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Highlights

- *DUX4* is a transcription factor expressed in early embryos and repressed in most adult tissues *via* multiple genetic and epigenetic mechanisms;
- *DUX4* aberrant expression caused by genetic abnormalities is linked to facioscapulohumeral dystrophy (FSHD);
- Recent data point to chromosomal rearrangements involving *DUX4* as driver in leukaemias and sarcomas;
- *DUX4* is reactivated in solid cancers;
- *DUX4* expression is regulated by several feedback loops;
- Several approaches targeting *DUX4* in FSHD and cancer are currently being developed

Control of *DUX4* expression in FSHD and cancer: from feedback loops to chromatin loops

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Abstract

DUX4, a gene encoding a transcription factor involved in early embryogenesis, is heavily repressed in most somatic tissues while its aberrant expression is linked to facioscapulohumeral muscular dystrophy (FSHD). Recently, the deregulation of *DUX4* expression was found in many cancers. While *DUX4* role in FSHD is well studied, its relevance in cancer is less explored. Here we discuss multiple levels of control of *DUX4* expression, including enhancer-promoter interactions, epigenetic modifications, non-coding RNAs and telomere positioning effect. We also connect disparate data on intrachromosomal contacts involving *DUX4* and emphasize feedback loops in *DUX4* regulation. Finally, we bridge data on *DUX4* in FSHD and cancer and discuss prospective approaches for future FSHD therapies and the potential outcomes of *DUX4* inhibition in cancer.

Keywords: FSHD; leukemia; Ewing-like sarcoma; rhabdomyosarcoma; *DUX4*; gene regulation; transcription; epigenetics

DUX4 expression in development and pathologies

DUX4 is a transcription factor containing two homeobox DNA binding domains and a C-terminal transactivation domain. The *DUX4* open reading frame (ORF) is present in multiple copies throughout the human genome. Most of the copies are located within 3.3kb **D4Z4** repeats, organized into long polymorphic macrosatellite arrays in the subtelomeric regions of chromosomes 4q35 and 10q26; many D4Z4-related sequences are also dispersed elsewhere in the genome, notably in the acrocentric chromosomes and heterochromatic regions on 1q12 [1,2]. *DUX4* is expressed in the early embryo consistent with its likely function in stimulating **zygotic genome activation (ZGA)** (reviewed in [3], and is then repressed in the majority of adult tissues, except for the testis [4] and the thymus [5].

Aberrant *DUX4* expression is considered to be one of the major causes of the facioscapulohumeral dystrophy (FSHD), an autosomal dominant disorder characterized by progressive and asymmetric muscle weakness [6]. Recently, chromosomal *DUX4* rearrangements were implicated in the pathogenesis of **rhabdomyosarcoma (RMS)** [7], **Ewing-like sarcoma** [8] and **B-cell precursor acute lymphoblastic leukemia (BCP-ALL)** [9]. All these translocations result in the production of putative chimeric proteins implicated in oncogenesis (reviewed in [10]) (see Key Figure 1). Thus, understanding how *DUX4* expression is regulated in physiological and pathological conditions is essential for development of potential treatment strategies.

DUX4 expression and its consequences

Physiological conditions

DUX4 transcripts are detected from the oocyte stage to the 4-cell (4C) embryo and *DUX4* transcriptional targets are detected from 2C to 8C [11], concordant with the major wave of

zygotic genome activation (**ZGA**), which takes place at ~ 2C in mice and 4C-8C in humans [12]. Both mouse Dux and human DUX4 activate ZGA-associated genes [13,14], and DUX family proteins have been proposed to be key ZGA-inducers in placental mammals [11]. Nevertheless, more recent studies indicate that Dux is not strictly required for the survival of mouse embryos and DUX proteins are rather non-essential ZGA synchronizers [15–18], that possibly facilitate post-implantation development [18]. In adult human tissues, except for testis [4] and thymus [5], *DUX4* is heavily repressed and its post-embryonic function (if any) remains unknown.

Facioscapulohumeral muscular dystrophy

Facioscapulohumeral muscular dystrophy (FSHD) is the most extensively studied DUX4-associated pathology (see **Box 1**). The *DUX4* gene located within each D4Z4 repeat [19] is considered the major cause of FSHD [20]. DUX4 protein is cytotoxic in postnatal tissues and *DUX4* aberrant expression is associated with apoptosis [21–23], oxidative stress and DNA damage [21,24], myogenesis disruption [21,25–27], immune response [28] and impaired transcript quality control [29–31] in several cell types. Nevertheless, the exact mechanism connecting DUX4 production and specific pathological phenotypes observed in FSHD still remains to be elucidated.

Cancer

Genes aberrantly expressed in cancer may both induce oncogenic transformation, i.e. be cancer drivers, or be associated with it, i.e. serve as biomarkers. Depending on the cancer, DUX4 may be either a driver or a marker. In several cancers, chromosomal translocations produce chimeric genes with the participation of *DUX4*. In BCP-ALL, these translocations

lead to the insertion of *DUX4* ORF into the **immunoglobulin heavy chain (*IGH*)** gene locus [9,32–34] or into an intron of the **ETS-related gene (*ERG*)** [9]. In both *DUX4-IGH* and *DUX4-ERG* **fusion genes**, the *DUX4* homeodomains are preserved, while the part of the *DUX4* ORF encoding for a C-terminal transactivation domain is truncated and/or partially replaced by the non-coding parts of the host genes [9,32,33,35]. Thus, the resulting putative fusion proteins would retain the DNA-binding specificity of *DUX4*, but would not activate transcription in a *DUX4*-specific manner, which was indeed experimentally demonstrated for the *DUX4-IGH* protein in a cellular BCP-ALL model [36]. *DUX4*-involving rearrangements in BCP-ALL are accompanied by *DUX4* overexpression [9,33], as well as frequent production of an alternative *ERG* isoform with transforming properties, *ERGalt* [33], which is likely produced due to the interaction of *DUX4* homeodomains with the alternative transcription initiation site in *ERG* intron 6 [33,35]. Finally, the chemotaxis and cytokine environment may favour a relapse in BCP-ALL patients carrying *DUX4-IGH* rearrangements [37]. *DUX4* upregulates chemotaxis genes in myoblasts and stimulates mesenchymal stem cell migration in **CXCR4** (C-X-C motif Receptor 4)-dependent manner [38]. A similar cell migration mechanism may favour relapse in *DUX4-IGH* BCP-ALL patients.

In Ewing-like sarcomas, chromosomal translocation generates a fusion between *DUX4* and the capicua transcriptional repressor (***CIC***) gene [8,39,40], encoding for a strong tumor suppressor [41]. The resulting *CIC-DUX4* fusion protein is ~90% similar to the original *CIC*, but gains the C-terminal transactivation domain of *DUX4*, thus reversing its own function to activate the formerly repressed genes, i.e. *PEA3* genes of the ETS family [8] that promote cellular proliferation and migration [42]. Overexpression of the *ETV4* gene of the *PEA3* subgroup is observed in 90% of the *CIC-DUX4*-expressing tumors and distinguishes them from other sarcomas [8,43–45]. The pro-metastatic function of *ETV4* demonstrated in studies with *CIC* inactivation [46,47] may explain the rapid progression and increased metastatic

activity of *CIC-DUX4*-positive sarcomas [48]. Other transcriptional targets of *CIC-DUX4* include cell cycle regulators, notably *CCNE1* [49] and negative regulators of MAPK-ERK signaling, such as *DUSP6* [44].

In one case of embryonic RMS, t(4;22) translocation led to the *EWSRI-DUX4* fusion [7], which might have contributed to a specific striated muscle differentiation phenotype of this tumor.

DUX4 is reactivated in many solid cancers, where it inhibits the induction of MHC1 (major histocompatibility complex 1) by gamma interferon [50]. This decreases antigen presentation by cancer cells and reduces recognition by cytotoxic T cells, contributing to cancer cells immune evasion and resistance to immune checkpoint blockade [50]. *DUX4* is also one of the markers predicting the transition of sinonasal papilloma to carcinoma [51]. On the other hand, in colon adenocarcinoma, *DUX4* may inhibit growth and proliferation of cancer cells as it interacts with the cell cycle regulator cyclin-dependent kinase 1 (**CDK1**), blocking its activity [52]. When ectopically overexpressed in multiple cell-types, *DUX4* causes cellular toxicity and cell death [21–23], though this phenomenon has not been observed in cancer cells so far, probably because of the insufficient level of its expression and/or the alterations of the *DUX4* ORF following chromosomal aberrations. Thus, an increasing body of evidence links *DUX4* to cancers of different origin, both as a driver and as a marker.

Regulation and silencing of *DUX4* expression

Gene expression is regulated by hierarchical interactions of various control elements. Enhancers activate transcription via interaction with promoters essential for transcription initiation. Insulators block the enhancer-promoter interactions. The three-dimensional folding of these control elements in the nuclear space orchestrates regulation of gene expression. All

these elements are present in the 4q35 locus and participate in the regulation of the *DUX4* gene in physiological conditions, FSHD and cancer (**Figure 2**).

Promoters

The D4Z4 repeat where the *DUX4* gene is located has long been considered “junk” DNA (reviewed in[53]) until an open reading frame for a double homeobox gene with a cryptic promoter sequence was discovered inside each D4Z4 unit [2,19,54]. The *DUX4* transcripts are generated from the most distal D4Z4 unit, stabilized by the polyA provided by an adjacent 4qA sequence (see Box 1). Three major splice isoforms of the polyadenylated *DUX4* transcript exist: two *DUX4-fl* transcripts containing the entire *DUX4* ORF and one or two introns spliced in the 3'- UTR, and a *DUX4-s* transcript utilizing the cryptic splice site inside the *DUX4* ORF that conserves the double-homeobox domain without the C-terminal part of *DUX4* [4,55]. *DUX4-s* transcripts are observed in both healthy and FSHD muscles and are not associated with the pathology, while *DUX4-fl* transcripts are specific to FSHD [4,55]. The switch from the canonical to the cryptic splice site leading to the predominant *DUX4-s* production is possibly correlated with the accumulation of repressive chromatin modifications in the region [4]. **Nonsense-mediated decay (NMD)** eliminates *DUX4-fl* mRNA transcripts due to the presence of a spliced intron in the 3' UTR of *DUX4* [29]. Importantly, NMD is inhibited in myoblasts ectopically expressing *DUX4* [29].

Enhancers

Though each D4Z4 repeat contains a functional promoter [19,54], the muscle specificity of FSHD suggests the presence of additional tissue-specific regulatory elements modulating the transcription from the D4Z4 units. A strong myo-specific enhancer interacting with KLF15 transcription factor is located inside the D4Z4 repeat [56,57], but, surprisingly, this enhancer

activates the genes proximal to D4Z4, *DUX4c* and *FRG2*, but not *DUX4* located within the D4Z4 unit itself [57]. Two muscle-specific enhancers named **DME1** and **DME2** (for *DUX4* Myogenic Enhancer) are situated proximally to the D4Z4 array (**Figure 2**) [58]. They interact directly with the *DUX4* promoter in both healthy and FSHD myoblasts, but *DUX4* activation is only observed following their association with the hypomethylated promoter in FSHD cells. DME1 and DME2 contain binding sites for several myogenic transcription factors: PAX7, Myogenin and MYOD [58].

Promoter function is conserved in some pathological conditions, e.g. in FSHD and some cancers where a 5' part of the gene fusion is composed of the *DUX4* gene. On the contrary, partner promoters (e.g. that of *CIC*) are used when the 3' part of the *DUX4* gene is involved in the fusion. DME1 and 2 only participate in the pathological regulation of *DUX4* expression in FSHD and possibly some cancers where *DUX4* overexpression is not linked to translocations. In other cases, enhancers from the host regions, e.g. *IGH* μ enhancer, activate expression of *DUX4* and its fusion variants.

Insulators

Insulators mediate long-distance intra- and inter-chromosomal interactions *via* loop formation and epigenetic modifications [59]. A region adjacent to the D4Z4 array harbors the **FR-MAR** insulator (FSHD-related nuclear matrix attachment region) [56,60,61] (**Figure 1**). This region separates the chromatin in the 4q35 locus into two distinct domains, so that the enhancers (DME1, DME2 and the enhancer within the D4Z4 repeat) and their target genes (*DUX4c*, *FRG2* and *DUX4*) can no longer interact. In FSHD myoblasts, the FR-MAR insulator is non-functional: the distinct loops fuse into one and the insulator activity is abolished, thus permitting expression of several genes within the locus [56,61]. The presence of specific

haplotypes (4A161, 4A159 and 4A168) within the insulator sequence together with the methylation of a specific CpG is important for the formation of the two distinct chromatin domains and the insulator function [60]. It is currently unclear whether the insulator is involved in *DUX4* regulation in cancer.

Epigenetic regulation of DUX4 expression

DNA methylation

The CpG-rich D4Z4 array is located close to the telomere [62] in the nuclear periphery [63] and is highly methylated in most somatic tissues, including skeletal muscle [64]. Two genes associated with DNA methylation: *SMCHD1* and *DNMT3B*, are often mutated in FSHD. Thus, it is tempting to hypothesize that the hypomethylation of the D4Z4 array on chromosome 4 is one of the reasons underlying the FSHD pathology. Nevertheless, the methylation level of the D4Z4 array is highly variable in both healthy and FSHD1 individuals and seems to correlate with the number of D4Z4 units rather than the disease status (reviewed in [65]). In contrast, hypomethylation resulting from the *SMCHD1* or *DNMT3B* mutations in FSHD2 seems to be the major cause of *DUX4* derepression, though the strict correlation between *SMCHD1/DNMT3B* variants, the extent of D4Z4 hypomethylation and FSHD2 symptoms has not been established yet.

Hypo- or hypermethylation of the D4Z4 repeats was observed in a number of cancers [66,67], tumor cell lines [68] and in human bladder carcinoma cells treated with DNA-methyltransferase inhibitor zebularine [69], although most of these studies employed PCR-based techniques which were not specific to a particular D4Z4 array. A specific study of the 4q35-associated D4Z4 array demonstrated that D4Z4 repeats were hypomethylated in FSHD

and sperm cells, while both hypo- and hypermethylation was observed in cancer cells [70]. Interestingly, this study revealed methylation-resistant regions within the first 2.2 kb of each D4Z4 unit. These regions were not methylated in cancers with otherwise hypermethylated D4Z4 repeats and contained G-runs (several consecutive guanines) that could form **G-quadruplexes (GQs)** *in vitro* [70]. These structures might be important for the intra-array looping within the D4Z4 array of normal length, while in the contracted D4Z4, this looping would be disrupted allowing for the long-distance interactions [70]. The GQs are present in the promoter zone, the coding region, and the enhancer region of *DUX4* [71]. Treatment with a GQ-stabilizing compound berberine downregulates expression of *DUX4* and its target genes *ZSCAN4*, *TRIM43* and *MBD3L2* in FSHD myoblasts and reduces the amount of DUX4 protein in mice with exogenous *DUX4* expression [71].

Histone modifications

DUX4 transcription is also affected by histone modifications. Generally, histone acetylation is linked to transcriptional activation, while methylation can both activate (*e.g.* H3K4me2) and repress (*e.g.* H3K9me3 and H3K27me3) transcription. H3K9me3 is present within D4Z4 repeats on chromosomes 4 and 10 in healthy individuals but lost in FSHD1 patients [72,73]. The ratio of repression vs. activation marks (H3K9Me3 vs. H3K4Me2) is reduced in myoblasts and fibroblasts from FSHD patients; the lowest values correspond to severe symptoms [74]. In some regions of 4q35, both repressive and activation marks coexist: H3K27me3 and H3K27Ac were detected simultaneously at the DME1 enhancer [58].

Proteins

Certain proteins were reported to maintain the D4Z4 silencing. Each D4Z4 unit contains a region named **DBE** (D4Z4 binding element) proposed to confer copy-number-dependent

repressive activity to the D4Z4 array [75]. DBEs recruit HMGB2 chromatin-associated protein, YY1 transcriptional repressor and EZH2 protein of the Polycomb group (PcG) [75–78]. PcG proteins act to preserve the repressed chromatin state through DNA methylation, histone deacetylation and H3K27 trimethylation [79]. Intriguingly, a significant reduction of EZH2 and H3K27me3 repressive mark in the 4q35 region was observed in FSHD1 patients suggesting the loss of Polycomb-mediated silencing in the FSHD locus [77]. Recently, **NuRD (Nucleosome Remodeling Deacetylase)** and **CAF-1 (Chromatin Assembly Factor 1)** protein complexes were also reported to play a role in *DUX4* gene repression by binding to D4Z4 repeats in healthy human muscle cells [80]. This repression is partially relieved in FSHD cells due to the DUX4-induced activation of MBD3L2 protein [80] which can replace the MBD2 and MBD3 subunits of the NuRD complex, thus possibly disrupting NuRD recruitment to its DNA targets [81].

Non-coding RNAs in DUX4 regulation

Non-coding RNAs also participate in *DUX4* regulation. Long non-coding RNA (lncRNA), *DBE-T* transcribed from the DBE of the most proximal D4Z4 unit and the NDE (Non Deleted Element), an element proximal to D4Z4 that is maintained in FSHD patients whatever is the extent of the D4Z4 contraction, may contribute to *DUX4* derepression in FSHD cells by interacting with the ASH1L protein of the Trithorax group (**trxG**) (**Figure 3**) [77]. TrxG proteins act antagonistically to those of the Polycomb group and activate genes that are normally repressed by the PcG proteins, including *DUX4*. *DBE-T* transcription might be affected by the chromatin conformation of 4q35 which is altered upon D4Z4 contraction. Inhibition of DNA-methyltransferases and histone deacetylases caused a mild de-repression of *DBE-T* in the CHO hybrid cells containing human chromosome 4 [82]. Specific non-coding RNAs may also participate in pathogenesis of the *DUX4* BCP-ALL subtype where a

strong correlation exists between expression of lncRNAs and the genes involved in the activation of TGF- β and Hippo signaling pathways [83].

D4Z4 units produce microRNA-sized fragments as a result of bi-directional transcription [55]. These fragments may generate siRNAs and activate the endogenous RNA interference pathway to maintain repeat-mediated epigenetic repression of the D4Z4 array. Targeting exogenous siRNAs to the region upstream of the *DUX4* transcription start site caused *DUX4* suppression in FSHD muscle cells and was accompanied by **AGO2** (Argonaute 2) recruitment and H3K9 methylation in the targeted region [84]. In contrast, knockdown of AGO2 or DICER, essential components of the RNAi pathway, induced *DUX4* expression in healthy muscle cells, suggesting that RNAi is indeed required for the D4Z4 silencing [84]. Interestingly, *DUX4* induction following AGO2 knockdown is dependent on the D4Z4 repeat number: *DUX4* was reactivated in the cells from two unaffected individuals carrying 13 D4Z4 repeats, but not in the cells from an individual carrying 74 D4Z4 units [84]. miRNome/transcriptome analysis revealed 29 microRNAs differentially expressed between the control and FSHD primary myoblasts, including those that potentially target *DUX4* [85].

Telomere length and DUX4 regulation

The 4q35 region is subtelomeric and the expression of its genes may be affected by telomere length. Indeed, *DUX4* and *FRG2* expression in the myoblasts and myotubes of FSHD1 patients is inversely proportional to telomere size; this could explain the age-related severity of FSHD [86]. Importantly, this correlation was not observed in healthy individuals, indicating that a large D4Z4 array might isolate the genes of the 4q35 region from the telomere, thus protecting them from a transcriptional deregulation due to telomere shortening. The effect of telomere shortening was greater on *DUX4*, the gene closest to the telomere, than on *FRG2* which is approximately 70Kb more proximal, and the most proximal of the three

genes, *FRG1*, was not at all affected [86]. Telomeres can also participate in long-range chromosome looping and thus influence gene expression over large distances (Telomere Position Effect-Over Long Distances - **TPE-OLD**) [87]. A telomere loop participates in the regulation of the *SORBS2* gene in the 4q35 locus. *SORBS2* is upregulated in the FSHD myoblasts with short telomeres, while its expression is low in healthy myoblasts and in FSHD myoblasts with long telomeres suggesting that the expression of this gene is altered by TPE-OLD in pathogenic conditions [88].

3D organization of the 4q35 locus

Different chromatin conformations allow genes to interact with each other and the control elements affecting their transcription (reviewed in [89]). 4q35 genes are involved both in short-range and long-range **cis-interactions**. In normal cells, many short-range interactions are observed, e.g. D4Z4 interacts with *TUBB4Q*, *FRG1*, and *DUX4c*; *DUX4c* interacts with *FRG1*, *FRG2* and the 4qA/B region [76,90,91]. Long-range interactions in normal cells are few; they include contacts of the D4Z4 region with *ZFP42*, *FAM149A*, and *SORBS2*; *SORBS2* with *WWC2*, *FRG1* and the telomere [88,90,92]. In FSHD cells, many of the short-range contacts disappear, while some new long-range contacts emerge (**Figure 4**). Notably, 4qA/B interacts with *ANT1* and *D4S139* interacts with *FAT1* in FSHD cells [90–92]. It should be noted that there are discrepancies in the datasets from different studies. They can be explained by differences in experimental approaches used but also by the presence of the D4Z4 on other chromosomes [88]. In general, however, all results converge on a transition from short-range interactions in normal cells towards long-range interactions, in agreement with a general derepression of the locus (**Figure 4**). These changes in chromatin architecture lead to transcriptional upregulation of many genes in the 4q35 region.

In conclusion, *DUX4* is tightly regulated at the genetic and epigenetic levels to assure its timely expression in early development and repression in somatic cells. Failure to repress the *DUX4* in skeletal muscles leads to FSHD onset. In cancer, the *DUX4* gene is translocated to other chromosomes and is found in a different genomic context. In these cases, *DUX4* expression is due to the presence of control elements in the partner regions; they also provide the poly-A signal, essential to *DUX4* mRNA stabilization. The mechanisms of recently discovered *DUX4* overexpression in a panel of cancers [50] merit a separate investigation.

Feedback loops in DUX4 regulation

Even though most recent studies agree on the *DUX4* involvement in FSHD pathology, this concept still raises questions. *DUX4* is believed to be expressed stochastically at a very low level [4], and is often undetectable in the biopsies of FSHD patients. How can such a rare event trigger the disease onset?

Multiple evidence suggests that *DUX4* can amplify its own gene expression in a number of **feedback loops**. *DUX4* induces oxidative stress and renders the cells more susceptible to it [21,24,25,93]. Inversely, oxidative stress can enhance *DUX4* expression [94] (**Figure 5**). Once transcribed, *DUX4-fl* mRNA is likely to be a substrate of NMD [29]. At the same time, *DUX4* disrupts NMD by stimulating the proteolytic degradation of UPF1 protein [29], which plays a central role in the NMD pathway (reviewed in [95]). Thus, *DUX4* stimulates its own production, *via* a double-negative feedback loop. A similar feedback can be observed in the interplay between *DUX4* and the NuRD complex which represses *DUX4* by binding to D4Z4 repeats [80]. NuRD recruitment and subsequent *DUX4* repression could be disrupted by MBD3L2 protein, and the gene encoding for MBD3L2 is itself a *DUX4* target [80]. Another feedback loop concerns the CIC-*DUX4* fusion protein in Ewing sarcoma. **ERK** (Extracellular signal-regulated kinase) interacts with the wild-type CIC [96] subjecting it to the proteasome-

mediated degradation [47,97,98]. The same is true for the CIC-DUX4 fusion protein, which retains the putative ERK-interaction domains [44]. DUSP6 is a specific negative regulator of ERK (reviewed in [99]), and *DUSP6* expression is transactivated by CIC-DUX4, generating a feedback loop which enhances CIC-DUX4 protein stability. DUSP6 inhibition limits the growth of tumors bearing *CIC-DUX4* translocations in a mice xenograft model [44].

A negative feedback loop controls the murine *Dux* expression during ZGA. *Dux* binds to LINE1-encoding genomic loci activated at the 4C/8C stage [100]. LINE1 RNA can interact with Nucleolin and Kap1 to repress *Dux* expression [101].

Positive (or double-negative) feedback loops are often associated with bistability and the processes where a definite decision should be made, including cellular differentiation, cell cycle progression and apoptosis. Assuming its role in ZGA, it is not surprising that *DUX4* can be regulated in a bistable manner: its expression is self-amplifying in the cleavage-stage embryo to ensure the complete developmental transition, while in the adult tissues it is totally repressed. Bistability is often accompanied by **hysteresis**, which reduces the system noise sensitivity and further ensures the complete transition. In human myoblasts ectopically expressing *DUX4*, *DUX4* induces the expression of the alternative histone variants, H3.X and H3.Y, which incorporate into the regions of its target-genes [102]. This likely helps to maintain an open chromatin structure in these regions, resulting in a greater perdurance of *DUX4* target genes expression and their enhanced activation following the subsequent *DUX4* exposure [102]. Interestingly, this “memory” effect may be more profound for the muscle fibers due to their multinucleated nature. Abnormal *DUX4* activation in a predisposed pathological condition may reactivate the amplifying feedback-loops, triggering a “pseudo-transition” in an inappropriate context, thus interfering with the normal physiological processes. If this is the case, we can expect more positive feedback loops to be soon

discovered in DUX4 signaling. Importantly, these loops can become promising therapeutic targets to control *DUX4* expression and prevent disease progression.

Therapeutic DUX4 inhibition in disease

FSHD

Several approaches have been developed to inhibit *DUX4* in skeletal muscles. They have been recently reviewed [103], therefore we will just outline these strategies to discuss their relevance for *DUX4*-related cancer treatment. Targeting *DUX4* mRNA by using antisense oligonucleotides (**AOs**), phosphorodiamidate morpholino oligomer (**PMO**) or locked nucleic acid (**LNA**) gapmer AOs targeting different parts of the *DUX4* ORF is the most straightforward approach to selectively repress *DUX4* [27,104–109]. Another strategy to silence *DUX4* is to reinforce *D4Z4* epigenetic or transcriptional repression. This can be done using RNAs targeting the *DUX4* upstream region [84] or dCas9-KRAB transcriptional inhibitor targeted to the *DUX4* promoter and exon 1 [110]. In both FSHD1 and FSHD2 myoblasts, *DUX4* expression can be repressed by ectopic expression of *SMCHD1* [111]. Several small molecules decreasing *DUX4* expression in FSHD muscle cells were identified, they include inhibitors of the bromodomain and extra-terminal (BET) family of proteins, agonists of the beta-2 adrenergic receptor [112,113] and inhibitors of p38 mitogen-activated protein kinase [114,115], although clinical trials of these compounds have not yet produced encouraging results [103]. Finally, a possible, though complicated, therapeutic strategy would be to target DUX4 protein. DUX4 toxicity is mainly attributed to its C-terminal domain, which can activate transcription in part by recruiting histone acetyltransferases p300 and **CBP** (CREB binding protein), resulting in local H3K27 acetylation [116]. A p300 inhibitor

protects cells from DUX4-mediated toxicity by reversing the global imbalance in H3 acetylation and reducing the ability of DUX4 to activate its target genes [117].

Cancer

As expression of DUX4 and chimeric proteins seems to be the major cancer driver in *DUX4*-related ALL [36] and sarcomas [44,49], it would be logical to target DUX4 in these cancers, although transcription factors were considered to be elusive targets [118], in part because many TF are essential for survival of normal cells in the organism. *DUX4* is not expressed in adult tissues and thus represents a relatively straightforward target. DUX4 can be inhibited, for example, by using siRNA to the homeodomain part of the *DUX4* ORF, conserved in most cancer cells. Another potential and more specific approach would be to target DUX4 fusion proteins. A recently developed approach for *in vivo* CRISPR/Cas9 targeting of fusion oncogenes [119] may prove useful when targeting *DUX4* fusions in BCP-ALL and sarcoma. Alternatively, the strategies targeting different domains of DUX4 protein and its interactors, such as the p300 inhibitor mentioned above could be applied to inhibit the activity of DUX4 fusion proteins in cancer cells. Other approaches include the inhibition of downstream targets of DUX4 fusion proteins, e.g. *DUSP6* [44] or the CCNE-CDK2 complex [49] in CIC-DUX4 sarcoma. Treatment of a Kitra-SRS CIC-DUX4 sarcoma cell line with the IGF-1R inhibitor, linsitinib, attenuated cell growth *in vitro* and in xenografts [120]. The Kitra-SRS cell line was also used to screen FDA-approved drugs to identify several cancer growth-inhibiting genotoxic drugs (Doxorubicin, Epirubicin, Topotecan and Camptothecin), two proteasome inhibitors (carfilzomib, bortezomib) and an inhibitor of multiple tyrosine kinases (ponatinib) [120]. Thus, a combination of approaches developed to suppress DUX4 in muscle cells and classical cancer chemotherapy may rapidly produce clinical advances in the treatment of *DUX4*-expressing tumors.

Concluding remarks

DUX4 is involved in a variety of activities in normal and pathological conditions. The effects of *DUX4* expression are tissue-specific as different subsets of genes are regulated by DUX4 in different cell types. As DUX4 is toxic to somatic cells, multiple mechanisms control its expression, from long-range enhancer-promoter interactions to feedback loops. Some of them are well known and are already targeted for development of new therapies. The feedback loops in *DUX4* regulation may be new interesting targets for therapy. Some factors and pathways that regulate DUX4 remain to be discovered (see Outstanding questions). Understanding the relationship between multiple mechanisms of DUX4 control is important for our understanding and treatment of *DUX4*-related pathologies.

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Glossary

BCP-ALL - B-cell precursor acute lymphoblastic leukemia is the most common cancer in children, with an annual incidence of ~35 per million. A distinct subset of BCP-ALL is associated with translocations involving *DUX4*.

Cis-interaction - interaction between elements located on the same molecules (chromosomes). *Trans*-interaction - interaction between elements located on different molecules (chromosomes).

D4Z4 - a macrosatellite repeat array in the 4q35 and 10q26 subtelomeric area. Each copy of D4Z4 includes a *DUX4* gene and several transcription control elements.

ETS - a family of transcription factors containing an Ets (Erythroblast Transformation Specific) domain.

Ewing-like sarcomas are a subgroup of small round blue cell sarcomas that lack a hallmark translocation between a gene of the RNA-binding TET family (*EWSR1* or *FUS*) with a gene of the ETS-transcription family (*FLI1*, *ERG*, *ETV1*, *ETV4*, or *FEV*). A subset Ewing-like sarcomas is associated with translocations involving *DUX4*.

Fusion (chimeric) protein - proteins that result from joining of two or more genes that originally coded for separate proteins.

Feedback loop - a mechanism whereby a product resulting from several reactions affects the previous steps in order to enhance or repress the overall effect of the entire process. A double-negative feedback loop is in fact a positive feedback loop made of two negative interactions, so that the two factors repress each other.

GQs (G-quadruplexes) - specific secondary DNA or RNA structures consisting of guanine-rich chains linked by Hoogsteen-type hydrogen bonds and stabilized at the center by a monovalent cation.

Hysteresis - the effect of dependence of the state of a system on its history;

NMD (Nonsense-mediated decay) - a process controlling the quality of RNA, which degrades RNAs with premature termination codons.

NuRD (Nucleosome Remodeling Deacetylase) - a protein complex with ATP-dependent chromatin remodeling and histone deacetylase activities, regulating gene transcription, genome integrity and cell cycle progression;

RMS (Rhabdomyosarcoma) - Mostly pediatric cancer that develops from skeletal muscle cells

ZGA (zygotic genome activation) - the initiation of gene expression after fertilization.

Box 1. DUX4 and facioscapulohumeral muscular dystrophy

DUX4 has been mostly studied in the context of facioscapular humeral muscular dystrophy (FSHD), an autosomal dominant disease characterized by age-dependent muscle weakness and wasting [121]. The FSHD genetic abnormality is localized in the subtelomeric region 4q35 of chromosome 4. Its major form (FSHD1; 95% of FSHD cases) results from a combination of several genetic factors: a decrease in the number of D4Z4 units below 11 [4] and a distal haplotype 4qA [122]. It is also strongly associated with polymorphic sequences located proximally to the D4Z4 array [123]. D4Z4 contraction causes chromatin derepression and DNA hypomethylation within the short D4Z4 array allowing for the *DUX4* transcription from the most distal D4Z4 unit. The 4qA haplotype provides an intron and a polyadenylation site, stabilizing the full-length *DUX4* mRNA which is otherwise degraded. Together these lead to the production of toxic DUX4 protein in skeletal muscles. A simple sequence length polymorphism (**SSLP**), located proximally to D4Z4 harbours an insulator [56,60,61], which is less efficient in FSHD myoblasts [60]. FSHD2 comprising ~5% of total cases is phenotypically indistinguishable from FSHD1. It results from mutations in methylation-associated genes. 85% of the FSHD2 patients carry mutations in *SMCHD1* [124], encoding for a protein involved in gene repression and X-chromosome inactivation [125]. Some cases are caused by DNA-methyltransferase 3B (*DNMT3B*) mutations [126,127]. Both *SMCHD1* and *DNMT3B* mutations lead to *DUX4* derepression and its transcription from the distal D4Z4 unit. Both FSHD2 and FSHD1 are associated with the presence of the 4qA haplotype on at least one copy of chromosome 4. In some severe FSHD cases, both D4Z4 contraction and *SMCHD1* mutations are present [128,129]. Regardless of the primary cause, all FSHD types seem to converge on DUX4 protein production.

Box 2. Evolution of DUX family genes

The genes of the Double homeobox (DUX) family are unique to placental mammals [130]. One group of DUX genes is composed of intron-containing *DUXA*, *DUXB* and *DUXC* in many placental mammals [131]. Another intron-containing variant, *Duxbl* (*DUXB*-like) exists only in mice and rats with its pseudogene forms also found in primates [130]. The second group is represented by intronless *DUX4* in primates and *Afrotheria* (elephant, hyrax and tenrec) and *Dux* in mice and rats [131].

Among double homeobox genes, only *DUX4*, *Dux* and *DUXC* possess a conserved C-terminal domain, responsible for transcription activation [130,132]. Similarly to *DUX4* in *Afrotheria* and primates, copies of mouse *Dux* are organized in tandem repeat arrays [132].

The intron-containing *DUXC* macrosatellite was likely present in the genome of the most recent common ancestor of all placental mammals, while the intronless *DUX4* and *Dux* arose later on in a series of independent retrotransposition events [131].

Clinician's corner

Facioscapulohumeral dystrophy

FSHD is the third most common myopathy, with a frequency ranging from 1 in 20000 to 1 in 8000 individuals. The disease affects skeletal muscles, typically those of the face (facio), shoulders (scapula) and upper arms (humerus), but can eventually spread to the other muscles of the body. The first symptoms usually manifest in adolescence or early adulthood, though the onset of FSHD is highly variable. The molecular mechanisms leading to FSHD are outlined in **Box 1**. Currently there is no effective FSHD treatment; the potential therapeutic approaches are extensively discussed in a recent review [103].

B-cell precursor acute lymphoblastic leukemia

BCP-ALL is the most common cancer in children, with an annual incidence of ~35 per million. The first symptoms may include bleeding, fatigue, or appetite loss. The disease is then confirmed by blood test and cytogenetic analysis as BCP-ALL results from chromosomal translocations. One subset of BCP-ALL is associated with translocations involving *DUX4*. The 5-year survival rate is ~90% for children younger than 15 years, ~75% for adolescents aged 15 to 19 years and ~50% for adults [133]. Current treatment options include chemotherapy with combinations of drugs, including vincristine, prednisone, cyclophosphamide, doxorubicin, L-asparaginase, cytarabine and methotrexate.

Ewing-like sarcomas

EWS are rare and mostly pediatric soft tissue sarcomas with poor prognosis resulting from gene fusions that are distinct from that of the *FET-ETS* family rearrangements specific for Ewing sarcoma. A subset of Ewing-like sarcomas is associated with a *CIC-DUX4* translocation. In general, CIC-fused Ewing-like sarcomas have an aggressive clinical course with frequent metastases and have a 5-year survival rate of less than 50% [133]. Treatment

options include combinations of chemotherapy, such as cyclophosphamide and topotecan or irinotecan and temozolomide with or without vincristine as well as radiotherapy.

Rhabdomyosarcoma

RMS is a malignant tumor of striated muscle origin and the most prevalent soft tissue sarcoma in children, comprising ~4.5% of all childhood cancers [134]. The common symptoms are lump and swelling, and the more specific manifestations depend on the tumor location and size. The frontline therapy is currently based on the multi-modal approach combining surgical resection, radiation therapy and chemotherapy with vincristine, actinomycin D and cyclophosphamide or ifosfamide. Such therapy is effective for over 90% of the patients with low-grade localized tumors, though the survival rates for patients with more severe metastatic or recurrent cases remain low (~20-30%) (reviewed in [135]).

Figure Legends

Key figure 1. *DUX4* expression in early development, FSHD and cancer.

Figure 2. *Regulatory elements and their target genes within the 4q35 region.* Each D4Z4 unit contains an enhancer activating *FRG2* and *DUX4c* genes located proximally to the D4Z4 array; two muscle specific *DUX4* enhancers, *DME1* and *DME2*, reside proximally to D4Z4; these regulatory elements are isolated from each other by an insulator (*FR-MAR*), which is less efficient in FSHD cells; *SSLP* - simple sequence length polymorphism, located within the *FR-MAR* sequence; the distance from the first proximal D4Z4 unit is indicated in kb.

Figure 3. *Non-coding RNAs in D4Z4 silencing.* D4Z4 units are bi-directionally transcribed generating microRNA-sized fragments which potentially produce siRNAs, activating endogenous RNA interference pathway and contributing to D4Z4 silencing. These siRNAs are less abundant upon D4Z4 contraction. Proteins of the Polycomb group bind to the DBE element inside each D4Z4 unit to repress the D4Z4 array. Chromatin relaxation on a contracted allele allows the production of a FSHD-specific lncRNA DBE-T, which attracts the *ASH1L* protein of the Trithorax group, acting antagonistically to PcG proteins.

Figure 4. *Chromatin folding and gene contacts within the 4q35 locus.*

Figure 5. *Feedback loops in DUX4 regulation.* Clockwise: *DUX4* is a substrate of NMD, but it also inhibits NMD. *DUX4*-induced oxidative stress can enhance *DUX4* expression. NuRD complex which represses *DUX4* by binding to D4Z4 repeats. NuRD recruitment and subsequent *DUX4* repression could be disrupted by *MBD3L2* protein, and the gene encoding for *MBD3L2* is itself a *DUX4* target.

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Outstanding questions

- How is *DUX4* regulated in the cleavage embryo?
- What is the role of *DUX4* in adult tissues where it is normally expressed (testis and thymus) and how would therapeutic *DUX4* inhibition affect these tissues?
- How does sporadic *DUX4* expression cause a chronic disease?
- How can the muscle specificity of *DUX4*-associated pathology be explained?
- What is the role of *DUX4* in cancer and how *DUX4* inhibition affects tumor cells?
- Why cancers associated with *DUX4* are mostly pediatric?
- Can feedback loops be targeted to modulate *DUX4* expression in pathological conditions?









