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REVIEWS

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## DUX4, a Zygotic Genome Activator, Is Involved in Oncogenesis and Genetic Diseases

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**Abstract**—After fertilization, the genome is transcriptionally quiescent to allow zygote reprogramming that relies on the RNA and proteins accumulated in the oocyte and ensures the transition from the differentiated germ cells to a totipotent state. Reprogramming is followed by zygotic genome activation (ZGA). *DUX4* gene encoding for a double homeobox transcription factor is one of the key ZGA drivers in humans. Its expression, essential for embryo development, is subject to precise temporal regulation and is normally observed only at early cleavage stages. *DUX4* is efficiently silenced in most somatic tissues via numerous epigenetic mechanisms, while its aberrant expression in skeletal muscle causes facioscapulohumeral muscular dystrophy (FSHD). *DUX4* expression following chromosomal rearrangements is also observed in a subset of leukemias and sarcomas; it leads to anti-cancer immune activity suppression.

**Keywords:** DUX4, zygotic genome activation (ZGA), oncogenesis, muscular dystrophy

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### INTRODUCTION

After fertilization, the newly formed genome is comprised of maternal and paternal genetic material, each with its own chromatin organization, and needs to be remodeled to a globally accessible state before it can be transcribed. Zygote reprogramming occurs immediately after fertilization while the fertilized oocyte is transcriptionally quiescent (Newport and Kirschner, 1982; Tadros and Lipshitz, 2009). This reprogramming relies on RNAs and proteins accumulated in the oocyte before fertilization and ensures the transition of the oocyte and the spermatozoon which have formed the zygote to a totipotent state so that they can give rise to different cell types. The process known as maternal-to-zygotic transition (MZT) (Tadros and Lipshitz, 2009) follows reprogramming. MZT includes zygotic genome activation (ZGA) and gradual degradation of maternal products. After the MZT, the newly formed zygotic genome takes entire control of transcription in the developing embryo.

The mechanisms driving ZGA are not entirely understood; ZGA is at least partially regulated through the change in nucleocytoplasmic ratio (Newport and Kirschner, 1982) which can promote titration of some highly expressed non-specific maternal repressors, inhibiting transcription from zygotic DNA (Amodeo et al., 2015; Jevtić and Levy, 2015, 2017;

Joseph et al., 2017). Rapid cell cycles in the early embryo of many species leave almost no time for transcription between divisions (Shermoen and O’Farrell, 1991; Rothe et al., 1992). As division slows down and cell-cycle lengthens, ZGA becomes possible (Kimelman et al., 1987). ZGA is also associated with chromatin remodelling. Finally, essential transcription factors inherited in the form of maternal mRNA need time to be polyadenylated, translated and accumulated at certain levels to activate transcription (Veenstra et al., 1999; Guven-Ozkan et al., 2008). Here we will review the family of DUX transcription factors that participate in mammalian ZGA (De Iaco et al., 2017).

### DUX GENE ORIGIN AND EVOLUTION

*DUX* gene family is represented by intronless *DUX4* in primates and *Afrotertia* (elephant, hyrax and tenrec), *Dux* in mice and rats, as well as intron-containing *Duxa*, *Duxb* and *Duxc* in other mammals (Leidenroth et al., 2012). One more intron-containing variant, *Duxbl* (DUXB-like), is found only in mice and rats, though its pseudogene forms are also found in primates (Leidenroth and Hewitt, 2010). *DUX4* is present in the human genome in multiple copies which are organized into large (3.3 kb) macrosatellite tandem repeats (D4Z4 arrays) in the subtelomeric regions of

chromosome 4q and 10q (Gabriëls et al., 1999). D4Z4 is also present elsewhere in the human genome as individual copies. Similarly, many copies of murine *Dux* are embedded within 4.9 kb repeats (Clapp et al., 2007).

All DUX proteins possess two DNA-binding homeodomains (HDs; HD1 and HD2) separated by a linker. Though HDs typically bind DNA in dimers, the presence of several HDs within a single protein is quite unusual and is not observed outside of placental mammals (*Eutheria*). *Duxc*, *Dux* and *DUX4* share a conserved C-terminal transactivation domain (Leidenroth and Hewitt, 2010; Mitsuhashi et al., 2018).

According to the present evolutionary model, the DUX family appeared following a duplication of a part of a gene containing a single homeobox (Leidenroth and Hewitt, 2010) so that the resulting gene contained two tandemly arranged homeoboxes (Lee et al., 2018). The genome of the most recent common ancestor of all placental mammals contained the *Duxc* double homeobox gene, then *DUX4* and *Dux* genes arose via multiple independent retrotransposition events (Leidenroth et al., 2012).

The two DUX homeodomains belong to the Paired-homeobox (PAX) branch of homeodomain family, but their similarity to each other is much stronger than to any of the other PAX branch members. This supports the hypothesis that the DUX family most probably radiated out following a duplication in the progenitor of eutherian mammals (Leidenroth et al., 2012; Lee et al., 2018). A single-homeobox gene (sDUX) identified in non-mammalian genomes is likely to be a homologue of the mammalian DUX ancestor (Leidenroth and Hewitt, 2010).

DUX4 is a transcription factor. Its HD domains bind to a specific DNA sequence and this activates gene expression via the DUX4 transactivation domain. The human DUX4 consensus motif is 5-TAATCTAATCA-3 (Geng et al., 2012; Yu Zhang et al., 2016). As the two DUX4 homeodomains are very similar to each other, it was first suggested that they bind the two identical TAAT cores one after another in a head-to-tail orientation (Dong et al., 2018). However, the recent crystal structure of the DUX4 N-terminus (15–155 residues, including both DUX4 homeodomains) in complex with its DNA consensus motif revealed that HD1 and HD2 recognize different target sequences—5-TAAT-3 and 5-TGAT-3 respectively and bind DNA in a head-to-head fashion (Lee et al., 2018).

### DUX GENES AND ZGA

As a transcription factor, DUX4 activates expression of several hundred genes. *DUX* genes (human *DUX4* and murine *Dux*) are expressed prior to zygotic activation as a part of the first (minor) wave of ZGA. It has been proposed that DUX transcription factors are the key inducers of ZGA in mammals (De Iaco

et al., 2017; Hendrickson et al., 2017). *DUX4* RNA has been detected from the oocyte to four-cell (4C) stage, and the transcripts of DUX4 putative targets including other ZGA-associated genes such as *ZSCAN4*, *ZFP352* and endogenous retroviral elements (MERVL in mice and HERVL in humans) are present from two-cell (2C) to eight-cell (8C) stages peaking at 8C (De Iaco et al., 2017), which corresponds to the major wave of ZGA in humans (Vassena et al., 2011).

Ectopic *Dux* expression in mouse embryonic stem cells (mESCs) converts them into two-cell embryo-like ('2C-like') state. *Dux*-expressing mESCs reactivate expression of "2C" genes and repeat elements, lose the pluripotency associated POU5F1 protein and chromocenters (heterochromatic aggregations) and acquire chromatin landscape characteristic of the two-cell embryos as determined by ATAC-seq (Hendrickson et al., 2017). Alternately, CRISPR/Cas9 knockout of *Dux* in mouse zygotes ex vivo was shown to prevent their transition from 2C. KO zygotes did not exhibit ZGA-specific transcriptional changes and failed to form morula/blastocyst (De Iaco et al., 2017).

However, more recent studies demonstrate that *Dux* depletion in vivo is much less harmful and the complete loss of *Dux* is compatible with mouse development (Chen and Zhang, 2019; Guo et al., 2019). Homozygous *Dux*  $-/-$  mice survive to adulthood, albeit they display a slightly reduced frequency than expected from the Mendelian distribution and reduced developmental potential. RNA-seq profiles of *Dux* knockout mouse embryos at late 1C and 2C stages do not differ significantly from those of WT (Chen and Zhang, 2019). Single-cell RNA-seq of 2C embryos at early, middle and late 2C-stages demonstrates that while a subset of ZGA genes is indeed downregulated in *Dux* KO embryos at the early 2C stage (as compared to WT early 2C), these genes are robustly upregulated at the late 2C stage (compared to early 2C in *Dux* KO embryos) indicating that their transcription, though delayed, is activated even in the absence of *Dux* (Guo et al., 2019). These data suggest that *Dux* may be an important but not essential ZGA factor rather than initiator of expression of ZGA transcripts. Other transcription factors and/or chromatin remodelers may also contribute to successful ZGA in murine 2-cell embryos, while ES cell system might not be an appropriate model for totipotent state studies. Furthermore, *Dux* transcripts are not detected in mouse embryos after the late 2C (Guo et al., 2019; Deng et al., 2014) and the artificial injection of in vitro transcribed *Dux* mRNA into mouse blastomeres at late 2C arrests their development mainly at 4C stage (Guo et al., 2019). The arrested embryos robustly express *Zscan4* and MERVL, the signatures of 2C embryo. In conclusion, DUX4 expression is characteristic of 2C stage and is likely to accelerate ZGA while its precise temporal regulation is essential for proper development.

## POST-ZYGOTIC *DUX4* REPRESSION AND PATHOLOGICAL EXPRESSION IN ADULT TISSUES

Mechanisms ensuring *DUX* repression at precise points of embryonic development and its further repression in somatic tissues are not entirely understood, though the importance of successful *DUX4* silencing in somatic tissues is beyond doubt. In adult humans, *DUX4* is normally expressed only in testis (Snider et al., 2010) and presumably thymus (Das and Chadwick, 2016), and its aberrant expression in other tissues is associated with facioscapulohumeral muscular dystrophy (FSHD), B-cell leukemia (Dib et al., 2019), anti-cancer immune activity suppression and anti-cancer therapy resistance (Chew et al., 2019).

Human *DUX4* is believed to be silenced via repeat-mediated epigenetic repression, as it is embedded in D4Z4 macrosatellite repeat array, situated in the subtelomeric region of chromosome 4 (4q35). This array is highly polymorphic in size and contains 11–200 3.3 kb repeat units, each containing an open reading frame for the *DUX4* gene. D4Z4 is silenced at multiple levels via DNA methylation, histone modification, and association of repressive chromatin proteins. Most repeat arrays are monitored by the cellular genome surveillance machinery and if their length is over a certain threshold, they are silenced (Mitsuda and Shimizu, 2016). In these conditions, the proper length of D4Z4 is essential for effective *DUX4* repression.

Pathological shortening of D4Z4 array is associated with an autosomal dominant genetic disease: facioscapular humeral muscular dystrophy 1 (FSHD1). Individuals with FSHD1 bear a reduced number of D4Z4 repeats (1–10), allowing for a more open chromatin state, and a permissive 4qA allele providing a functional polyadenylation signal stabilizing *DUX4* mRNA, transcribed from the distal D4Z4 unit.

Individuals with FSHD2, a contraction-independent form of FSHD constituting ~5% of the overall disease cases (de Greef et al., 2010), possess the normal number of D4Z4 repeats but bear mutations in essential chromatin regulators, notably *SMCHD1* (structural maintenance of chromosomes hinge-domain protein 1), leading to an incomplete D4Z4 array methylation (Dion et al., 2019) and a similar relaxed D4Z4 chromatin state enabling *DUX4* expression.

Notably, chromatin relaxation alone is not sufficient for disease development, since *DUX4* mRNA undergoes alternative splicing. Initial *DUX4* mRNA possesses 2 splicing sites in the 3' UTR generating two *DUX4fl* transcripts, encoding for a full-length *DUX4* protein (Dixit et al., 2007; Snider et al., 2009), but these transcripts are highly unstable and undergo further splicing by the splice donor site in the coding region. This generates a further transcript—*DUX4s*—encoding for a protein retaining DNA-binding homeodomains, but lacking a C-terminal transactivation domain, mainly responsible for cytotoxicity (Snider

et al., 2010). *DUX4s* is frequently observed in both FSHD and healthy individuals and is not associated with muscle pathology. The disease-permissive 4qA haplotype creates a functional polyadenylation site flanking the very distal D4Z4 repeat and is required for both FSHD1 and FSHD2 development. This polyA stabilizes the full length *DUX4*, transcribed from the distal unit, allowing for a cytotoxic full-length protein production.

Another level of complexity is added by the presence of the D4Z4 array on chromosome 10, which is 99% homologous to the 4q D4Z4 and also exhibits length variations, though no canonical polyA signal at its distal boundary has been identified. 10q D4Z4 contractions are non-pathogenic, emphasizing the importance of a specific chromosomal background to cause FSHD. A recent proof-of principle study has shown that exchanging of the short 4q arm with a long 10q arm via CRISPR-Cas induced translocations can improve differentiation potential and reverse some of the pathological expression patterns in FSHD muscle cells (Ma et al., personal communication).

In several pathologies including leukemia, rhabdomyosarcoma (RMS) and Ewing-like sarcoma, translocations involving the 4q chromosome lead to the fusion of *DUX4* with other proteins. In acute lymphoblastic leukemia (ALL) the insertions of *DUX4* into the *IGH* (immunoglobulin heavy chain) locus (Lilljebjörn et al., 2016; Yasuda et al., 2016) leading to production of *DUX4* with a truncated C-terminus or into an intron of *ERG* oncogene (ETS-related gene) leading to production of an *ERG-DUX4* chimeric protein (Sirvent et al., 2009). The *DUX4*-containing protein expression is probably due to the presence of control elements in the partner regions which also provides the poly-A signal, essential to *DUX4* mRNA stabilization. In tumor cells of Ewing-like sarcoma, the t(4;19)(q35;q13) chromosomal translocation generates a fusion between the *DUX4* C-terminus and *CIC* (capicua transcriptional repressor) gene. This fusion results in the production of *CIC-DUX4* chimeric protein where *DUX4* confers to *CIC* a high transcriptional activity leading to the deregulation of the PEA3 subclass of ETS family genes (Kawamura-Saito et al., 2006). Another type of translocation involving *DUX4* gene in embryonic RMS has been reported with a t(4;22)(q35;q12) translocation as the sole cytogenetic modification that leads to the *EWSR1-DUX4* chimeric protein production (Sirvent et al., 2009).

## PATHOLOGICAL CONSEQUENCES OF ABERRANT *DUX4* EXPRESSION IN FSHD AND CANCER

*DUX4* is a transcription factor; therefore its aberrant expression observed in myoblasts (Dixit et al., 2007; Kowaljew et al., 2007; Snider et al., 2010), biopsies of FSHD patients (Snider et al., 2010), and during embryonic development (Ferreboeuf et al., 2014) may

have serious consequences for the cells. Indeed, *DUX4* overexpression induces pathological effects such as hypersensitivity to oxidative stress accompanied by a decrease in expression of enzymes involved in the metabolism of glutathione (Winokur et al., 2003; Bosnakovski et al., 2008; Barro et al., 2010; Saada et al., 2016), atrophy of myotubes in vitro by inducing *Atrogin1* and *Murf1* (Vanderplanck et al., 2011), apoptosis by activating caspase 3 (Kowaljew et al., 2007) and by a p53-dependent mechanism (Wallace et al., 2011), and deregulation of myogenic differentiation (Dmitriev et al., 2013) marked by a decrease in *MYOD* expression (Winokur et al., 2003; Celegato et al., 2006). In addition, *DUX4* induces expression of genes expressed in germ cells (Geng et al., 2012) and that of a paired-type homeodomain transcription factor, *PITX1* (Dixit et al., 2007) which is involved in the segmentation of the embryo and the development of the hindlimbs (Szeto et al., 1999) controlling the morphology of their muscle, tendon and bones (DeLaurier et al., 2006). Conditional up-regulation of *PITX1* induces skeletal muscle atrophy in mice (Pandey et al., 2012).

So far, the role of *DUX4* in the development of leukemia has not been completely elucidated. However, it has been demonstrated that *DUX4* is involved in the loss of function of wild-type *ERG*. Indeed, *DUX4* induces expression of *ERG* negative-dominant isoform, ERGalt, which is essential for leukemogenesis and increases the transcriptional activity in the region, making it more susceptible to deletion via the recombination-activating genes (RAGs) (Jinghui Zhang et al., 2016). *DUX4* expression induces DNA damage (Dmitriev et al., 2016), providing a possible explanation for the frequent deletion of *ERG* which is a hallmark of this ALL subtype. Moreover, expression of *IGH-DUX4* but not *DUX4* in pro-B cells generates B cell leukemia in transplanted immunodeficient mice proving that *DUX4* gains an oncogenic potential following the chromosomal rearrangement (Yasuda et al., 2016).

In the Ewing-like sarcoma, *DUX4* fuses with *CIC* gene encoding a transcriptional repressor that functions as a primary downstream sensor of RTK/ERK (receptor tyrosine kinase/extracellular signal-regulated kinase) pathway (Tseng et al., 2007; Jin et al., 2015). Chimeric *CIC-DUX4* protein exhibits strong transcriptional activity and induces a unique gene expression profile, characterized by the upregulation of the PEA3 transcription factor group (Kawamura-Saito et al., 2006). PEA3 proteins regulate several genes involved in tumorigenesis such as matrix metalloproteinases, which play an important role in metastasis (Reviewed in (De Launoit et al., 2006)).

In the embryonic RMS, the translocation results in the fusion of *DUX4* to *EWSR1* gene (Sirvent et al., 2009), a situation very similar to the one observed in Ewing-like sarcoma where another transcription fac-

tor, *ERG*, is fused to *EWSR1* (Ida et al., 1995). The danger of this type of translocation consists in the production of a fusion protein with aberrant properties.

In addition to rearrangement involving the *DUX4* gene in various sarcomas and leukemia, epigenetic alterations of 4q35, the FSHD-associated locus, have been described in other cancers (Tsumagari et al., 2008; Katargin et al., 2009). A study comparing FSHD to 35 cancers expression profiles has shown that cancer-related genes are differentially expressed in FSHD (Dmitriev et al., 2014). Interestingly, the FSHD transcription signatures strongly resemble those of Ewing-like sarcoma. Cancer predisposing conditions frequently observed in FSHD patients including inflammation, fibrosis, oxidative stress (Arahata et al., 1995; Barro et al., 2010; Dmitriev et al., 2016) and DNA damage (Dmitriev et al., 2016) may account for the similarity between FSHD and cancer cell expression profiles.

*DUX4* may contribute to cancer progression via its numerous targets, many of which, as well as *DUX4* itself, take part in early developmental program, and thus their expression is characteristic of totipotent cells. For example, *DUX4* target ZSCAN4 normally required for telomere extension in embryonic stem cells (Zalzman et al., 2010), is active in many *DUX4*-expressing cancers, possibly promoting tumor replicative potential. Another *DUX4* target, CCNA (A-type cyclin), is involved in male meiosis (Liu et al., 1998) and is abnormally expressed in the majority of myeloid and undifferentiated hematological malignancies (Krämer et al., 1998). CCNA-overexpressing mice exhibit altered myelopoiesis and develop acute myeloid leukemia (Liao et al., 2001).

Finally, *DUX4* has recently been linked with anti-tumor immune activity suppression. *DUX4*-expressing cancers are characterized by low anti-tumor cytolytic activity and resistance to checkpoint blockade therapy, relying on cytotoxic T cell recognition of antigens presented by major histocompatibility complex (MHC) class I on malignant cells. *DUX4* expression inhibits interferon- $\gamma$ -mediated induction of MHC1 leading to suppressed antigen presentation in *DUX4*-mediated immune invasion (Chew et al., 2019). RNA-seq data from metastatic melanoma patients receiving anti-CTLA-4 checkpoint blockade therapy (Van Allen et al., 2015) revealed significantly increased *DUX4* expression in non-responsive patients as compared to responsive subjects.

## CONCLUSIONS

*DUX4* is an embryonic transcription factor accelerating ZGA in mammals. It is expressed at precise stages of embryonic development and must be silenced in the somatic tissues of an adult organism. Abnormal post-natal *DUX4* expression re-activates an early developmental program characteristic of toti-

potent cells and results in expression deregulations and differentiation abnormalities, leading to FSHD and several types of cancers.

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### CONFLICT OF INTEREST

The authors declare no conflict of interests.

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