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Original Article

Testing the effects of FSHD candidate gene expression in vertebrate muscle development

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Abstract: The genetic lesion leading to facioscapulohumeral muscular dystrophy (FSHD) is a dominant deletion at the 4q35 locus. The generally accepted disease model involves an epigenetic dysregulation in the region resulting in the upregulation of one or more proximal genes whose overexpression specifically affects skeletal muscle. However, multiple FSHD candidate genes have been proposed without clear consensus. Using *Xenopus laevis* as a model for vertebrate development our lab has studied the effects of overexpression of the FSHD candidate gene ortholog, frg1 (FSHD region gene 1), showing that increased levels of frg1 systemically led specifically to an abnormal musculature and increased angiogenesis, the two most prominent clinical features of FSHD. Here we studied the overexpression effects of three other promising FSHD candidate genes, DUX4, DUX4c, and PITX1 using the same model system and methods for direct comparison. Expression of even very low levels of either DUX4 or pitx1 early in development led to massive cellular loss and severely abnormal development. These abnormalities were not muscle specific. In contrast, elevated levels of DUX4c resulted in no detectable adverse affects on muscle and DUX4c levels did not alter the expression of myogenic regulators. This data supports a model for DUX4 and PITX1 in FSHD only as pro-apoptotic factors if their expression in FSHD is confined to cells within the myogenic pathway; neither could account for the vascular pathology prevalent in FSHD. Taken together, increased frg1 expression alone leads to a phenotype that most closely resembles the pathophysiology observed in FSHD patients.

Keywords: facioscapulohumeral muscular dystrophy, FSHD, DUX4, DUX4c, PITX1, FRG1

Introduction

FSHD is now recognized as one of the most prevalent forms of muscular dystrophy in adults (http://www.orpha.net). Prominent features of this myopathy are the progressive weakening of the skeletal muscles in the face, shoulder girdle, and the upper arms, and these muscular aspects are often combined (>50% of patients) with retinal vasculopathy [1, 2]. The genetic lesion leading to the most prominent form of FSHD (FSHD1A), accounting for ~98% of FSHD patients, is an autosomal dominant contraction of the D4Z4 repeat array at chromosome 4q35 below 11 copies [3, 4]. This contraction leads to hypomethylation of the D4Z4 repeats, which has been proposed to lead downstream to the misregulation of one or more of the 4q35 localized genes including *FRG1*, *ANT1*, *FRG2*, *DUX4*, and *DUX4c* [5]. However, none of these candidate genes has consistently been shown to exhibit significantly altered RNA expression levels in affected FSHD muscle biopsies compared to unaffected controls [6-13]. Multiple issues complicate these expression analyses including large differences within an affected muscle and potentially at the site of biopsy, the bias focusing on FSHD gene misexpression exclusively in the skeletal muscle lineage, and the potential that FSHD gene misexpression occurs during cell differentiation [10, 14-16]. Thus, without knowing when and where in human muscle development gene misexpression leading to FSHD occurs, the cause of the FSHD pathophysiology

has remained controversial.

To circumvent the ambiguity of RNA expression analyses, we have taken a developmental approach to the problem by first addressing the normal function of an FSHD candidate gene during development and then assaying the effect of overexpression of an FSHD candidate gene on vertebrate development. The system for these studies is the early development of *Xenopus laevis*. Our initial analysis focused on understanding the function and expression of one candidate gene, *frg1* [17, 18]. *FRG1* is a highly conserved gene of unknown function that is overexpressed in FSHD patient derived myoblasts undergoing myogenic differentiation [15]. These studies found that frg1 is required for the normal development of the vertebrate musculature and vasculature [17, 18]. Consistent with a role in FSHD pathology, systemically elevated levels of frg1 led to phenotypes specifically in the vertebrate musculature and vasculature which strongly correlated to the two most common symptoms of FSHD, dystrophic muscle and increased angiogenesis [17, 18]. Thus, developmentally, FRG1 overexpression fits the criteria for being causal for FSHD pathology.

We have continued our analysis with three additional FSHD candidate genes, *DUX4*, *DUX4c*, and *PITX1* [6, 19, 20]. *DUX4* and *DUX4c* are encoded within open reading frames (ORFs) of different 4q35 D4Z4 repeat units within or near the FSHD deletion [21, 22]. Although D4Z4 repeat arrays exist in multiple loci in the genome [23], RNAs originating specifically from the 4q35 localized D4Z4/DUX4 and D4Z4/DUX4c loci are increased in certain FSHD patient-derived muscle cells [6, 19, 20]. A normal cellular or developmental role for the 4q35 DUX4 protein, if any, has not been described; however, expression of the currently accepted 4q35 derived DUX4 protein is highly toxic to all cells leading to a rapid onset of apoptosis [19, 24]. This apoptotic effect of DUX4 expression is postulated to be from direct competition with the regulatory targets of PAX3/PAX7 and is inhibited by elevated expression of PAX3 or PAX7 [24]. Interestingly, in a cell culture system, DUX4 has been shown to bind the promoter and activate expression of *PITX1*, a non-4q35 localized FSHD candidate gene whose expression has been found to be upregulated in FSHD muscle, providing an alternative mechanism for DUX4-mediated pathology [6]. *DUX4c*, located within a partial D4Z4 unit 42 kb proximal to the FSHD-associated D4Z4 array, is identical to DUX4 through their N-terminal double homeobox domains however they have differing C-terminal amino acid sequences [22]. DUX4c expression has been detected in muscle cells where it is proposed to act as a myogenic regulator and inhibitor of myoblast differentiation [20, 25].

In this study, we assayed the effects of expression of human DUX4 and DUX4c, as well as the *X. laevis* ortholog of *PITX1* on early vertebrate development, with particular attention to muscle growth and differentiation. We show that DUX4 expression and pitx1 overexpression both lead to massive cellular loss that is not muscle specific. With DUX4 in particular the cellular loss occurred at extremely low expression levels and was cell-type independent indicating that this protein is highly toxic to all vertebrate cells and this toxic effect was not specific to muscle. DUX4c expression did not lead to any observable change in muscle development or differentiation or changes in the expression of the myogenic regulators myf5 or myoD in *Xenopus*. Contradictory to what has been reported in cell culture, we found that both DUX4 and DUX4c significantly reduced expression levels of pitx1 transcripts in our animal model. Together with our previous studies on frg1, this presents the first analysis for direct comparison of the effects of expression of the main FSHD candidate genes in a developing vertebrate system.

Materials and methods

Frog husbandry

Adult *X. laevis* were purchased from *Xenopus* Express. All procedures were carried out in accordance with established UIUC IACUC approved protocols for animal welfare.

Plasmid constructs and RNA production

The vectors pCIneo DUX4 and pCIneo DUX4c were generously provided by Dr. Alexandra Belayew [20, 21]. The plasmids for *EGFP, myoD*, *pax3*, and *myf5* RNA have been previously described [17]. The *pitx1* cDNA was produced by RT-PCR using primers 5' GTGATTGACATGGATT CCTTTAAAGG 3' AND 5' TCAACTGTTATATTGGCA AGCATTGAG 3', cloned into pGEM T-Easy (Promega) and sequenced. The cDNA was subcloned into the EcoRI and XbaI sites of

pcDNA3.1 (Invitrogen). Production of *EGFP* mRNA was performed as previously described [17]. For DUX4, DUX4c and pitx1 mRNA, constructs were linearized and capped mRNA was generated using T7 RNA polymerase and the mMessage mMachine kit (Ambion, Inc).

Xenopus embryo injections

In vitro fertilized embryos were generated as described [17]. Embryos were microinjected after completion of the two cell stage, as indicated by the beginning of the second cleavage, in 1X MMR with 3% Ficoll and incubated at 19° C. Between 3-6 hours after injection, embryos were transferred to 0.1X MMR with 3% Ficoll. After 24-36 hours embryos were either peeled and fixed for stage 18-22 embryos or cultured in 0.1X MMR until the desired stage. After neural tube closure all injected embryos were sorted based on left, right or bilateral fluorescence. *DUX4* mRNA was injected at 500 pg, 250 pg, 100 pg, 10 pg, 1 pg, and 0.5 pg along with 500 pg *EGFP* mRNA. *DUX4c* mRNA was injected at 1 ng along with 500 pg *EGFP*. *pitx1* was injected at 150 pg and 50 pg along with 500 pg *EGFP*. Control *EGFP* mRNA injections were performed at 500 pg.

TUNEL assay

TUNEL staining of whole-mount *Xenopus* embryos was carried out using a protocol adapted from Hensey and Gautier [26]. All procedures were carried out at room temperature unless noted otherwise. Embryos were fixed for 1 hr. in MEMFA, (100 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO4, 4% formaldehyde). Embryos were washed in methanol 2 x 30 min. and stored in methanol at -20°C. For rehydration, half of methanol was replaced with PBS and washed 5 x 5min. The embryos were washed with PBT (0.2% Tween-20 in PBS), 2 x 15 min., followed by 2 x 15 min. washes in PBS. Embryo pigment was removed by treatment for 1-2 hours in 1% H_2O_2, 5% Formamide, and 0.5X SSC under bright light, and washed 3 x 15 min. in PBS. Embryos were transferred to terminal deoxynucleotidyl transferase, (TdT), buffer (Invitrogen) and washed for 30 min. End labeling was carried out overnight in TdT buffer containing 0.5 mM digoxygenin-dUTP (Roche Diagnostics), and 150 U/ml TdT (Invitrogen). Embryos were then washed 2 x 1 hr. in PBS/1 mM EDTA, at 65°C, followed by 4 x 1 hr. in PBS. Detection and chromogenic reaction was carried out as previously described [27]. Embryos were viewed and stored following rehydration in 1X PBS.

In situ hybridizations

Embryos were staged according to Nieuwkoop and Faber [28], fixed 1-2 hrs in MEMFA, washed 2 x 30 min in 100% methanol and stored in 100% methanol at -20°C until use. The *EGFP*, *Xenopus myoD*, *pax3*, and *myf5* antisense probes generated as previously described [17]. The *pitx1* probe was generated by linearizing pGEM *pitx1* with SalI and using T7 RNA polymerase transcription to generate digoxigenin (DIG) -11-UTP (Roche Diagnostics) antisense RNA probes. *In situ* hybridizations were performed according to standard methods [27] and detected with alkaline phosphatase (AP) linked anti-DIG antibody (Roche Diagnostics) and the chromogenic substrates BCIP (5-Bromo-4-chloro -3-indolyl phosphate, toluidine salt) and NBT (Nitro blue tetrazolium chloride) (Roche Diagnostics). Embryos were refixed overnight in Bouin's fixative, followed by washing in 70% ethanol/30% PBS-Tween 0.1%, and pigment was removed by treatment for 1-2 hours in 1% H_2O_2, 5% Formamide, and 0.5X SSC under bright light. Embryos were then washed in methanol 10 minutes and transferred to 1mM EDTA in PBS or glycerol for analysis and photography.

Immunohistochemistry

Embryos were staged and fixed as above, rehydrated in PBS-DT (1% DMSO, 1% Tween-20) and washed for 15 min in PBS-DT. Samples were blocked in 0.1M glycine, 2% milk, 1% BSA, 1% Tween-20 and 1% DMSO for 4 hours at room temperature or overnight at 4°C. Primary antibodies were diluted in blocking solution as follows: Skeletal muscle marker (12/101) diluted 1:3 or NCAM (4d) diluted 1:20 were incubated with embryos overnight at 4°C and detected using a HRP secondary (GE Healthcare) with a DAB staining kit from (Roche Diagnostics). For paraffin sectioning, tadpoles immunostained for 12/101 or NCAM were dehydrated through an EtOH series, placed in 50/50 EtOH/Xylene for 10 minutes, washed twice with 100% Xylene, embedded in paraffin, positioned, and sectioned using a microtome. The 12/101 monoclonal antibody, developed by J.P. Brockes, and the NCAM 4d monoclonal antibody, developed

by U. Rutishauser, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained at The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

qRT-PCR

For each sample, total RNA was purified from 10 pooled embryos using Trizol reagent (Invitrogen) per manufacture's protocol. RNA was then treated with 1U RQ1 DNase (Promega) per 1ug RNA for 30 min. at 37°C. cDNA synthesis was preformed using 1ug of total RNA, 50ng of random hexamer, and Superscript III (Invitrogen) per manufacture's suggested method. Relative transcript levels were determined using 1ul of (1:20 diluted) cDNA (in triplicate), iQ SYBR Supermix, and gene specific primers (myoD: 5' TGCCAAGAGTCCAGATTTCC 3', 5' CAGGTCTTCAAAGAAACTCATGTC 3'; myf5: 5' GCTTATCTAGTATTGTGGATCGG 3', 5' CTGGTT TGTTGGGTGTAAGG 3'; pitx1: 5' CATGAGCA-GAAGTGATTGAC 3', 5' GTAAAGTGAGTCCTTTGTC TCC 3'; gapdh: 5' GGTGAAGGTTGGAATTAACGG 3', 5' GATCAGCTTGCCATTCTCAG 3') on a Bio-Rad iCycler IQ machine. Experiments were preformed at least 3 times. Data analyses were preformed using the comparative Ct method and error bars are ± standard error of the mean. Changes were determined using the two tailed student's t-test and considered significantly different at a *P-value* <0.05.

Results

Expression of DUX4 and pitx1 lead to cellular loss while DUX4c expression has minimal effect on development

The early development of *X. laevis* was used as a model system to determine the effects of FSHD candidate genes DUX4, DUX4c, and pitx1 expression levels on vertebrate development. In normal human tissues, DUX4 and DUX4c expression is undetectable and the proteins are neither required for nor involved in any known normal cellular function. The human 4q35 DUX4 and DUX4c genes are not conserved outside of their double homeobox domains and *Xenopus* do not possess any orthologs. Therefore, we assayed the effects of the presence of DUX4 or DUX4c during muscle development compared to a background of no expression. Conversely, the pitx1 transcription factor, as was the case with frg1, is highly conserved between mammals and *Xenopus* in protein sequence, function, and developmental expression [29]. For this gene, we assayed the effect of increasing its expression during development as well as expression outside its normal developmental profile.

To determine the effects of DUX4 and DUX4c expression, and elevated levels of pitx1 during development, one side of early four cell-stage *X. laevis* embryos were injected with the corresponding mRNAs as well as mRNA encoding the *EGFP* marker mRNA. The developing embryos resulted in animals overexpressing the desired proteins on the injected side (confirmed by fluorescence) while co-developing a control, uninjected side. To study any tissue-specific effects on muscle, titration experiments with *DUX4*, *pitx1*, and *DUX4c* mRNA were performed and the numbers of abnormally developing embryos were observed. Abnormal development was scored as any abnormality observed during development, regardless of severity. Similar to previous data from cultured myoblasts, we found that our initial injection level of 100 pg *DUX4* mRNA was highly toxic to the embryos (**Figure 1A** & **1K**). Embryos were arrested by stage 9 with an overall apoptotic appearance throughout the entire embryo. This severe phenotype occurred in all embryos injected with 100 ng (n = 13), 50 pg (n = 26), and 25 pg (n = 19) examined, although developmental arrest occurred slightly later with less *DUX4* mRNA (**Figure 1A** & **1K**). This severe apoptotic phenotype resulting in early developmental arrest was never observed in EGFP injected (n = 119) or uninjected (n = 600) controls (**Figure 1J** & **1K**). Unlike 100 pg, injection of 10 pg *DUX4* mRNA (n = 426) resulted in toxicity generally restricted to the injected side 72 hours post-injection (**Figure 1B**). The heavy cellular loss in the injected side led to a small embryo and eventual arrest, likely due to gastrulation or neurulation defects caused by DUX4 expression. Further reducing the level to 1 pg *DUX4* mRNA (n = 166) resulted in a low percentage of animals which were able to progress through and complete closure of their neural tubes (**Figure 1C**); however, all 1 pg *DUX4* injected embryos tested showed massive apoptosis by TUNEL staining (**Figure 1D**). A further 2-fold reduction to 0.5 pg *DUX4* mRNA (n = 213) resulted in a significant increase of normal developing embryos, suggesting the effect of

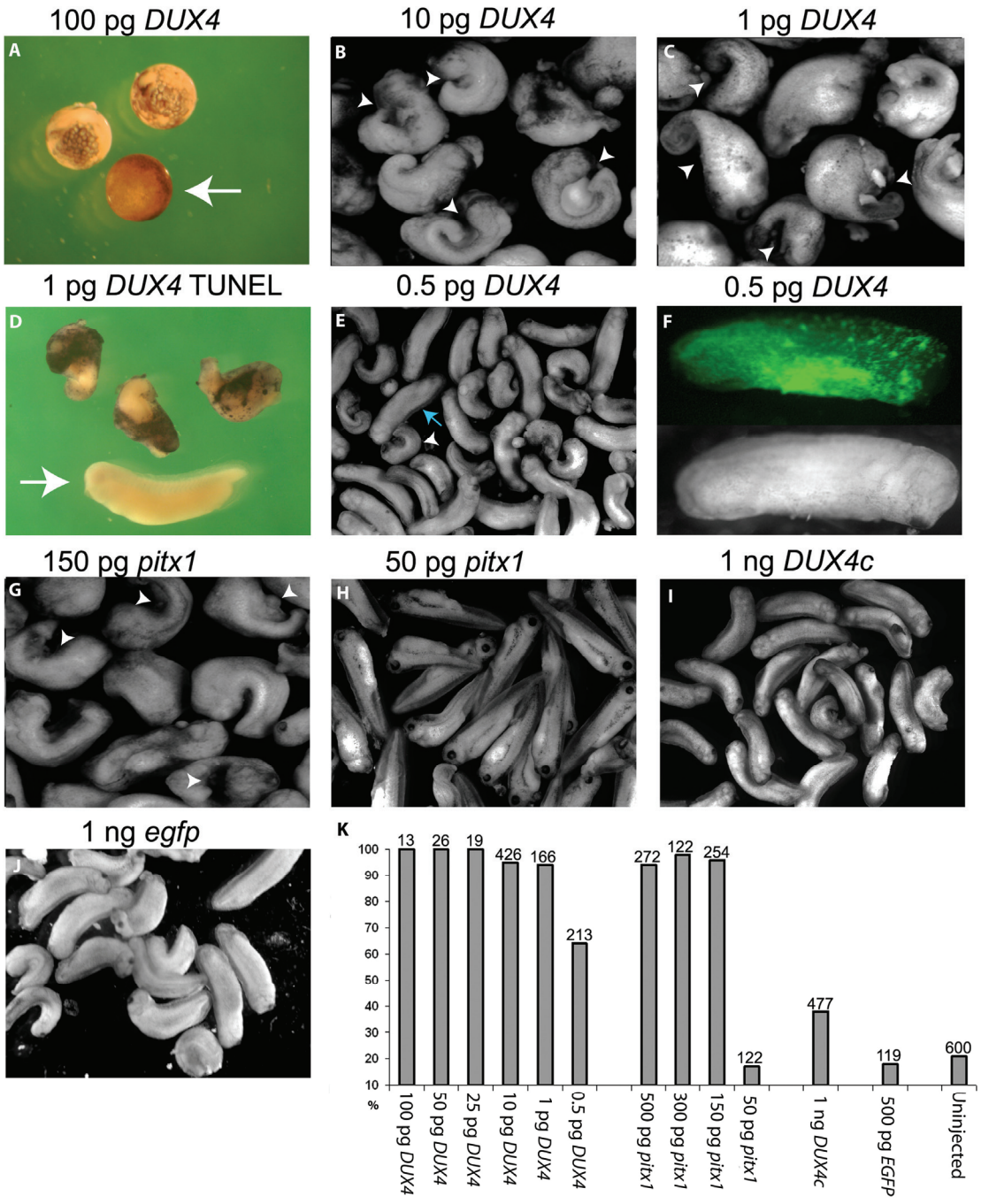

Figure 1. DUX4 and pitx1 induce developmental abnormalities. A) Injection of 100 pg *DUX4* mRNA leads to early developmental arrest (stage 9) and apoptosis compared to control embryos (arrow). B and C) Injection of10 pg and 1 pg of *DUX4* mRNA lead to developmental abnormalities on only the injected side of embryos (white arrowhead). D) TUNEL assay on 1 pg DUX4 mRNA injection shows apoptosis in embryos (blue staining) compared to control injections (white arrow). E) Injection of 0.5 pg *DUX4* mRNA allows some normal development (blue arrow) compared with abnormal developing animals (white arrowhead). F) Normally developing 0.5 pg *DUX4* mRNA injected embryo is indicated by expression of co-injected EGFP (top) compared with the uninjected, non-fluorescing side (bottom, shown as a bright field image). G) Injection of 150 pg *pitx1* mRNA results in developmental abnormalities on the injected side (white arrow head). H) Embryos injected with 50 pg *pitx1* mRNA are developmentally normal (compared with G). I and J) The effect of 1ng DUX4c mRNA injection (I) is similar to EGFP controls (J). K) Summary of percent abnormal embryos observed in mRNA injection experiments with number of animals analyzed for each set.

DUX4 is either cellular toxicity or nothing (**Figure 1E**). Even in these embryos, the fluorescence of the EGFP appeared patchy (**Figure 1F**), suggesting that though they appeared to have progressed through development normally, DUX4 had still led to cellular loss. Therefore, consistent with other systems, DUX4 expression is extremely cytotoxic in developing *Xenopus* [19, 24].

DUX4 is capable of inducing *PITX1* expression in mouse C2C12 cell culture suggesting PITX1 misexpression as a possible mechanism for DUX4-mediated pathology [6]. To determine the effect of aberrantly induced pitx1 expression, we bypassed DUX4 expression and microinjected mRNA encoding *Xenopus* pitx1 into embryos as above. Similar to injections of *DUX4* mRNA, we found that both 500 pg (n = 272) and 300pg (n = 122) of *pitx1* mRNA led to early embryo arrest while injection of 150 pg *pitx1* (n = 254) led cellular loss specifically on the injected side leading to a curled phenotype and gastrulation or neurulation defects (**Figure 1G** & **1K**). Injections of 50 pg *pitx1* mRNA (n = 122) had no obvious effects on development (**Figure 1H** & **1K**). Thus, while pitx1 overexpression is cytotoxic, developing embryos are much more tolerant of pitx1 overexpression than DUX4 expression suggesting that DUX4 cytotoxicity is not mediated through the activation of *pitx1*.

The analysis of the third FSHD candidate gene, DUX4c, produced results that were a stark contrast to the effects of DUX4 and pitx1. Microinjections of as much as 1ng of DUX4c mRNA (n=477) only lead to a modest increase over uninjected background levels of developmental abnormalities, yet still far fewer and less in severity than seen with a 2000-fold lower amount of *DUX4* mRNA (**Figure 1I** & **1K**). Because DUX4c shares an identical double homeobox domain with DUX4, the fact that a 2000-fold increase in mRNA produced only minor developmental problems indicates that a strictly competitive interaction with Pax3 and Pax7 for DNA regulatory targets is not responsible for the DUX4 phenotype.

Cytotoxicity mediated by DUX4 and pitx1 is not muscle specific

The symptoms associated with FSHD are primarily muscular and often combined with a less prominent vascular component. Taking into account the generally accepted model whereby the FSHD1A deletion leads to an epigenetic upregulation of gene expression, one would expect the effect of a viable candidate gene's systemic expression to be primarily seen in tissues affected in FSHD as is the case with *frg1*. In order to determine if muscle was specifically affected by DUX4, pitx1, or DUX4c expression, injected embryos were analyzed for differentiated muscle and neurologic tissue by immunostaining with the 12/101 or NCAM antibodies, respectively (**Figure 2** & **3**). Due to the severe loss of tissue in the 1 pg *DUX4* and 150 pg *pitx1* injected animals, the figures depict some of the best developing and staining animals, as most failed to form a complete neural tube closure and were consequently too curled to capture the staining pattern in photographs.

Immunostained animals were qualitatively determined to exhibit normal, depleted, or absent levels of immunostaining on the mRNA injected side (**Figure 2**). Embryos scored as depleted included those exhibiting highly dispersed but significant staining, missing somites and somite disruptions, and a significantly thinner somite area.

DUX4 injected embryos were first analyzed for affects on the developing muscle. Qualitative inspection of 1 pg *DUX4* injected embryos stained with 12/101 (n = 45, missing n = 22, depleted n = 23) indicate a severe loss of muscle tissue by the lack of 12/101 immunostaining specifically on the injected side of the embryo (**Figure 2A'** and **2B'** compared to **Figure 2A** and **2B**). However, transverse paraffin sectioning revealed these animals had a weak appearance of staining due to a build up of cellular debris between the 12/101 stained muscle near the notochord/neural tube and the lateral edge of the injected side (**Figure 2B** and **2F**). The area of the myotome on the injected side was significantly decreased due to cellular loss. We find a large increase in the number of embryos with normal 12/101 staining when we inject 0.5 pg *DUX4* mRNA (n = 87, missing n = 27, depleted n = 31) and this increase in normally stained embryos corresponded well to the number of normally developing embryos (**Figure 2I'** compared to **2I, 2J**, **Figure 3**).

Muscle is the primary affected tissue in FSHD. To determine if DUX4 was similarly cytotoxic to tissues unaffected in FSHD, neurons were as-

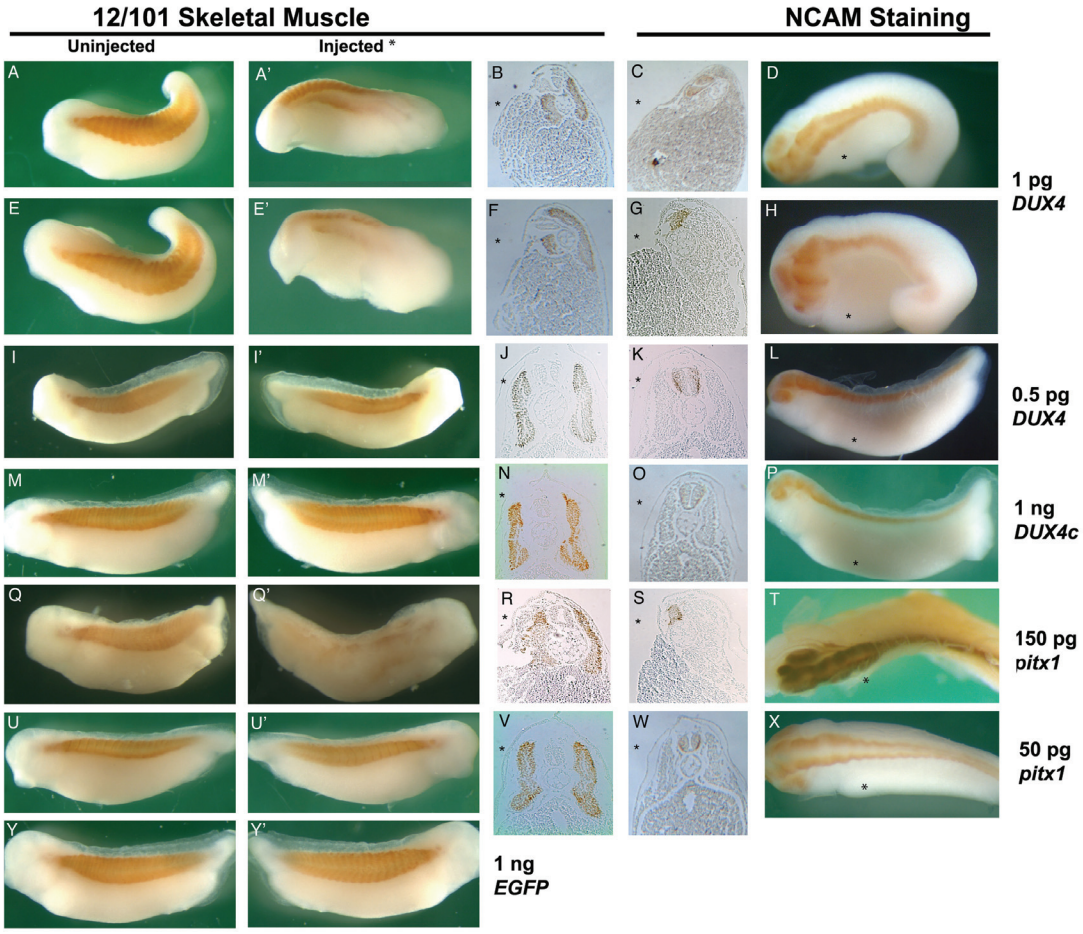

Figure 2. Expression of DUX4 and pitx1 but not DUX4c affect muscle and neural tissue development. A-H) *DUX4* (1 pg) injected embryos show depletion of 12/101 (A, B, E, and F) and NCAM (C, D, G, and H) immunostaining and cellular loss specifically on the mRNA injected side (*) in wholemount (A, A', D, E, E', and H) and transverse sections (B, C, F, and G). Wholemount (I, L) and transverse sectioned (J, K) *DUX4* (0.5 pg) injected embryos have normal 12/101 (I, J) and NCAM (K, L) staining and myotome development on the injected side (*). Wholemount (M, P) and transverse sectioned (N, O) *DUX4c* (1 ng) injected embryos have normal 12/101 (M, N) and NCAM (O, P) staining and myotome development on the injected side (*). Wholemount (Q, T) and transverse sectioned (R, S) *pitx1* (150 pg) injected embryos show depletion of 12/101 (Q, R) and NCAM (S, T) immunostaining, abnormal myotome development and cellular loss specifically on the injected side (*). Wholemount (U, X) and transverse sectioned (V, W) U-X) *pitx1* (50 pg) injected embryos show no depletion of 12/101 (U, V) and NCAM (W, X) immunostaining and exhibit normal myotome development with no cellular loss on the injected side (*). Wholemount (Y) 1 ng *EGFP* (1 ng) injected embryos have normal 12/101 staining when compared to the uninjected side.

sayed in 1 pg *DUX4* injected embryos (n = 24). Immunostaining for NCAM, which at stage 34 of *Xenopus* development is confined to neural tissue, resulted in levels of missing (n = 13) and depleted (n = 11) NCAM staining similar to that of 12/101 (**Figure 3**). In several animals the NCAM staining was specifically depleted in the area of the eye (**Figure 2D** & **2H**) and transverse sections revealed that often the neural tube staining was depleted as well (**Figure 2C** & **2H**). We conclude that the cellular loss mediated by DUX4 is not restricted to muscle.

Embryos injected with *pitx1* mRNA were similarly analyzed for muscle and neuronal defects. The injection of *pitx1* led to developmental abnormalities similar to those seen with DUX4 albeit at a much higher concentration of mRNA. As with the DUX4 injections, 150 pg *pitx1* mRNA injections (n = 64) led to depletion (n = 32) and

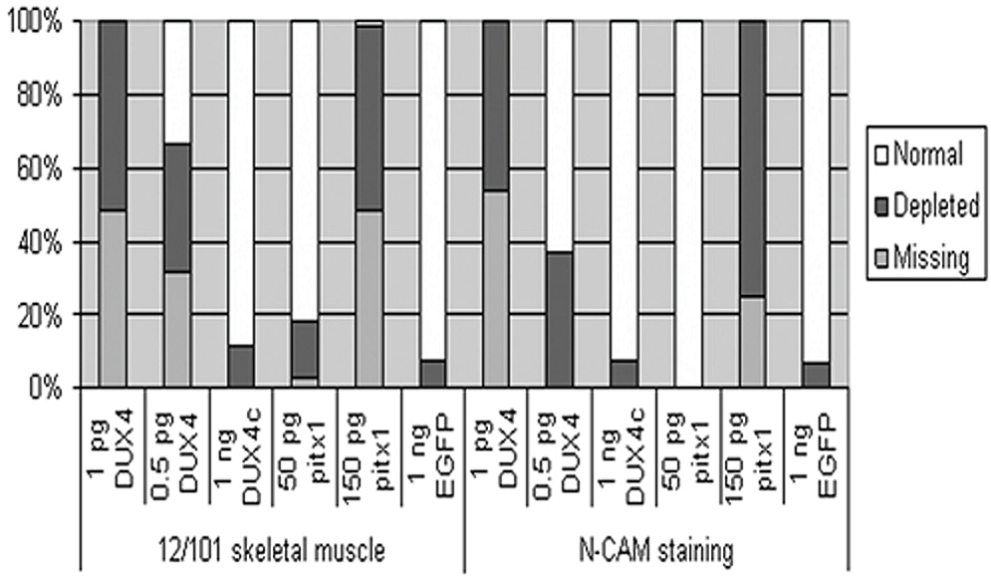

Figure 3. Summary of DUX4, DUX4c, and pitx1 mediated affects. Graphic summary of results from Figure 2, showing percent of animals with missing, depleted, or normal 12/101 and NCAM immunostaining.

loss (n = 31) of 12/101 immunostaining from the injected side (**Figure 2Q**' compared to **2Q**, **2R**). This effect was dose dependent, with 50 pg *pitx1* mRNA (n = 71) causing almost no loss of 12/101 immunostaining (missing n = 2, depleted n = 11) (**Figure 2U'** compared to **2U**, **2V**). Similarly, the 150 pg *pitx1* injection (n = 8) led to depleted (n = 6) or missing (n = 2) NCAM immunostaining (**Figure 2S** & **2T**), while 50 pg *pitx1* injection (n = 19) did not (**Figure 2W** &**2X**).

Despite the lack of any significant gross developmental defects (**Figure 1I**), DUX4c injected embryos were assayed for muscular and neuronal alterations. Differences were expected in the myotome based on previous findings of DUX4c mediated inhibition of myoblast differentiation [20, 25]. However, when stained with 12/101, 1 ng *DUX4c* mRNA injected embryos (n = 89) still displayed normal immunostaining (**Figure 2M'** compared to 2M, 2N) with abnormalities (missing n = 0, depleted n = 10) comparable to *EGFP* mRNA injected controls (n = 41, missing n = 0, depleted n = 3) (**Figure 2Y**, **2Y'**, **Figure 3**). Similarly, the NCAM staining of DUX4c injected embryos (**Figure 2O** & **2P**) had abnormalities (n = 14, missing n = 0, depleted n = 1) similar to NCAM stained *EGFP* mRNA injected controls (n = 15, missing n = 0, depleted n = 1) (**Figure 3**). We conclude that DUX4c levels have no effect on the developing musculature or neurons.

DUX4 eliminates myoD, myf5, and pax3 expression profiles

DUX4 expression led to the loss of 12/101 immunostaining indicating differentiated muscle was degraded or missing. To determine if DUX4 expression leads to a loss of muscle cell precursors, *in situ* hybridizations with probes against *myoD*, *myf5*, or *pax3* were performed (**Figure 4**). In stage 34 embryos from the 1 pg *DUX4* mRNA injections, the expression of all three of these markers was missing in a large majority (myoD n = 60/62; myf5 n = 12/20; pax3 n = 23/28) (**Figure 4A'**, **4B'**, & **4C'** compared to **4A**, **4B**, & **4C**). Assaying earlier in development, stage 20 *DUX4* injected embryos were missing expression of *pax3* (n = 11/20) (**Figure 4G**). Although the markers *myf5* and *pax3* were depleted, the entire tissue, including neural tissue was affected. When taken into consideration with our previous findings that the DUX4-meidated defects began at gastrulation (**Figure 1A**) and we conclude that the DUX4-mediated cellular loss occurs prior to stage 20 and therefore is clearly not muscle specific.

Unlike DUX4, DUX4c had little to no observable

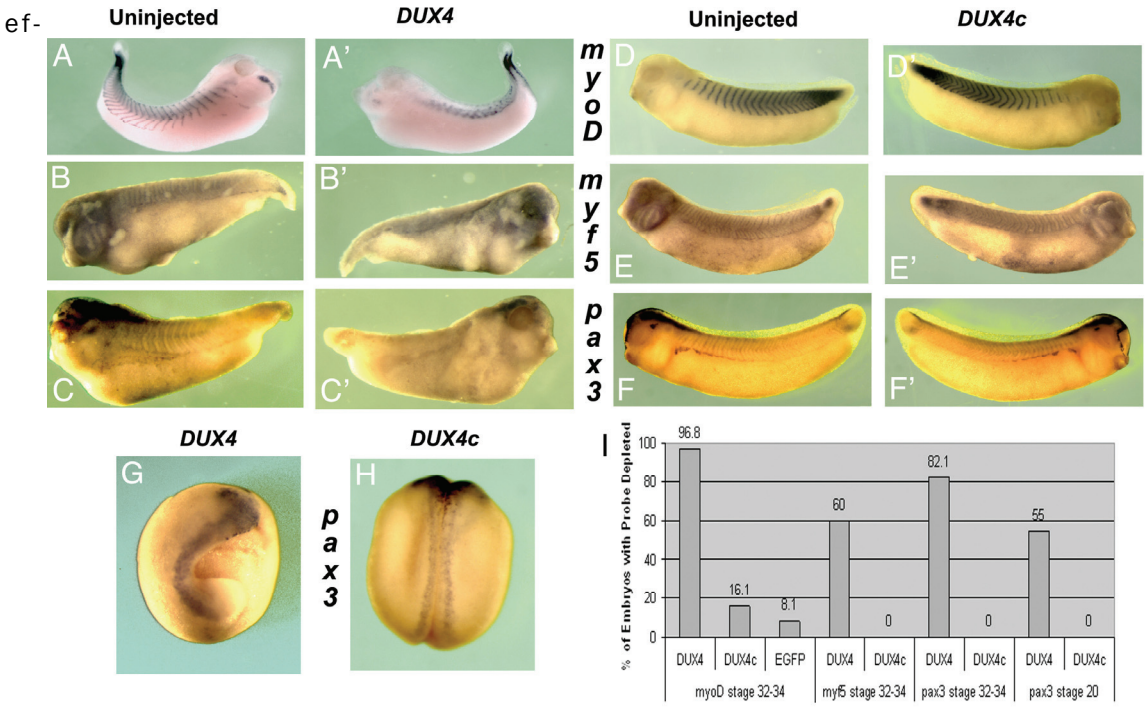

Figure 4. Effects of DUX4 and DUX4c on *myoD*, *myf5*, and *pax3* mRNA expression patterns. A-F) Stage 32-34 embryos were assessed for observable defects produced by DUX4 (A-C) and DUX4c (D-F) by *in-situ* hybridization for myogenic regulators. Injection of *DUX4* (1 pg) mRNA into developing embryos decreased myoD (A, A'), myf5 (B, B"), and pax3 (C, C') staining specifically on the injected side due to cellular loss. Injection of *DUX4c* (1 ng) mRNA into embryos produces no observable changes in myoD (D, D'), myf5 (E, E'), or pax3 (F, F') staining intensities when compared to the uninjected side. G) Stage 20 embryos injected with *DUX4* (1 pg) mRNA show depletion of pax3 staining before skeletal muscle development. H) Stage 20 embryos injected with *DUX4c* (1 ng) mRNA does not affect pax3 staining. I) Summary graph showing the percentage of observed *in situ* probe depleted embryos injected with DUX4 and DUX4c.

fect on the expression of *myoD*, *myf5*, and *pax3* (myoD n = 5/31; myf5 n = 0/8; pax3 n = 0/30) (**Figure 4D'**, **4E'**, & **4F'** compared to **4D**, **4E**, & **4F**, respectively). Similarly, no change was observed in *pax3* staining at stage 20 in embryos injected with 1 ng *DUX4c* mRNA (n = 0/12) (**Figure 4H**). Quantitative reverse transcription PCR (qRT-PCR) was used to determine the transcript levels of the myogenic regulators myoD or myf5 when injected with 1 ng *DUX4c* mRNA. Neither stage 20 embryos (n = 30) nor stage 34 embryos (n = 20) have statistically significant differences in *myoD* or *myf5* transcript levels when compared to *EGFP* mRNA injected controls normalized to gapdh (**Figure 5**). Thus, DUX4c has no effect on muscle precursors or myogenic regulators. Moreover, we found no significant changes of myogenic regulators by qRT-PCR on remaining intact tissue from 1 pg *DUX4* stage 34 injected embryos, further suggesting DUX4 has a strictly apoptotic role (**Figure 5**).

DUX4 and DUX4c expression reduce endogenous pitx1 expression

FSHD affected muscle has elevated levels of *PITX1* transcript and in cell culture assays DUX4 activates transcription of *PITX1* [6]. Endogenous *pitx1* expression was examined by *in situ* hybridization in 1 pg *DUX4* and 1 ng *DUX4c* injected stage 34 *X. laevis* embryos. During early *X. laevis* development (prior to hind-limb development), observable *pitx1* expression is confined to the cement and pituitary glands, and this expression pattern is not altered by expression of either DUX4 (**Figure 6A'** and **6A**; n = 23) or DUX4c (**Figure 6B'**and **6B**; n = 27). Although there is no statistically significant change in stage 20 embryos, surprisingly, analysis of

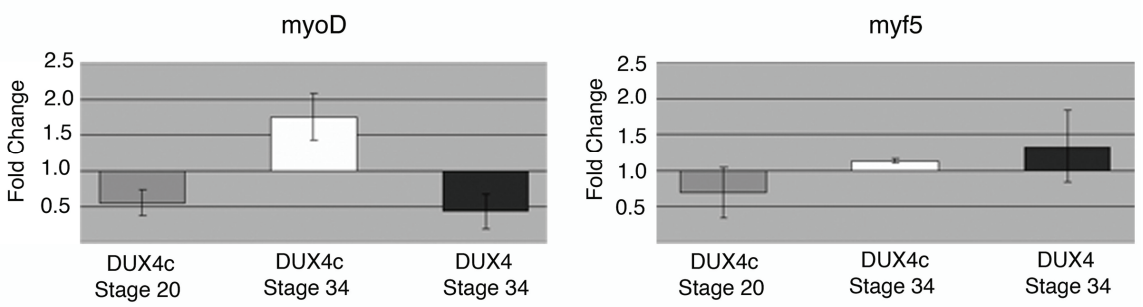

Figure 5. Global levels of *myoD* and *myf5* mRNA in DUX4 and DUX4c injected embryos. Levels of mRNA were quantified by qRT-PCR for *myoD* and *myf5* in *DUX4c* (1 ng) or DUX4 (1 pg) injected embryos. Fold change is normalized to *gapdh* levels and compared to *EGFP* mRNA at a relative level of 1; error bars are ± standard error of the mean.

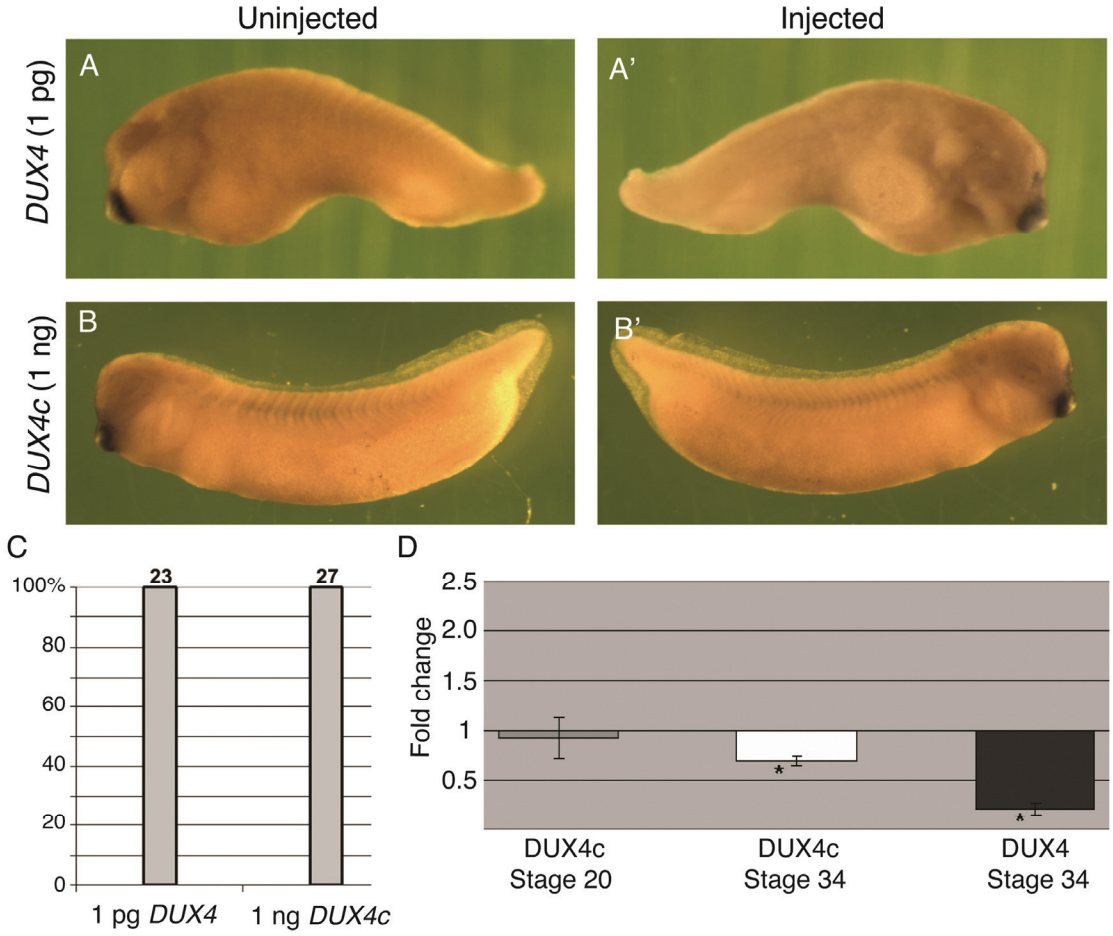

Figure 6. Neither DUX4 nor DUX4c can induce *pitx1* expression. A-B) *In situ* hybridizations for *pitx1* indicate that neither embryos injected with *DUX4* nor *DUX4c* mRNA upregulate pitx1. C) Graphic summary showing none of the embryos tested by *in situ* hybridization had obvious upregulation of *pitx1*, as illustrated by percent embryos without increased probe, with numbers of animals assayed. D) Quantification of *pitx1* mRNA in *DUX4c* (1 ng) or *DUX4* injected embryos as determined by qRT-PCR. Fold change is normalized to *gapdh* and compared to *EGFP* at a relative level of 1; error bars are ±standard error of the mean. Significance (p-value < 0.05) is indicated by (*).

stage 34 transcript levels by qRT-PCR showed that *pitx1* expression was actually reduced by DUX4c and DUX4 when compared to EGFP controls (*p-value* < 0.05) (**Figure 6D**). We conclude that neither DUX4 nor DUX4c expression activates *pitx1* expression in vivo.

Discussion

Multiple candidate genes have been proposed as causal of FSHD pathophysiology based on selective upregulation in FSHD patient-derived tissues or cell lines [6, 8, 10, 13, 30]. However inconsistent results, likely due in part to variations within and between FSHD patient biopsies as well as culturing conditions, have made it unclear which gene(s) are misexpressed. Previously we used *X. laevis* as a developing vertebrate model to analyze the effects of altering the expression levels of *frg1*. In the current study, we have used this same system to similarly analyze the effects of three additional FSHD candidate genes, *DUX4, DUX4c* and *pitx1*, on vertebrate muscle development.

X. laevis is a well-defined model system for muscle development with many advantages including external development, cells grow and differentiate under normal growth and environmental conditions giving rise to all tissues, and gene expression is easily manipulated through microinjection or transgenesis. In addition, *Xenopus* and humans share high levels of conservation of tissue organization, developmental processes, genes, and proteins. For example, the conservation of some muscle and FSHD associated proteins between human and *Xenopus* are as follows: FRG1 (80% identity, 88% similar), PITX1 (77% identity, 84% similar), PAX3 (91% identity, 96% similar) including 100% conservation of the homeodomain (aa 220-277), PAX7 (88% identity, 94% similar) including 100% conservation of the homeodomain (aa 217-274), MYOD1 (65% identity, 75% similar), and MYF5 (69% identity, 84% similar), as determined by alignments using NCBI BLAST Alignp. In contrast, DUX4 and DUX4c belong to the DUX family of double homeobox domain proteins but do not have *Xenopus* orthologs. In fact, when considering the entire protein sequence, including that residing outside their homeodomains, they both completely lack evolutionary conservation and appear to be unique to humans. Even the most closely related DUX4 ORFs in mice [31-34] show levels of sequence similarity (31% amino acid identity for the Duxbl protein aligned to DUX4 using the ClustalW function of BioEdit Sequence Alignment Editor software) far below what is expected for mouse to human conservation. Without any clear ortholog available, the human sequences for *DUX4* and *DUX4c* were used for these studies.

We have confirmed the toxic effect of DUX4 expression in the context of normal vertebrate development during stages of active myogenesis. By differential staining of neuronal and muscle tissues in DUX4 injected *Xenopus* embryos, we observe massive cellular loss of both tissue types and thus toxicity to not be muscle specific. Moreover, DUX4 injected *X. laevis* embryos show heavy TUNEL staining, indicating the function of DUX4 to induce apoptosis is conserved in *Xenopus*. Interestingly we have identified an extremely low threshold level of *DUX4* mRNA (0.5 pg) required for developmental abnormalities in *Xenopus,* at which point embryos appear to be either apoptotic or normal on the injected side. Although "non-toxic levels" of DUX4 in myoblast cell culture has been shown to impair differentiation [24], we observed no change in myotome development unless it was accompanied with generalized tissue apoptosis. This observation suggests that DUX4 has an "all or nothing" effect in *Xenopus*; we observe either severe developmental consequences or no effect on normal development of the organism.

The toxic effects of DUX4 are proposed to be due to the similarity of the homeodomains with those of myogenic regulators involved in development and regeneration such that DUX4 competes for their binding to regulatory sites [24]. The DUX4 homeodomain sequences are similar to *ortodenticle* (*otx*) and *paired* (*prd*) classes of proteins [35-37]. Otx and Pax proteins represent two families containing otx and prd homeodomains, respectively. Otx1 and Otx2 function in the nervous system [38], while the Pax3 and Pax7 proteins have known functions in the development of skeletal muscle and Pax7 in the maintenance of muscle satellite cells [39]. In support of the DUX4 competition model, MyoD, a well characterized target of Pax3 activation during development and Pax7 in adult myogenesis [40-42], was shown to be rapidly down regulated by DUX4 in a inducible C2C12 myoblast cell line system [24]. Considering *Xenopus* pax3 and pax7 homeodomains are 100% conserved with human PAX3 and PAX7, if DUX4 is function-

ing as a competitor of both PAX3 and PAX7 target genes, and consequently an antipodal regulator of myogenic genes in myoblasts, we would expect this competition to also be conserved in our study. In phenotypic DUX4 injected embryos, we observed a reduced level of *myoD* and *myf5* staining by *in situ* hybridization when compared to EGFP controls. Furthermore, DUX4 injected embryos showed that *pax3* transcripts, the upstream regulator of myoD, was absent by stage 20 of *Xenopus* development indicating DUX4 toxicity precedes muscle development. These data lead us to conclude that the DUX4 injected *Xenopus* phenotype is likely due to massive apoptosis on the injected side of the individuals and not resulting from muscle cell specific competition for pax3/7 targets. Taking into account that an aberrant increase in apoptosis is not generally considered to be part of the muscle pathology in FSHD [43, 44], this data could be consistent with DUX4 having a role in FSHD muscle pathology provided DUX4 is either only expressed at very low levels if at all under normal conditions and is only overexpressed in the muscle cell precursors of FSHD patients and not any other cells.

DUX4c, located within a truncated and inverted D4Z4 repeat located just centromeric from the FSH1A locus, has been shown to be up-regulated in FSHD [20, 22]. The gene encodes an ORF identical to DUX4 except for differing in the last 82 amino acids which are substituted with 32 unrelated amino acids. Interestingly, the C-terminal substitution leaves DUX4c with the exact homeodomains found in DUX4. This, in theory, would enable DUX4c to interact with all of the same genetic targets of DUX4. Therefore, considering 100% conservation of pax3/7 homeodomains from human to *Xenopus,* expression of DUX4c should also compete with the pax3 and pax7 for myogenic target genes and thus lead to myogenic abnormalities in *Xenopus*. Interestingly, 1ng *DUX4c* mRNA injections (2000 fold over DUX4 threshold levels) produce only a slight increase in abnormal *Xenopus* development, further indicating the DUX homeodomains do not compete with pax3 or pax7 for myogenic target genes. In two previous studies, DUX4c has been shown to inhibit myoblast differentiation and down-regulate MyoD [20, 25]. Oddly, both studies investigate effects of DUX4c on Myf5 expression in identical myoblast cell lines and find opposite results; one finding Myf5 is down-regulated [25], while the other shows an up regulation of Myf5 [20]. Interestingly, differences between FSHD and control DUX4c levels were only observed in myotubes, after the effects on myoblast differentiation would have passed [20]. We observed no obvious changes in staining patterns for myoD or myf5 in DUX4c injected *Xenopus* embryos by in-situ hybridization and mRNA levels are not significantly different from that of EGFP injected controls by qRT-PCR. This study on the effects of DUX4c on myogenic regulators in a vertebrate going through muscle development leads us to conclude that DUX4c expression has no overt effects on muscle development and is not consistent with DUX4c expression having a role in FSHD pathology.

The FSHD candidate PITX1 is a member of the paired family of homeodomain transcription factors [45]. Multiple studies focused on PITX1 shows it is involved in specification of hind limb identity, as well as left-right symmetry [46-51]. It was recently shown that DUX4 could activate transient expression of a reporter gene fused to the *PITX1* promoter as well as the endogenous *PITX1* gene in transfected C2C12 cells [6]. Although the sequence of the *pitx1* promoter is unknown in *X. laevis*, the fact that DUX4 maintains its characteristic ability to induce apoptosis suggests it is interacting with its conserved targets. Taking into consideration that DUX4 and DUX4c contain identical homeodomains we tested their potential to regulate *pitx1*. At stage 34 pitx1 is characteristically expressed at high levels in the cement gland [29, 52, 53]. We found no obvious increase of *pitx1* in DUX4 or DUX4c injected embryos. Interestingly, we did see a statistically significant (*p-value* <0.05) decrease in *pitx1* mRNA in DUX4c or DUX4 injected stage 34 tadpoles assayed by qRT-PCR. We conclude that neither DUX4 nor DUX4c induce *pitx1* in *Xenopus* and DUX4-mediated apoptosis is not mediated through activation of *pitx1*. To directly test the effects of overexpression of pitx1, we circumvented the issues related to DUX4 expression by directly increasing pitx1 through microinjection and determine the effects on *Xenopus* development. As with DUX4, we observed severe, general (not muscle cell specific) developmental abnormalities when pitx1 was overexpressed in *Xenopus*, agreeing with previous reports [53]. Like DUX4, these abnormalities likely arise from the induction of apoptosis, as increased pitx1 expression has been shown to directly lead to increased p53

expression and cellular loss [54]. This result is consistent with PITX1 playing a role in FSHD assuming it is only overexpressed in muscle lineages of FSHD patients.

At this point, the mechanism of FSHD pathophysiology remains unknown. In total we tested the effects of systemic overexpression of four FSHD candidate genes on vertebrate development in our *Xenopus* system. We have found systemic overexpression of DUX4c has little effect while DUX4 and pitx1 produce a general cytotoxicity to all cell types in developing embryos. FSHD is likely an epigenetic disorder but it is not known if the cause of FSHD is a misregulation of a gene specifically restricted to skeletal muscle and its precursors or if there is a global misregulation of a gene with skeletal muscle myogenesis being specifically susceptible. Only if it is the former, and DUX4 and PITX1 were exclusively overexpressed in skeletal muscle precursors, could they have a role in FSHD pathology. We know no mechanism whereby DUX4 or PITX1 cytotoxicity could produce the vasculature phenotype strongly associated with FSHD. In respect to DUX4c we conclude it likely has no role in FSHD pathology, however, it is possible multiple candidates including DUX4c could function together to produce a synergistic effect ultimately resulting in FSHD-like pathology. This compares poorly to FRG1 from our previous studies where systemic overexpression of frg1 could recapitulate both major symptoms of FSHD in *Xenopus*, dystrophic muscle and increased angiogenesis [17, 18]. Taken together, the functional and phenotypic data point to *FRG1* as the most likely candidate whose misexpression, either systemically or specifically during myogenesis, leads to FSHD pathology.

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References

[1] Fitzsimons RB, Gurwin EB and Bird AC. Retinal vascular abnormalities in facioscapulohumeral muscular dystrophy. A general association with genetic and therapeutic implications. Brain 1987; 110 (Pt 3): 631-648.

[2] Padberg GW, Brouwer OF, de Keizer RJ, Dijkman G, Wijmenga C, Grote JJ and Frants RR. On the significance of retinal vascular disease and hearing loss in facioscapulohumeral muscular dystrophy. Muscle Nerve 1995; 2: S73-80.

[3] van Deutekom JC, Wijmenga C, van Tienhoven EA, Gruter AM, Hewitt JE, Padberg GW, van Ommen GJ, Hofker MH and Frants RR. FSHD associated DNA rearrangements are due to deletions of integral copies of a 3.2 kb tandemly repeated unit. Hum Mol Genet 1993; 2: 2037-2042.

[4] Wijmenga C, Hewitt JE, Sandkuijl LA, Clark LN, Wright TJ, Dauwerse HG, Gruter AM, Hofker MH, Moerer P, Williamson R and et al. Chromosome 4q DNA rearrangements associated with facioscapulohumeral muscular dystrophy. Nat Genet 1992; 2: 26-30.

[5] van Overveld PG, Lemmers RJ, Sandkuijl LA, Enthoven L, Winokur ST, Bakels F, Padberg GW, van Ommen GJ, Frants RR and van der Maarel SM. Hypomethylation of D4Z4 in 4q-linked and non-4q-linked facioscapulohumeral muscular dystrophy. Nat Genet 2003; 35: 315-317.

[6] Dixit M, Ansseau E, Tassin A, Winokur S, Shi R, Qian H, Sauvage S, Matteotti C, van Acker AM, Leo O, Figlewicz D, Barro M, Laoudj-Chenivesse D, Belayew A, Coppee F and Chen YW. DUX4, a candidate gene of facioscapulohumeral muscular dystrophy, encodes a transcriptional activator of PITX1. Proc Natl Acad Sci U S A 2007; 104: 18157-18162.

[7] Gabellini D, D'Antona G, Moggio M, Prelle A, Zecca C, Adami R, Angeletti B, Ciscato P, Pellegrino MA, Bottinelli R, Green MR and Tupler R. Facioscapulohumeral muscular dystrophy in mice overexpressing FRG1. Nature 2006; 439: 973-977.

[8] Gabellini D, Green MR and Tupler R. Inappropriate gene activation in FSHD: a repressor complex binds a chromosomal repeat deleted in dystrophic muscle. Cell 2002; 110: 339-348.

[9] Jiang G, Yang F, van Overveld PG, Vedanarayanan V, van der Maarel S and Ehrlich M. Testing the position-effect variegation hypothesis for facioscapulohumeral muscular dystrophy by analysis of histone modification and gene expression in subtelomeric 4q. Hum Mol Genet 2003; 12: 2909-2921.

[10] Rijkers T, Deidda G, van Koningsbruggen S, van Geel M, Lemmers RJ, van Deutekom JC, Figlewicz D, Hewitt JE, Padberg GW, Frants RR and

van der Maarel SM. FRG2, an FSHD candidate gene, is transcriptionally upregulated in differentiating primary myoblast cultures of FSHD patients. J Med Genet 2004; 41: 826-836.
[11] Snider L, Asawachaicharn A, Tyler AE, Geng LN, Petek LM, Maves L, Miller DG, Lemmers RJ, Winokur ST, Tawil R, van der Maarel SM, Filippova GN and Tapscott SJ. RNA transcripts, miRNA-sized fragments and proteins produced from D4Z4 units: new candidates for the pathophysiology of facioscapulohumeral dystrophy. Hum Mol Genet 2009; 18: 2414-2430.
[12] Winokur ST, Chen YW, Masny PS, Martin JH, Ehmsen JT, Tapscott SJ, van der Maarel SM, Hayashi Y and Flanigan KM. Expression profiling of FSHD muscle supports a defect in specific stages of myogenic differentiation. Hum Mol Genet 2003; 12: 2895-2907.
[13] Klooster R, Straasheijm K, Shah B, Sowden J, Frants R, Thornton C, Tawil R and van der Maarel S. Comprehensive expression analysis of FSHD candidate genes at the mRNA and protein level. Eur J Hum Genet 2009; 17: 1615-1624.
[14] Barro M, Carnac G, Flavier S, Mercier J, Vassetzky Y and Laoudj-Chenivesse D. Myoblasts from affected and non affected FSHD muscles exhibit morphological differentiation defects. J Cell Mol Med 2008;
[15] Bodega B, Ramirez GD, Grasser F, Cheli S, Brunelli S, Mora M, Meneveri R, Marozzi A, Mueller S, Battaglioli E and Ginelli E. Remodeling of the chromatin structure of the facioscapulohumeral muscular dystrophy (FSHD) locus and upregulation of FSHD-related gene 1 (FRG1) expression during human myogenic differentiation. BMC Biol 2009; 7: 41.
[16] Morosetti R, Mirabella M, Gliubizzi C, Broccolini A, Sancricca C, Pescatori M, Gidaro T, Tasca G, Frusciante R, Tonali PA, Cossu G and Ricci E. Isolation and characterization of mesoangioblasts from facioscapulohumeral muscular dystrophy muscle biopsies. Stem Cells 2007; 25: 3173-3182.
[17] Hanel ML, Wuebbles RD and Jones PL. Muscular dystrophy candidate gene FRG1 is critical for muscle development. Dev Dyn 2009; 238: 1502 -1512.
[18] Wuebbles RD, Hanel ML and Jones PL. FSHD region gene 1 (FRG1) is crucial for angiogenesis linking FRG1 to facioscapulohumeral muscular dystrophy-associated vasculopathy. Dis Model Mech 2009; 2: 267-274.
[19] Kowaljow V, Marcowycz A, Ansseau E, Conde CB, Sauvage S, Matteotti C, Arias C, Corona ED, Nunez NG, Leo O, Wattiez R, Figlewicz D, Laoudj-Chenivesse D, Belayew A, Coppee F and Rosa AL. The DUX4 gene at the FSHD1A locus encodes a pro-apoptotic protein. Neuromuscul Disord 2007; 17: 611-623.
[20] Ansseau E, Laoudj-Chenivesse D, Marcowycz A, Tassin A, Vanderplanck C, Sauvage S, Barro M, Mahieu I, Leroy A, Leclercq I, Mainfroid V, Figlewicz D, Mouly V, Butler-Browne G, Belayew A and Coppee F. DUX4c is up-regulated in FSHD. It induces the MYF5 protein and human myoblast proliferation. PLoS One 2009; 4: e7482.
[21] Gabriels J, Beckers MC, Ding H, De Vriese A, Plaisance S, van der Maarel SM, Padberg GW, Frants RR, Hewitt JE, Collen D and Belayew A. Nucleotide sequence of the partially deleted D4Z4 locus in a patient with FSHD identifies a putative gene within each 3.3 kb element. Gene 1999; 236: 25-32.
[22] Wright TJ, Wijmenga C, Clark LN, Frants RR, Williamson R and Hewitt JE. Fine mapping of the FSHD gene region orientates the rearranged fragment detected by the probe p13E-11. Hum Mol Genet 1993; 2: 1673-1678.
[23] Winokur ST, Bengtsson U, Vargas JC, Wasmuth JJ, Altherr MR, Weiffenbach B and Jacobsen SJ. The evolutionary distribution and structural organization of the homeobox-containing repeat D4Z4 indicates a functional role for the ancestral copy in the FSHD region. Hum Mol Genet 1996; 5: 1567-1575.
[24] Bosnakovski D, Xu Z, Gang EJ, Galindo CL, Liu M, Simsek T, Garner HR, Agha-Mohammadi S, Tassin A, Coppee F, Belayew A, Perlingeiro RR and Kyba M. An isogenetic myoblast expression screen identifies DUX4-mediated FSHD-associated molecular pathologies. EMBO J 2008; 27: 2766-2779.
[25] Bosnakovski D, Lamb S, Simsek T, Xu Z, Belayew A, Perlingeiro R and Kyba M. DUX4c, an FSHD candidate gene, interferes with myogenic regulators and abolishes myoblast differentiation. Exp Neurol 2008;
[26] Hensey C and Gautier J. A developmental timer that regulates apoptosis at the onset of gastrulation. Mech Dev 1997; 69: 183-195.
[27] Harland RM. In situ hybridization: an improved whole-mount method for Xenopus embryos. Methods Cell Biol 1991; 36: 685-695.
[28] Nieuwkoop PD and Faber J. Normal table of Xenopus laevis (Daudin) : a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. New York: Garland Pub., 1994.
[29] Chang W, KhosrowShahian F, Chang R and Crawford MJ. xPitx1 plays a role in specifying cement gland and head during early Xenopus development. Genesis 2001; 29: 78-90.
[30] Reed PW, Corse AM, Porter NC, Flanigan KM and Bloch RJ. Abnormal expression of mu-crystallin in facioscapulohumeral muscular dystrophy. Exp Neurol 2007; 205: 583-586.
[31] Bosnakovski D, Daughters RS, Xu Z, Slack JM and Kyba M. Biphasic myopathic phenotype of mouse DUX, an ORF within conserved FSHD-related repeats. PLoS One 2009; 4: e7003.
[32] Clapp J, Mitchell LM, Bolland DJ, Fantes J, Corcoran AE, Scotting PJ, Armour JA and Hewitt JE. Evolutionary conservation of a coding function for D4Z4, the tandem DNA repeat mutated in

facioscapulohumeral muscular dystrophy. Am J Hum Genet 2007; 81: 264-279.
[33] Kawazu M, Yamamoto G, Yoshimi M, Yamamoto K, Asai T, Ichikawa M, Seo S, Nakagawa M, Chiba S, Kurokawa M and Ogawa S. Expression profiling of immature thymocytes revealed a novel homeobox gene that regulates double-negative thymocyte development. J Immunol 2007; 179: 5335-5345.
[34] Wu SL, Tsai MS, Wong SH, Hsieh-Li HM, Tsai TS, Chang WT, Huang SL, Chiu CC and Wang SH. Characterization of genomic structures and expression profiles of three tandem repeats of a mouse double homeobox gene: Duxbl. Dev Dyn
[35] Bopp D, Burri M, Baumgartner S, Frigerio G and Noll M. Conservation of a large protein domain in the segmentation gene paired and in functionally related genes of Drosophila. Cell 1986; 47: 1033-1040.
[36] Finkelstein R, Smouse D, Capaci TM, Spradling AC and Perrimon N. The orthodenticle gene encodes a novel homeo domain protein involved in the development of the Drosophila nervous system and ocellar visual structures. Genes Dev 1990; 4: 1516-1527.
[37] Hewitt JE, Lyle R, Clark LN, Valleley EM, Wright TJ, Wijmenga C, van Deutekom JC, Francis F, Sharpe PT, Hofker M and et al. Analysis of the tandem repeat locus D4Z4 associated with facioscapulohumeral muscular dystrophy. Hum Mol Genet 1994; 3: 1287-1295.
[38] Simeone A, Puelles E and Acampora D. The Otx family. Curr Opin Genet Dev 2002; 12: 409-415.
[39] Buckingham M. Myogenic progenitor cells and skeletal myogenesis in vertebrates. Curr Opin Genet Dev 2006; 16: 525-532.
[40] Olguin HC and Olwin BB. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev Biol 2004; 275: 375-388.
[41] Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, Mansouri A, Cumano A and Buckingham M. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. J Cell Biol 2006; 172: 91-102.
[42] Tajbakhsh S, Rocancourt D, Cossu G and Buckingham M. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell 1997; 89: 127-138.
[43] Winokur ST, Barrett K, Martin JH, Forrester JR, Simon M, Tawil R, Chung SA, Masny PS and Figlewicz DA. Facioscapulohumeral muscular dystrophy (FSHD) myoblasts demonstrate increased susceptibility to oxidative stress. Neuromuscul Disord 2003; 13: 322-333.
[44] Sandri M, El Meslemani AH, Sandri C, Schjerling P, Vissing K, Andersen JL, Rossini K, Carraro U and Angelini C. Caspase 3 expression correlates with skeletal muscle apoptosis in Duchenne and facioscapulo human muscular dystrophy. A potential target for pharmacological treatment? J Neuropathol Exp Neurol 2001; 60: 302-312.
[45] Tremblay JJ, Goodyer CG and Drouin J. Transcriptional properties of Ptx1 and Ptx2 isoforms. Neuroendocrinology 2000; 71: 277-286.
[46] Lanctot C, Moreau A, Chamberland M, Tremblay ML and Drouin J. Hindlimb patterning and mandible development require the Ptx1 gene. Development 1999; 126: 1805-1810.
[47] Szeto DP, Rodriguez-Esteban C, Ryan AK, O'Connell SM, Liu F, Kioussi C, Gleiberman AS, Izpisua-Belmonte JC and Rosenfeld MG. Role of the Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development. Genes Dev 1999; 13: 484-494.
[48] Logan M and Tabin CJ. Role of Pitx1 upstream of Tbx4 in specification of hindlimb identity. Science 1999; 283: 1736-1739.
[49] Cole NJ, Tanaka M, Prescott A and Tickle C. Expression of limb initiation genes and clues to the morphological diversification of threespine stickleback. Curr Biol 2003; 13: R951-952.
[50] Shapiro MD, Marks ME, Peichel CL, Blackman BK, Nereng KS, Jonsson B, Schluter D and Kingsley DM. Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature 2004; 428: 717-723.
[51] Tanaka M, Hale LA, Amores A, Yan YL, Cresko WA, Suzuki T and Postlethwait JH. Developmental genetic basis for the evolution of pelvic fin loss in the pufferfish Takifugu rubripes. Dev Biol 2005; 281: 227-239.
[52] Hollemann T and Pieler T. Xpitx-1: a homeobox gene expressed during pituitary and cement gland formation of Xenopus embryos. Mech Dev 1999; 88: 249-252.
[53] Schweickert A, Deissler K, Blum M and Steinbeisser H. Pitx1 and Pitx2c are required for ectopic cement gland formation in Xenopus laevis. Genesis 2001; 30: 144-148.
[54] Liu DX and Lobie PE. Transcriptional activation of p53 by Pitx1. Cell Death Differ 2007; 14: 1893-1907.