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DUX4, the rockstar of embryonic genome activation?

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ABSTRACT During the initial days of development, the embryo gradually shifts from reliance on maternally provided RNAs and proteins to regulation of its own development. This transition is marked by embryonic genome activation (EGA). While the factors driving human EGA remain poorly characterized, accumulating evidence suggests that double homeobox 4 (DUX4) is an important regulator of this process. Despite advances in single-cell methods which have allowed studies in early human embryos, fundamental questions regarding the function and regulation of DUX4 persist. Here, we review current knowledge of DUX4 with a focus on EGA in humans.

KEYWORDS: Double Homeobox 4 (DUX4), embryo development, embryonic genome activation, gene regulation, non-coding genome

Introduction

The oocyte-to-embryo transition culminates in the degradation of maternal transcripts and in embryonic genome activation (EGA) (Schulz and Harrison, 2019; Vastenhouw *et al*., 2019). The transcriptome changes prominently during the oocyte-to-four cell and four-to-eight cell transitions that are considered as the minor and major EGA stages, respectively (Braude, 1988; Petropoulos *et al*., 2016; Tesařék, 1988; Töhönen *et al*., 2015; Vassena *et al*., 2011; Xue *et al*., 2013; Yan *et al*., 2013). In addition to the activation of key developmental genes, the non-coding genome that contributes to genome regulation becomes extensively transcribed (Bouckenheimer *et al*., 2016; Paloviita and Vuoristo, 2022). The activation of the embryonic transcriptome is intricately linked with major alterations in the epigenome and chromatin architecture (Chen *et al*., 2019; Liu *et al*., 2019; Wu *et al*., 2018; Xia *et al*., 2019). It is conceivable that EGA takes place only in a favorable epigenomic landscape that generates adequate conditions for timely gene regulation. How these processes are regulated in human fertilized oocytes and pre-implantation embryos, and whether the factors involved exhibit redundancy, remain poorly understood.

DUX4 belongs to a group of double homeobox genes that are unique to placental mammals. These genes are characterized by two proximal homeoboxes that encode DNA-binding homeodomains (Gabriëls *et al*., 1999). The primate specific *DUX4* is believed to have originated through retro-transposition of the ancestral *DUXC* gene, followed by the loss of *DUXC* from the primate genome (Leidenroth *et al*., 2012). *DUX4* mRNA is enriched in human zygotes and cleavage-stage embryos (De Iaco *et al*., 2017; Hendrickson *et al*., 2017; Liu *et al*., 2019; Töhönen *et al*., 2017; Vuoristo *et al*., 2022). Silencing of *DUX4* in human embryos leads to inefficient degradation of maternal transcripts and incomplete EGA, which implies a potential role of DUX4 as an EGA regulator (Liu *et al*., 2022; Vuoristo *et al*., 2022). Human embryonic stem cells (hESCs) have been used to elucidate possible roles of selected EGA factors given the shortage of supernumerary embryos donated for research and the fact that experiments in human embryos are challenging due to ethical and technical limitations (Gawriyski *et al*., 2023; Hendrickson *et al*., 2017; Madissoon *et al*., 2016; Vuoristo *et al*., 2022; Zou *et al*., 2022). In hESCs, ectopic expression of *DUX4* activates both coding and non-coding genes that are typically active in early human embryo at the time of EGA (Hendrickson *et al*., 2017; Taubenschmid-Stowers *et al*., 2022; Vuoristo *et al*., 2022; Yoshihara *et al*., 2022). These findings collectively suggest a pivotal role for DUX4 in regulating human EGA. In this review, we aim to provide the latest insight into DUX4 and discuss its significance in the context of human EGA.

The peculiar *DUX4* repeat locus

The *DUX4* open reading frames are located in the subtelomeric region of chromosome 4, within a macrosatellite repeat region known as *D4Z4* (Gabriëls *et al*., 1999) (Fig. 1). While the *D4Z4* repeat array is typically epigenetically repressed in most tissues, it becomes transiently derepressed in human embryos possibly due to global epigenome reprogramming (De Iaco *et al*., 2017;

Abbreviations used in this paper: DUX4, Double Homeobox 4; EGA, Embryonic genome activation; FSHD, Facioscapulohumeral muscular dystrophy; TE, Transposable element.

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Hendrickson *et al*., 2017; Liu *et al*., 2019; Vuoristo *et al*., 2022; Xia *et al*., 2019). The repetitive nature, high GC content, and low expression level of *DUX4* pose challenges for sequencing and annotation of this genomic region, particularly when working with human embryos that are available in limited numbers. Most of our current knowledge about *DUX4* stems from research on its involvement in the pathogenesis of facioscapulohumeral muscular dystrophy (FSHD) (Campbell *et al*., 2018). FSHD is caused by derepression of *D4Z4* locus which likely leads to a burst of *DUX4* expression in a subset of affected muscle cells resulting in cell death (Rickard *et al*., 2015; Snider *et al*., 2010). The derepression of *D4Z4* locus is caused by either a reduction of *D4Z4* repeat units alone (FSHD1), or defects in *D4Z4* chromatin repressor SMCHD1 (FSHD2), or both (FSHD1+2) (Hewitt, 2015; Sacconi *et al*., 2019). Interestingly, a nearly identical *D4Z4* repeat array exists on chromosome 10, but the contraction of this repeat array does not cause FSHD presumably due to the lack of a permissive polyadenylation signal (Lemmers *et al*., 2010). The toxic effect of DUX4 in FSHD pathophysiology is not yet completely understood but the mechanisms likely involve the activation of the MYC-mediated apoptotic pathway and the double-stranded RNA innate immune response pathway (Shadle *et al*., 2017), as well as repression of nonsense-mediated decay (NMD) (Campbell *et al*., 2023; Feng *et al*., 2015; Jagannathan *et al*., 2019). Notably, DUX4 is cytotoxic not only for muscle cells but also for various other cell types (Kowaljow *et al*., 2007; Resnick *et al*., 2019; Rickard *et al*., 2015; Wallace *et al*., 2011; Yoshihara *et al*., 2022). Given that in human embryos MYC expression is only upregulated at the time of major EGA stage, the first two days of development take place without one of the main factors behind DUX4-induced cell death. This could be one of the reasons why human embryos tolerate short-term DUX4 expression.

Although each repeat unit in the *D4Z4* array contains the *DUX4* open reading frame, the current conception is that functional *DUX4* transcripts originate from the last repeat unit (Fig. 1). This is explained by the position of the polyadenylation signal, which is distal to the repeat array and thus transcribed exclusively as part of the last repeat (Dixit *et al*., 2007). Consequently, individuals that have a contracted *D4Z4* repeat region but lack the distal polyadenylation signal exhibit a normal muscle phenotype (Lemmers *et al*., 2010). In addition to the *DUX4* mRNA isoforms that are transcribed from the *D4Z4* array on chromosome 4 and use the conventional polyadenylation signal, numerous isoforms that utilize alternative polyadenylation sites have been described to originate from both chromosome 4 and 10. These isoforms likely result from alternative splicing, however the mechanisms that control the splicing of *DUX4* transcripts remain obscure. Moreover, it is unclear which mRNA isoforms give rise to functional DUX4 proteins. Testis and some cancer cell lines express *DUX4* transcripts that most likely produce a complete DUX4, as indicated by immunofluorescence stainings and the activation of DUX4 target genes (Smith *et al*., 2023; Snider *et al*., 2010). In contrast, various somatic tissues, including healthy muscle, express low levels of capped and polyadenylated *DUX4* transcript isoform, which probably produces a truncated protein that lacks the transcription activation domain and, consequently, the ability to activate DUX4 targets (Snider *et al*., 2010). *DUX4* mRNA isoforms present in human embryos have not been described.

The properties of DUX4

The DUX4 protein contains an N-terminal DNA binding domain and C-terminal transcription activation domains (Lee *et al*., 2021; Mitsuhashi *et al*., 2018; Vuoristo *et al*., 2022). The N-terminal DNA-binding domain of DUX4 includes two homeodomains, HD1 and HD2, that arose from an internal duplication of a single homeodomain and consequently, exhibit a high degree of similarity (Leidenroth and Hewitt, 2010). A primate specific mutation changing arginine to glutamate in HD1 have led to the different target DNA sequence preferences of HD1 and HD2; 5'-TAAT-3' and 5'-TGAT-3', respectively (Lee *et al*., 2018). DUX4 has been shown to regulate a

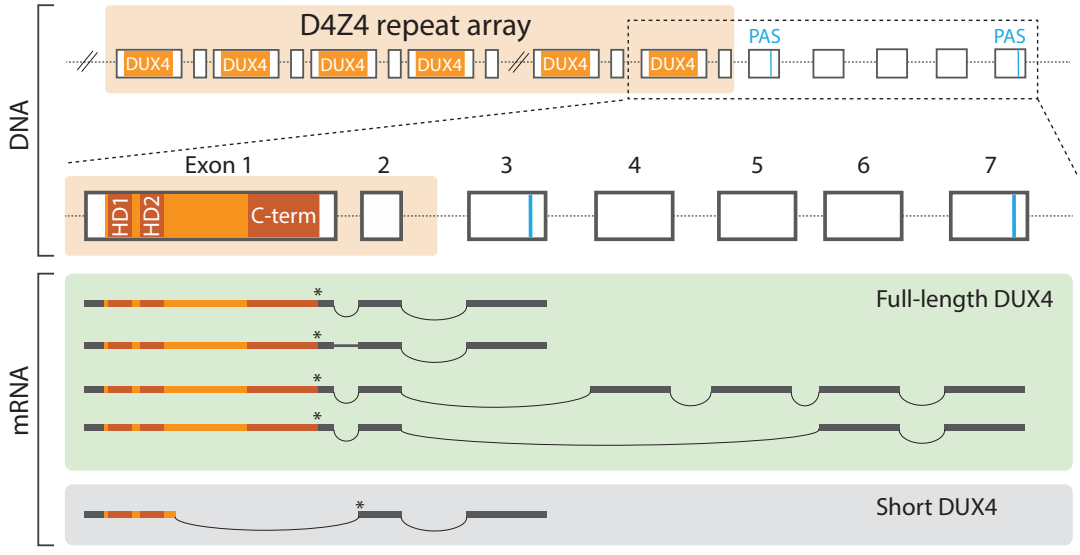

Fig. 1. The D4Z4 repeat array and DUX4 mRNA isoforms. The protein coding DUX4 transcripts are thought to originate from the last D4Z4 repeat unit and to utilize exons distal to the repeat array that provide canonical polyadenylation signals (PAS). DUX4 transcript isoforms are generated through alternative splicing, but the regulation of this process is poorly understood. In facioscapulohumeral muscular dystrophy (FSHD) muscle, DUX4 mRNA contains exons 1-2-3, and the first intron is alternatively spliced (Snider *et al.*, 2010). Testicular tissue expresses DUX4 mRNA isoforms with exons 1-2-4-5-6-7 and 1-2-6-7 (Snider *et al.*, 2010). Healthy myoblasts use a cryptic splice site within the first exon and express a DUX4 mRNA isoform that is predicted to give rise to a truncated DUX4 (short DUX4 or DUX4-s) that contains the N-terminal homeodomains, but lacks the C-terminus (Snider *et al.*, 2010). Additional DUX4 mRNA isoforms that utilize alternative polyadenylation signals are likely to exist (Smith *et al.*, 2023). The asterisk (*) indicates the location of the translation stop codon. Abbreviations: C-term, C-terminus; DUX4, double homeobox 4; HD, homeodomain; PAS, polyadenylation signal.

set of transposable elements (TEs) (Vuoristo *et al*., 2022; Young *et al*., 2013). Hence, the unique HD1 target sequence in primates, as opposed to other mammals, may have evolved as a consequence of co-evolution with species-specific TEs (Lee *et al*., 2018).

While all the human DUX family members, including DUX4, DUXA and DUXB, contain the N-terminal DNA-binding domain, only DUX4 possesses the conserved C-terminal domain (Leidenroth and Hewitt, 2010). The C-terminus of DUX4 contains a nine amino acid transactivation domain (9aaTAD) (Mitsuhashi *et al*., 2018) and a KIX-binding motif (KBM) (Vuoristo *et al*., 2022). The C-terminal domain is essential for DUX4-mediated transcriptional activation and cytotoxicity (Bosnakovski *et al*., 2008; Choi *et al*., 2016; Mitsuhashi *et al*., 2018). Consequently, DUXA and DUXB, which lack the C-terminal domain, are incapable of activating transcription and do not cause cytotoxicity (Bosnakovski *et al*., 2023). The region between the N-terminal homeodomains and C-terminal transactivating domain is predicted to be intrinsically disordered (Mitsuhashi *et al*., 2018). While many transcription factors contain an intrinsically disordered region (IDR), the precise nature of their function is not well understood (Ferrie *et al*., 2022). The deletion of the region between homeodomains and C-terminus does not affect the activation of the known DUX4 target gene *ZSCAN4* (Choi *et al*., 2016; Mitsuhashi *et al*., 2018) nor does it protect from the DUX4-elicited cell death in myoblasts (Choi *et al*., 2016) and HEK293 cells (Mitsuhashi *et al*., 2018). However, further studies are required to determine whether the IDR lacking form of DUX4 can fully recapitulate the function of full-length DUX4.

DUX4 in EGA regulation

DUX4 was first associated with embryogenesis when it was observed to activate early developmental genes in muscle cells of FSHD patients (Geng *et al*., 2012). Since then, experiments conducted in various cell types, including human myoblasts (Jagannathan *et al*., 2016; Resnick *et al*., 2019; Rickard *et al*., 2015), human pluripotent stem cells (De Iaco *et al*., 2017; Hendrickson *et al*., 2017; Taubenschmid-Stowers *et al*., 2022; Vuoristo *et al*., 2022; Whiddon *et al*., 2017; Yoshihara *et al*., 2022), and most recently various cancer cell lines (Smith *et al*., 2023), have consistently reported the activation of genes and TEs characteristic of early embryos upon induced or spontaneous *DUX4* expression. *DUX4* mRNA is enriched in zygotes and early cleavage stage embryos (De Iaco *et al*., 2017; Liu *et al*., 2019; Töhönen *et al*., 2017; Vuoristo *et al*., 2022). DUX4 protein shows nuclear accumulation in 2- and 4- cell stage embryos followed by rapid clearance by the 8-cell stage (Hendrickson *et al*., 2017; Vuoristo *et al*., 2022). Given the ethical and technical challenges associated with research involving human embryos, many insights into the DUX4 functions have been derived from the studies conducted using inducible *DUX4* transgene cell lines. Inspired by these findings, the scientific community has recognized DUX4 as one of the earliest regulators of EGA.

DUX4 activates the non-coding genome

In accordance with the timing of the nuclear localization of DUX4 (Vuoristo *et al*., 2022), the accessible genomic regions in 2-cell and 4-cell embryos are enriched with DUX4 binding motifs (Hendrickson *et al*., 2017; Liu *et al*., 2019; Wu *et al*., 2018). Accessible chromatin regions in human cleavage stage embryos are frequently located at distal sites (> 5 kb from the transcriptional start sites) of genes and enriched at TEs (Gao *et al*., 2018; Liu *et al*., 2019; Wu *et al*., 2018). TEs are mobile genetic elements constituting approximately half of the human genome (Franke *et al*., 2017). TEs are enormously diverse and divided into distinct classes and families based on their modes of transposition, structural features, and evolutionary relationship (Bourque *et al*., 2018). TEs promote genetic diversity by moving within the genome, which can lead to alterations in host genes and regulatory sequences. This mobility poses a significant risk to genome stability, and therefore several mechanisms have evolved to suppress TEs (Almeida *et al*., 2022).

Human TEs are transcriptionally and post-transcriptionally repressed by various chromatin remodellers, Krüppel-associated box (KRAB) domain-containing zinc finger proteins, and small RNAs (Almeida *et al*., 2022; Gainetdinov *et al*., 2017; Janssen *et al*., 2018). However, a substantial proportion of TEs, including human endogenous retroviral (HERV) elements, are transiently active during pre-implantation development (DiRusso and Clark, 2023; Göke *et al*., 2015; Grow *et al*., 2015; Liu *et al*., 2019; Pontis *et al*., 2019; Xu *et al*., 2022). For instance, MLT2A1 elements that are members of the HERV family, become accessible and transcribed at the 4-cell stage, retain these states for the 8-cell stage, but are subsequently repressed (Liu *et al*., 2019). Notably, *DUX4* overexpression in hESCs leads to the binding of DUX4 to 30% of the embryonically accessible MLT2A1 elements (Hendrickson *et al*., 2017; Liu *et al*., 2019). DUX4 also activates transcription of various other TEs (Geng *et al*., 2012; Hendrickson *et al*., 2017; Vuoristo *et al*., 2022; Young *et al*., 2013), and for example, the EGA genes *ZSCAN4* and *KHDC1P1* are regulated by DUX4-activated ERVL-MaLR overlapping enhancers in *DUX4*-expressing hESCs (Vuoristo *et al*., 2022). Some TEs have been evolutionarily co-opted to regulate species- and context-specific gene expression programs (Franke *et al*., 2017; Hashimoto *et al*., 2021; Liang *et al*., 2010; Macaulay *et al*., 2011; Pontis *et al*., 2019; Whiddon *et al*., 2017), and we speculate that some DUX4-induced TEs may have been co-opted to regulate human EGA transcripts.

Mechanisms of DUX4-mediated gene regulation

DUX4 has the capacity to modulate nucleosome structure by inducing the expression and incorporation of histone variants H3.X and H3.Y, which are associated with a relaxed chromatin state and enhanced transcription of the DUX4 target genes (Resnick *et al*., 2019). In myoblasts, induced DUX4 expression leads to the incorporation of H3.X/Y into highly transcribed DUX4 target genes, potentially contributing to the maintenance of an open chromatin conformation (Resnick *et al*., 2019). Studies on protein-protein interactions in myoblasts and HEK293 cells have shown that DUX4 can interact with histone acetyltransferases p300 and the CREB binding protein (CBP) (Choi *et al*., 2016; Vuoristo *et al*., 2022). These histone acetyltransferases are typically recruited to enhancers by transcription factors, resulting in local acetylation of H3K27 and subsequent expression of target genes (Raisner *et al*., 2018). DUX4 interacts with p300/CBP through its C-terminus, and the deletion of the last 98 amino acids of the DUX4 C-terminus disrupts the interaction with both p300 and CBP (Choi *et al*., 2016). The expression of several DUX4 target genes is reduced when the interaction of p300/CBP with DUX4 is disrupted by the DUX4 C-terminal deletion (Choi *et al*., 2016) or by p300/CBP inhibition (Bosnakovski *et al*., 2019), suggesting that transcriptional activation of certain DUX4 target genes depends on p300/CBP. Furthermore, DUX4 can interact with several chromatin modifiers and transcriptional

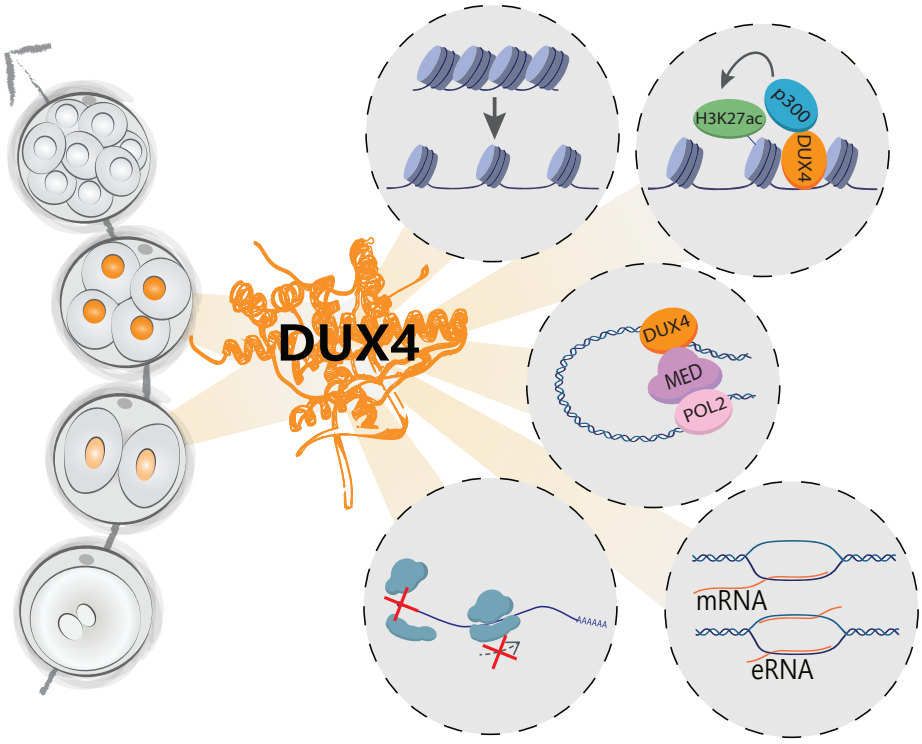

Fig. 2. A summary of the suggested mechanisms of action of DUX4. DUX4 accumulates in the nuclei of two- and four-cell stage human embryos and is subsequently cleared by the 8-cell stage (left) (Hendrickson ***et al.***, 2017; Vuoristo ***et al.***, 2022). DUX4 regulates chromatin accessibility (Resnick ***et al.***, 2019; Vuoristo ***et al.***, 2022), and interacts with various proteins, such as histone acetyltransferase p300 (Choi ***et al.***, 2016) and mediator complex members (Vuoristo ***et al.***, 2022), which are involved in transcriptional regulation. DUX4 activates several embryonic genome activation (EGA)-associated genes, repetitive elements (De Iaco ***et al.***, 2017; Geng ***et al.***, 2012; Hendrickson ***et al.***, 2017; Liu ***et al.***, 2019; Vuoristo ***et al.***, 2022) and putative enhancers (Vuoristo ***et al.***, 2022), and it was recently implicated in inhibition of translation initiation and elongation (Hamm ***et al.***, 2023). Abbreviations: DUX4, double homeobox 4; eRNA, enhancer RNA; H3K27ac, histone 3 lysine 27 acetylation; MED, mediator complex, mRNA, messenger RNA.

modifiers both stably and transiently (Vuoristo *et al*., 2022). DUX4 interacts with several mediator complex family members that relay regulatory signals from transcription factors to RNA polymerase II (Chen *et al*., 2021). DUX4 binds MED15 via the six amino acid KIX binding motif, which is located at the very end of the DUX4 C-terminus (Vuoristo *et al*., 2022). This interaction presents another direct mechanism of DUX4-mediated transcriptional modulation. Besides transcriptional regulation, DUX4 has been implicated in translational regulation. A recent study showed that induced *DUX4* expression leads to translational suppression of numerous mRNAs in myoblasts (Hamm *et al*., 2023). The DUX4-mediated regulation of translation is likely attributed to alterations in the activity of translation initiation regulators 4EBP1, eIF4E, and elongation factor eEF2, however, the intermediate mechanisms responsible for perturbing these factors remain unknown (Hamm *et al*., 2023). Taken together, DUX4 emerges as a versatile regulator, engaging with a variety of proteins to shape transcription and translation processes (summarized in Fig. 2). Further research is needed to elaborate implications of the DUX4 protein interactions and unravel the precise regulatory mechanisms downstream of DUX4.

Regulation of DUX4 expression

DUX4 transcripts have not been detected in oocytes, indicating that *DUX4* is embryonically expressed, however factors and processes responsible for triggering *DUX4* expression in human embryos remain unknown. In a study by Grow *et al*., an LTR element (LTR10C) near the *DUX4* gene was identified as a *DUX4* enhancer in myoblasts and induced pluripotent stem cells (iPSC) that were derived from FSHD patients (Grow *et al*., 2021). This enhancer was found to be bound by p53, a transcription factor that is activated in response to DNA damage, and as shown by CRISPR interference is required for full p53-dependent activation of the *DUX4* locus in FSHD iPSC (Grow *et al*., 2021). However, p53 binding alone is insufficient to cause activation of *DUX4* expression as the enhancer also becomes occupied by p53 in non-FSHD cells upon induction of DNA damage. Therefore, it is likely that p53-induced DUX4 expression entails additional prerequisites such as inefficient epigenetic repression, as is observed in FSHD (Grow *et al*., 2021). Notably, telomere shortening, a phenomenon observed both in FSHD (Stadler *et al*., 2013) and human embryos up to the 4-cell stage, may result in the loss of H3K9 methylated heterochromatin at the *DUX4* locus (Zhang *et al*., 2023). This decrease in epigenetic repression is suggested to facilitate p53 binding to the LTR10C enhancer subsequently leading to the activation of *DUX4* (Zhang *et al*., 2023).

EGA is required for zygotic development, but it is equally important that this unique transcription program is timely repressed as development progresses. The level of H3K9me3, which is strongly associated with densely packed and transcriptionally silenced heterochromatin, gradually accumulates during human pre-implantation development (van de Werken *et al*., 2014; Xia *et al*., 2019; Xu *et al*., 2022; Yu *et al*., 2022). While some TEs are marked by H3K9me3 throughout development, others gain H3K9me3 in a stage-specific manner (Xu *et al*., 2022; Yu *et al*., 2022), strongly implying their developmental stage-specific involvement in cis-regulatory functions during human embryogenesis. Mechanisms behind the selective temporal repression of specific genomic loci during human EGA remains poorly understood. Mouse DUX, a homolog of DUX4, has been suggested to contribute to the establishment of H3K9me3 by inducing the expression of DUXBL, which is subsequently recruited to DUX-bound regions with the TRIM24/33 complex to facilitate the silencing of the associated genes and TEs (Vega-Sendino *et al*., 2024). A recent study proposes an analogous mechanism in humans, where DUXA that is expressed in the 8-cell

stage embryos (Töhönen *et al*., 2015), potentially contributes to the repression of DUX4 target loci (Bosnakovski *et al*., 2023). DUXA DNA binding motifs are highly similar to those of DUX4 (Liu *et al*., 2019), however DUXA lacks the C-terminal transactivating domain, presumably abolishing its ability to activate transcription (Bosnakovski *et al*., 2023). Remarkably, when both DUX4 and DUXA are ectopically expressed in myoblasts, the expression levels of DUX4 target genes are significantly reduced (Bosnakovski *et al*., 2023). *DUXA* expression is activated by DUX4, which points to a feedback inhibition mechanism where DUXA suppresses DUX4 targets, possibly as a result of competitive target sequence binding by DUX4 and DUXA (Bosnakovski *et al*., 2023).

The mechanisms of *DUX4* repression in human embryos and somatic tissues remain unclear, however it is conceivable that multiple epigenetic mechanisms, such as DNA methylation and H3K9me3 mediated heterochromatin formation, contribute to this. In mouse 2-cell embryos, the relocation of *Dux* loci to the nucleolar periphery potentially drives the repression of *Dux* through the formation of perinucleolar heterochromatin, concurrently with the maturation of nucleoli (Xie *et al*., 2022; Yu *et al*., 2021). Also in human embryos, nucleoli maturation initiates around the time of EGA (Tesarik *et al*., 1986) and coincides with the repression of *DUX4* (Kresoja-Rakic and Santoro, 2019). However, whether nucleolar maturation contributes to *DUX4* repression in humans remains a topic for future research. Due to the location of *DUX4* in the subtelomeric region, it may be affected by the transcriptional repression induced by the spread of telomeric heterochromatin, named telomere position effect (TPE) (Lee *et al*., 2021). Zhang *et al.,* have provided evidence that telomere extension may contribute to the silencing of DUX4 in human embryos, supported by the observation that telomere length extends during major ZGA (Zhang *et al*., 2023) and longer telomeres lead to pronounced TPE (Baur *et al*., 2001).

Future prospects

Ever since DUX4 was recognized as a potent EGA factor it has raised broad interest across the research community. Earlier findings about DUX4 that were mainly focused on the etiology of FSHD, have become augmented by recent perceptions that DUX4 actively modulates embryonic gene expression and embryonic development. Yet several important questions about DUX4 and its implications in human oocyte-to-embryo transition and EGA remain unanswered. Current *DUX4* annotations rely on the *DUX4* sequences found in human somatic cells like myoblasts and therefore, it is possible that human zygotes express *DUX4* sequence variants. Cloning and sequencing of the full-length *DUX4* mRNA from zygotes and cleavage stage embryos using traditional PCR-based technologies may be complicated due to the repetitive nature and high GC content of this transcript (Jagannathan *et al*., 2016). Therefore, recent technological improvements in long read sequencing may prove to be useful in determining the *DUX4* mRNA sequence in human embryonic samples. Relatedly, recent findings according to which the canonical DUX4 interacts with numerous chromatin modifiers, RNA-binding proteins, and transcriptional modifiers open an interesting avenue to study which of these interactions take place in a context-dependent manner and how these interactions may pertain to embryonic development.

Our understanding about the implications of DUX4 in human development is restricted, mainly due to ethical and technical limitations related to experiments where human embryos are used. Downscaling the amount of input material needed to perform for instance chromatin profiling has enabled informative analyses about oocytes and preimplantation human embryos. As an example, LiCAT-sequencing that requires low input material (Liu *et al*., 2019) allowed Zhang *et al.,* to correlate chromatin accessibility at the *DUX4* regulatory region with *DUX4* expression levels and telomere length during EGA (Zhang *et al*., 2023). These types of approaches clarify the possible mechanisms behind the onset of *DUX4* expression in human development. While mutating of the *DUX4* transcription start site in human zygotes leads to embryo stalling by the 8-cell stage (Liu *et al*., 2022), the knockdown of *DUX4* leads to impaired oocyte-to-embryo transition (Vuoristo *et al*., 2022). This indicates that RNAi-mediated knockdown of DUX4 at zygotic stage may not reveal a complete *DUX4* phenotype. The EGA-associated coding transcripts are relatively well-known, however another interesting research avenue will be to investigate the implications of TEs that are activated at the time of EGA. Transcriptome and translatome profiling of preimplantation human embryos in combination with extensive functional experiments emphasized the importance of both maternal and zygotic transcription factors (Zou *et al*., 2022). These factors include TPRXL and OTX2 that are of maternal origin and undergo translation starting from the oocyte meiotic resumption (Zou *et al*., 2022). The OTX2 is highly expressed in human oocytes (Xue *et al*., 2013; Yan *et al*., 2013), and its binding motif is enriched at accessible chromatin regions in the early embryo (Liu *et al*., 2019), indicating that it functions temporally in parallel with DUX4. Simultaneous knockdown of TPRXL and embryonically expressed TPRX1 and TPRX2 results in developmental delay and impaired EGA, while knockdown of these factors individually caused milder phenotypes (Zou *et al*., 2022). EGA-associated factors presumably form a transcriptional circuitry in their specific epigenome landscape. Further research is imperative to understand how these factors function and to what extent each of these factors may be indispensable for development or whether they can compensate for one another. One of the challenges related to research on human EGA factors is their highly unique *in vivo* state, which is difficult to recapitulate in vitro. Recently acknowledged 8-cell embryo-like cellular model systems provide promising platforms for future studies (Mazid *et al*., 2022; Moya-Jódar *et al*., 2023; Taubenschmid-Stowers *et al*., 2022; Yoshihara *et al*., 2022; Yu *et al*., 2022), although they lack some of the key *in vivo* aspects such as the presence of maternal factors. As a summary, recent studies have emphasized the role of DUX4 as one of the active transcription factors during human EGA and focused on discovering how DUX4 may become activated, and how it regulates its target sequences. Future research is imperative to further elucidate mechanisms of DUX4 and other EGA factors in the context of human EGA and in cellular reprogramming.

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