 16, 61, 70, 71]. Investigation of the mononuclear cell infiltrates observed in the ACTA1-MCM;FLExDUX4 skeletal muscle histograms by immunostaining for CD11b (Itgam, Integrin subunit alpha M), a member of the CD18 integrin family of leukocyte adhesion receptors involved in leukocyte migration [72], and Ly6g (lymphocyte antigen 6 complex, locus (G), a marker for murine neutrophils [73, 74], confirmed the presence of pro-inflammatory cells in the affected muscle within six days of DUX4-fl induction (Fig 13C, 13D, 13G and 13H). These immune cell infiltrates were absent from healthy control muscles (Fig 13A, 13B, 13E, 13F and S13 Fig). By nine days, muscles were severely affected, with massive immune cell infiltration and loss of membrane integrity, as indicated by loss of dystrophin localization (Fig 14 and S12 Fig). Importantly, these immune
Phenotypes are consistent with FSHD pathology as identified by MRI and histopathology; thus, this model may be useful for characterizing the role of the immune system in FSHD.

**Discussion**

FSHD is a dominant genetic disease with a critical epigenetic component resulting in misexpression of the cytotoxic isoform of the old-world primate DUX4 gene [10]. Aberrant myogenic expression of DUX4-fl is considered to be the main driver of pathology in FSHD. However, due to the extreme cytotoxicity of DUX4-fl, FSHD has been very difficult to model in mice [23, 24, 75]. Here we report the generation and characterization of a conditional DUX4-fl transgenic mouse model, termed FLExDUX4. Crossing FLExDUX4 mice with cre driver lines of mice leads to increased DUX4-fl mRNA and protein expression. Use of muscle-specific cre-driver lines of mice results in double transgenic offspring that express DUX4-fl mRNA in skeletal muscles and develop an FSHD-like myopathic phenotype [4]. Thus, these mice should be

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Fig 9. TMX induction leads to DUX4-FL target gene expression in skeletal muscle of ACTA1-MCM;FLExDUX4 mice. Gastrocnemius muscles from 13-week-old mice were assayed 9 days post-IP injection with TMX, as indicated, and relative mRNA expression levels for DUX4-fl and three DUX4-FL target genes were determined. Each of the 3 individual mice per group (1, 2, 3) are shown. (A) DUX4-fl mRNA expression assayed by qRT-PCR does not significantly change between groups, despite increased protein expression (Fig 11). (B) Wfdc3 mRNA expression is upregulated in both double transgenic groups compared with FLExDUX4 alone; however, TMX treatment significantly induces Wfdc3 expression in ACTA1-MCM;FLExDUX4 compared with the untreated control ACTA1-MCM;FLExDUX4 mice. In contrast, (C) Trim36 and (D) Cxcr4 mRNA expression are relatively constant and only upregulated in response to TMX induction in ACTA1-MCM;FLExDUX4 mice. Significance was calculated using Welch’s t-test; n.s. = not significant (p > 0.05), * = p < 0.05, ** = p < 0.005.

https://doi.org/10.1371/journal.pone.0192657.g009

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of great use for investigating pathogenic mechanisms and testing therapeutic approaches for FSHD.

Key features of the FLEXDUX4 mouse model include the following: 1) the DUX4 transgene maintains the 3 exon, 2 intron gene structure, including the DUX4-fl5' UTR, endogenous PAS, and other important mRNA and RNA processing therapeutic targets, 2) the DUX4-fl mRNA expressed in skeletal muscle is spliced and polyadenylated as in FSHD patients, 3) hemizygous and homozygous FLEXDUX4 single transgenic mice are healthy, fertile, and have normal life spans, 4) FLEXDUX4 single transgenic mice express a very low level of DUX4 RNA in all tissues tested, and DUX4-fl mRNA in skeletal muscles, and 5) crossing with any number of cre-driver lines will allow investigators spatiotemporal control of DUX4-fl expression during development or in adults.

When FLEXDUX4 mice are crossed with cre-expressing lines of mice, severe phenotypes can be obtained. Crossing FLEXDUX4 mice with ACTA1-MCM mice [54], a skeletal muscle-specific, TMX-inducible cre-expressing strain, produces double transgenic offspring that themselves represent a mild model of FSHD, and can be induced to develop a severe DUX4-dependent FSHD-like myopathy with low levels of TMX injected IP into adult animals. Upon TMX induction, ACTA1-MCM;FLEXDUX4 mice exhibit decreased movement, an altered gait, and loss of hanging ability (S3–S10 Movies), indicating an overall decline in muscle function. Analysis of the skeletal muscles of ACTA1-MCM;FLEXDUX4 (+TMX) mice identified other important features characteristic of FSHD. In FSHD, DUX4-fl mRNA is expressed in a small

https://doi.org/10.1371/journal.pone.0192657.g010

Fig 10. TMX induction leads to mosaic expression of DUX4-FL protein in myonuclei of ACTA1-MCM;FLEXDUX4 mice. Gastrocnemius muscles from 13-week-old mice 6 days post-IP injection with TMX, as indicated, were assayed by immunohistochemistry for DUX4-FL protein, counterstained with wheat germ agglutinin (WGA) to outline myofibers, and DAPI to identify myonuclei. (A-D) ACTA1-MCM, (E-H) ACTA1-MCM;FLEXDUX4 no TMX, and (I-L) ACTA1-MCM;FLEXDUX4 + TMX. Arrows identify DUX4-FL positive myonuclei. Scale bar = 25μm.
fraction of myonuclei in skeletal muscles, and in myogenic cells in culture [5]. Similarly, our severe FSHD-like model shows mosaic DUX4-FL protein expression in myonuclei within several days of TMX administration (Figs 10 and 14 and S12 Fig). In humans, DUX4-FL is a transcriptional activator, and induction of DUX4-FL target genes is a signature of FSHD muscle [20]. Since DUX4-fl mRNA and protein levels are typically very low in FSHD myogenic cells, DUX4 target genes serve as sensitive surrogate markers for levels of DUX4-FL expression. While DUX4-fl mRNA and protein levels are also very low in this model, the mRNA levels of DUX4-responsive genes (Wfdc3, Trim36, and Cxcr4) are significantly increased following DUX4-fl induction (Fig 9). Although the murine and human DUX4-induced transcriptomes only partially overlap, measuring the expression of these murine DUX4 target genes will be useful for evaluating DUX4-fl levels during therapeutic interventions in this mouse model.

FSHD muscle biopsies and MRI data indicate an inflammatory immune response in muscles of many FSHD patients [15, 16, 61, 70] and histopathology shows fibro-fatty replacement in affected FSHD muscle [66]. Inflammatory cells are able to stimulate fibrotic activity during regeneration and promote fibrotic tissue remodeling in Duchenne muscular dystrophy [76, 77]. Similarly, in the dystrophic ACTA1-MCM;FLEXDUX4 TMX-induced mice, mosaic myogenic expression of DUX4-fl leads to an infiltration of inflammatory mononuclear cells, including neutrophils and macrophages (Figs 11, 13 and 14 and S12 Fig), and increased fibrosis (Fig 12). Thus, these FSHD-like mice may represent a useful model for investigating the

Fig 11. Induction of DUX4-fl induces a severe myopathy in skeletal muscles of ACTA1-MCM;FLEXDUX4 mice. H&E staining on skeletal muscle sections from 12-week-old ACTA1-MCM/+ (A-D), ACTA1-MCM;FLEXDUX4(E-H), and ACTA1-MCM;FLEXDUX4+TMX mice at 9 days post-injection (I-L). The tibialis anterior (A, E, I), gastrocnemius (B, F, J), and quadriceps (C, G, K) muscles are shown at 10X (bars = 500µm), and the tibialis anterior (D, H, L) is shown at 40X (bars = 50 µm). Low level skeletal muscle transgene recombination in the absence of TMX leads to a proportion of centralized nuclei, indicative of regenerating myofibers, in ACTA1-MCM;FLEXDUX4 muscles (H). TMX induction in ACTA1-MCM;FLEXDUX4 muscles leads to increased numbers of myonuclei expressing DUX4-FL, heterogeneous fiber size distribution, and mononuclear cell infiltration (L).

https://doi.org/10.1371/journal.pone.0192657.g011
**Fig 12.** Induction of DUX4-FL expression leads to fibrosis in skeletal muscles of *ACTA1-MCM;FLEXDUX4* mice. Picrosirius red staining on tibialis anterior (A-C) and heart (E-G), and summarized in (D and H), shows significantly increased fibrosis in skeletal muscles after 9 days of TMX treatment but not in the hearts of *ACTA1-MCM;FLEXDUX4* mice. (D and H) Combined results from muscle obtained from 3 mice per group, 5 non-consecutive cross-section sections analyzed per muscle. Significance (*p* < 0.01) was calculated using Welch’s t-test; n.s. = not significant. Bars = 500 μm and 100 μm (insets).

[https://doi.org/10.1371/journal.pone.0192657.g012](https://doi.org/10.1371/journal.pone.0192657.g012)

**Fig 13.** Induction of DUX4-fl leads to innate immune cell infiltration in skeletal muscles of *ACTA1-MCM;FLEXDUX4* mice. Gastrocnemius muscle sections from (A, E) *ACTA1-MCM*, (B-D and F-H) *ACTA1-MCM;FLEXDUX4* mice immunostained for CD11b (A-C, and E-G) or Ly6g (E and H) reveal immune cell infiltration upon TMX induction of DUX4-fl expression. Panels (A-D) 10X magnification, Scale bar = 100 μm, (E-H) 40X magnification, Scale bar = 25 μm.

[https://doi.org/10.1371/journal.pone.0192657.g013](https://doi.org/10.1371/journal.pone.0192657.g013)
role of the immune response in DUX4-mediated pathophysiology, including fibrosis, and for testing therapies targeting the immune system, such as anti-inflammatories.

Conclusions

This study represents the generation and initial characterization of the conditional cre-inducible FLexDUX4 line of mice to provide a foundation of information for investigators to further develop and utilize according to their individual needs. Full characterization of this model in respect to FSHD, including a global analysis of gene expression, muscle physiology, detailed muscle pathology, etc., is in progress and will be part of subsequent studies. However, since we made these mice freely available to the research community through Jackson Labs prior to any publication, and to prevent the wasting of time and resources in experimental design, it is important to disseminate relevant data on this model in a timely manner to researchers already using these mice or those interested in obtaining the mice prior to a full characterization. We show conclusively that FLexDUX4 mice are a highly versatile model for manipulating DUX4-fl expression levels in vivo. These mice can be used alone as a model of very low-level DUX4-fl mRNA expression in all myocytes without the development of adverse phenotypes, or, alternatively, in combination with cre-driver lines to force higher, pathogenic levels of DUX4-fl expression, as desired. Importantly, we characterized ACTA1-MCM;FLexDUX4 mice, with and without TMX, as valuable new tools for FSHD research and for pre-clinical testing of potential FSHD therapeutics targeting DUX4-fl mRNA, protein, and certain downstream

Fig 14. Induction of DUX4-fl leads to accumulation of immune cells, loss of membrane integrity, and a necrotic myopathy in skeletal muscles of ACTA1-MCM;FLexDUX4 mice. (A-C) Tibialis anterior muscles from myopathic ACTA1-MCM;FLexDUX4 mice 8 days post TMX treatment exhibit mosaic nuclear expression of DUX4-FL and (D, E) accumulation of CD11b+ cells around damaged and dying myofibers, distinguished by a loss of dystrophin localization (arrows). Scale bars = 25μm.

https://doi.org/10.1371/journal.pone.0192657.g014
effects. With the FLEXDUX4 mice already available from Jackson Labs, this initial analysis provides the platform for allowing the field to move forward with investigations of FSHD pathogenic mechanisms, and testing the efficacy of numerous potential therapeutic approaches targeting DUX4-fl mRNA and protein.

**Supporting information**

S1 Sequence. DUX4 transgene sequence.
(DOCX)

S2 Sequence. FLEXDUX4 mRNA sequence.
(DOCX)

S1 Fig. Silent DUX4 mutations eliminate DUX4-s mRNA while maintaining DUX4-fl mRNA.
(PDF)

S2 Fig. DNA methylation analysis of the DUX4 transgene shows no changes across generations.
(PDF)

S3 Fig. Female adult FLEXDUX4/+ mice exhibit a more severe alopecia than males.
(PDF)

S4 Fig. Homozygous FLEXDUX4/FLEXDUX4 mice have more severe phenotypes than hemizygous FLEXDUX4/+ mice.
(PDF)

S5 Fig. The DUX4 transgene does not aberrantly recombine in FLEXDUX4 mice in the absence of cre.
(PDF)

S6 Fig. The low levels of DUX4-fl mRNA expression in FLEXDUX4/+ mouse muscle do not come from bursting nuclei.
(PDF)

S7 Fig. Expression of Wifdc3, a murine DUX4-FL target gene, is not significantly induced in FLEXDUX4 hemi- and homozygous mice.
(PDF)

S8 Fig. Skeletal muscles of FLEXDUX4 mice have normal histology.
(PDF)

S9 Fig. UBC-creERT2;FLEXDUX4 mice undergo low levels of transgene recombination in the absence of TMX.
(PDF)

S10 Fig. DUX4-fl expression leads to enlarged lymph nodes, suggesting a DUX4-mediated immune response.
(PDF)

S11 Fig. TMX induction of DUX4-fl leads to physical decline and muscle loss in ACTA1-MCM, FLEXDUX4 mice.
(PDF)
S12 Fig. TMX induces DUX4-FL protein and mononuclear cell infiltration in
*ACTA1-MCM;FLExDUX4* double transgenic mice.
(PDF)

S13 Fig. Neutrophils do not accumulate in muscles of control mice.
(PDF)

S1 Movie. *UBC-creERT2;FLExDUX4* (12 wks) have kyphosis and difficulty with balance.
(MP4)

S2 Movie. *UBC-creERT2;FLExDUX4* mice (12wks) exhibit wild running.
(MP4)

S3 Movie. *ACTA1-MCM;FLExDUX4, ACTA1-MCM/+, and FLExDUX4/+* mice 8 days after exposure to TMX chow and only the *ACTA1-MCM;FLExDUX4* mice exhibit difficulty moving around the cage.
(MP4)

S4 Movie. *FLExDUX4/+* (mouse 7b, +TMX IP injection) shows normal movement.
(MP4)

S5 Movie. *ACTA1-MCM;FLExDUX4* (mouse 6b, No TMX) shows normal movement.
(MP4)

S6 Movie. *ACTA1-MCM;FLExDUX4* (mouse 3b, +TMX IP injection) displays impaired movement.
(MP4)

S7 Movie. *FLExDUX4/+* (mouse 7b, +TMX IP injection) hanging test for full 2 minutes.
(MP4)

S8 Movie. *ACTA1-MCM;FLExDUX4* (mouse 6b, No TMX) hanging test for full 2 minutes.
(MP4)

S9 Movie. *ACTA1-MCM;FLExDUX4* (mouse 3b, +TMX IP injection) hanging test <2 seconds.
(MP4)

S10 Movie. *ACTA1-MCM;FLExDUX4* (mouse 4b, +TMX IP injection) hanging test <2 seconds.
(MP4)

**Acknowledgments**

We thank Steven Blier, Jennifer Burgess, Chris Carrino, Chris Hughes, and Daniel P. Perez for their support in helping to raise world-wide awareness for FSHD and help us progress towards a cure. We thank Dr. Charis L. Himeda for helpful discussions and editing the manuscript.

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References


62.
65.
Klinge L, Eagle M, Haggerty ID, Roberts CE, Straub V, Bushby KM. Severe phenotype in infantile
67.
69.
Robinson SP, Langan-Fahey SM, Johnson DA, Jordan VC. Metabolites, pharmacodynamics, and phar-
64.
Guiraud S, Aartsma-Rus A, Vieira NM, Davies KE, van Ommen GJ, Kunkel LM. The Pathogenesis and
63.
66.
59.
Jegalian BG, De Robertis EM. Homeotic transformations in the mouse induced by overexpression of a
58.
57.
dystrophy revisited: Expansion of clinical phenotypes in patients with a very short EcoRI fragment. Neu-
55.
56.
Klinge L, Eagle M, Haggerty ID, Roberts CE, Straub V, Bushby KM. Severe phenotype in infantile
54.
53.
52.
51.
Fei R, Wagner J, Metzger D, Chambron P. Regulation of Cre recombinase activity by mutated estrogen
50.
49.
48.
47.
46.
45.
Schessl J, Zou Y, Bonnemann CG. Congenital muscular dystrophies and the extracellular matrix. Semin
44.
43.
MacDonald EM, Cohn RD. TGFbeta signaling: its role in fibrosis formation and myopathies. Curr Opin
42.
41.
facioscapulo-humeral muscular dystrophy is correlated with oxidative stress and mitochondrial dysfunc-
40.
39.
Wallace LM, Garwick-Coppens SE, Tupler R, Harper SQ. RNA Interference Improves Myopathic Pheno-
38.
37.
36.
35.
Wallace LM, Garwick-Coppens SE, Tupler R, Harper SQ. RNA Interference Improves Myopathic Phen-
34.
33.
ргo/10.1038/346425a0 PMID: 1974032.
32.
31.
Fleming TJ, Fleming ML, Malek TR. Selective expression of Ly-6G on myeloid lineage cells in mouse
bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6
30.
29.
28.
27.
26.
25.
24.