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—Original Article—

Transcription factor OTX2 silences the expression of cleavage embryo genes and transposable elements

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Abstract. Upon mammalian fertilization, zygotic genome activation (ZGA) and activation of transposable elements (TEs) occur in early embryos to establish totipotency and support embryogenesis. However, the molecular mechanisms controlling the expression of these genes in mammals remain poorly understood. The 2-cell-like population of mouse embryonic stem cells (mESCs) mimics cleavage-stage embryos with transient *Dux* activation. In this study, we demonstrated that deficiency of the transcription factor OTX2 stimulates the expression of ZGA genes in mESCs. Further analysis revealed that OTX2 is incorporated at *the Dux* locus with corepressors for transcriptional inhibition. We also found that OTX2 associates with TEs and silences the subtypes of TEs. Therefore, OTX2 protein plays an important role in ZGA and TE expression in mESCs to orchestrate the transcriptional network.

Key words: OTX2, Totipotency, Zygotic genome activation

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Zygotic genome activation (ZGA) occurs robustly at the 2-cell (2C) stage of mouse embryos, which is essential for development beyond the 2C stage [1]. During this process, thousands of genes and transposable elements are activated to replace maternally stored RNAs, driving embryonic programs. Among these transcripts, *Dux* gene [2], zinc finger and SCAN domain containing 4 (*Zscan4*) clusters [1], and endogenous retroviruses (ERVs) [3] are hallmarks of zygotic genome activation because of their expression specificity [4] and potential functions in totipotency establishment [5, 6]. The *Dux* gene, which encodes a double-homeodomain protein, is activated at the onset of ZGA in early embryos [7–9]. Although *Dux* is not essential for pre-implantation development, zygotic depletion of *Dux* leads to impaired early embryonic development and defective ZGA [10–12]. Despite intensive studies for decades, the regulatory network of ZGA genes is still largely unknown. Embryonic stem cells (ESCs), which are isolated from the inner cell mass (ICM) of blastocysts, can generate any cell type of the fetus but have extremely low efficiency in producing cell types of extraembryonic tissues [13]. In recent years, isolated mouse ESC (mESC)-derived 2C-like cells have been a useful model for exploring totipotency and early embryonic development [14–19]. The 2C-like cells express high levels of ZGA transcripts, including the murine endogenous retrovirus (ERV)-L (MERVL) family of retroviruses and *Zscan4* genes. In addition, these cells share some key epigenetic characteristics with 2C-stage embryos, such as high histone mobility [20] and chromatin accessibility [21]. Notably, *Dux* was transiently activated in 2C embryos and 2C-like mESC populations, and *Dux* knockout in mESCs prevents cells from cycling through the 2C-like state [7, 8].

OTX2 is a member of the OTX family expressed in mouse blastocysts and ESCs [22]. *Otx2*$^{-/-}$ mice exhibit embryonic lethality due to abnormal gastrulation, and *Otx2*$^{+/-}$ mice exhibit female-specific lethality and reduced male fertility [23–25]. Recently, the *Otx2* gene has been suggested to regulate the stem cell state of the ICM and epiblast [22]. The OTX2 protein exhibits heterogeneous expression in ESCs and facilitates the transition into the early primed state [22, 26, 27]. Furthermore, *Otx2* is required for mESC transition into epiblast stem cells (EpiSCs) and stabilizes the EpiSC [22]. However, the regulation of zygotic transcribed genes by OTX2 has not been reported.

mESCs are heterogeneous and contain subpopulations with distinct gene expression. A rare mESC subpopulation marked by the reactivation of endogenous retrovirus MERVL, called 2C-like ESCs, has been identified [28]. To examine the regulation of ZGA genes by OTX2 at the transcriptional level, we used mESCs and identified the upregulation of ZGA genes in the absence of the *Otx2* gene. Further analysis showed that OTX2 acts as a transcriptional barrier for the zygotic transcriptional program to inhibit Dux expression through incorporation at the *Dux* locus.

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Materials and Methods

Cell culture

mESCs were cultured on irradiated MEFs in N2B27 medium with 2i (PD0325901, 0.4 μM [Stemgent, San Diego, CA, USA] and CHIR99021, 3 μM [Stemgent]) and LIF (1000 u/ml) in tissue culture (TC) dishes pretreated with 7.5 μg/ml poly-L-ornithine (Sigma, St. Louis, MO, USA) and 5 μg/ml laminine (BD Biosciences, San Jose, CA, USA) [26].

RNA-seq dataset analysis

Raw reads [4, 26, 29] were processed with cutadapt v1.16 to remove adapters and perform quality trimming with default parameters, except for quality cutoff and minimum length, which were set at 20 each (GSE56138 and GSE22182). Cufflinks were used to calculate the FPKM values. The ZGA gene list was obtained from a previous report [21], and ZGA genes with abundance in MuERVL$^+$ versus MuERVL$^-$ populations of mESCs [4] above 1.5 fold were used for further analysis. Dot and box plots, heatmaps, and bar plots were generated using R. Processed RNAseq datasets of mESC transcriptome before and after *Dux* overexpression were also used [30].

ChIP-seq and ATAC-seq dataset analyses

Raw reads were processed with cutadapt v1.8.1 to remove adapters and perform quality trimming. Trimmed reads were mapped to the UCSC mm10 assembly using Bowtie with default parameters, and only uniquely aligned sequences were retained. The reported ChIP-seq and ATAC-seq results were obtained from GSE56138 [26], GSE17642 [31], GSE76823 [32], and GSE99746 [33].

Statistical analysis

The Wilcoxon rank sum test with continuity correction was used to calculate P-values and statistical significance was set at $P < 0.05$.

Results and Discussion

The transcription factor *Otx2* has been shown to act as a negative switch in the regulation of the transition from naive to primed pluripotency in mouse pluripotent stem cells [22, 26, 27]. Thus, we carefully examined the transcriptome of *Otx2* knockout mESCs [26]. Many typical ZGA genes were significantly upregulated upon *Otx2* depletion, including *Zscan4d*, *Sp110*, *Zfp560*, and *Zfp352* (Fig. 1A). In early mouse development, the *Zscan4* gene family is restrictedly enriched in the 2C stage, and these genes are typical ZGA genes that play important roles in driving the expression of cleavage embryo genes [34]. Here, we show that *Zscan4* gene family members are significantly activated when *Otx2* is deficient in mESCs (Fig. 1B). We then showed the enrichment of the genes in our ZGA gene list in the MuERVL$^+$ ESC population (Fig. 1C), and we found that the overall expression of ZGA genes was activated after Otx2 depletion (Fig. 1D).

To identify how ZGA genes are activated in the absence of the *Otx2* gene in mESCs, multiple reported ZGA gene regulators were examined, including ZGA activators and repressors [4, 16, 30, 35, 36] (Fig. 1E). For example, a recent study using overexpression screening showed that the maternal factors *Dppa2* and *Dppa4* activate *Dux* to drive the expression of downstream ZGA genes [36]. Notably, only the expression of *Dux* and *Zscan4* increased significantly in *Otx2* knockout mESCs, while *Zscan4* family genes are downstream targets of *Dux*. Therefore, *Otx2* removal in mESCs leads to specific upregulation of *Dux*, the master activator of ZGA genes.

We next examined the expression of representative maternal factors that were highly enriched in oocytes (Fig. 1F), and we did not find significantly different gene expression after *Otx2* depletion. *Otx2* has been reported to be an early factor in the differentiation of mESCs, and its activation was correlated with the downregulation of *Nanog* and *Oct4* during ESC differentiation [22]. *Otx2*, which is required for ESC transition into EpiSCs, stabilizes the EpiSC state by suppressing the mesendoderm-to-neural fate switch together with BMP4 and FGF2 in pluripotent cells [22]. Specifically, OTX2 binding was reported to be significantly enriched in the promoter/enhancer regions of *Oct4*, *Sox2*, and *Nanog* in EpiSCs [37]. Therefore, we also tested the expression of representative pluripotent regulators in mESCs (Fig. 1G) and found limited expression changes in these pluripotent regulators when *Otx2* was depleted. Taken together, our analysis showed that *Otx2* depletion did not significantly affect the maternal or pluripotency network.

To examine whether *Otx2* regulates ZGA regulators and ZGA genes directly, we analyzed the OTX2 ChIP-seq results in mESCs (Fig. 2A). We noticed significant enrichment of OTX2 occupancy at the *Dux* locus and no signal at *Zscan4* or other ZGA gene loci. We also identified similar binding patterns of two corepressors, SETDB1 [18] and RYBP [38], which were reported to inhibit ZGA genes (Fig. 2B). ATAC-seq results at the *Dux* locus in mESCs (Fig. 2B) also showed a peak at the *Dux* locus in mESCs. Taken together, our analysis indicates that the *Dux* locus is bound by OTX2-containing repressing complexes for transcriptional silencing, thereby blocking the activation of *Dux*-targeted ZGA genes.

To determine how *Dux* activity contributes to *Otx2*-mediated repression of ZGA, we compared the changes in the expression of ZGA genes in *Otx2* knockout and *Dux*-overexpressing mESCs (Fig. 2C). Generally, higher upregulation of ZGA genes upon *Otx2* knockout correlates with higher upregulation upon *Dux* overexpression. Therefore, we propose that *Dux* overexpression is the leading reason for the activation of ZGA genes in the absence of *Otx2*.

Transposable elements (TEs) help establish gene regulatory networks both in ESCs and embryonic development, including the long interspersed nuclear elements (LINEs), long terminal repeat (LTR) elements, and short interspersed nuclear elements (SINEs) [4, 39]. Our results showed that the number of reads of some TE subtypes was increased in *Otx2* KO mESCs compared to the control (Figs. 3A and B). According to OTX2 ChIP-seq data in mESCs, OTX2 was enriched in TE elements (Fig. 3C). Remarkably, reads at the ERV-K family and LTR retrotransposon (MaLR) in LTR elements accounted for most positions at LTR loci, and L1 accounted for most positions at LINE loci (Fig. 3C). Interestingly, the transition from the 2-cell stage and the development progression to the blastocyst stage depend on LINE-1 expression [14, 40]. In addition, we found that the changes in the L1 family in *Otx2* knockout mESCs are not associated with the evolutionary year of L1 (Fig. 3D). These results indicate that OTX2 is also associated with TEs and regulates TE expression.

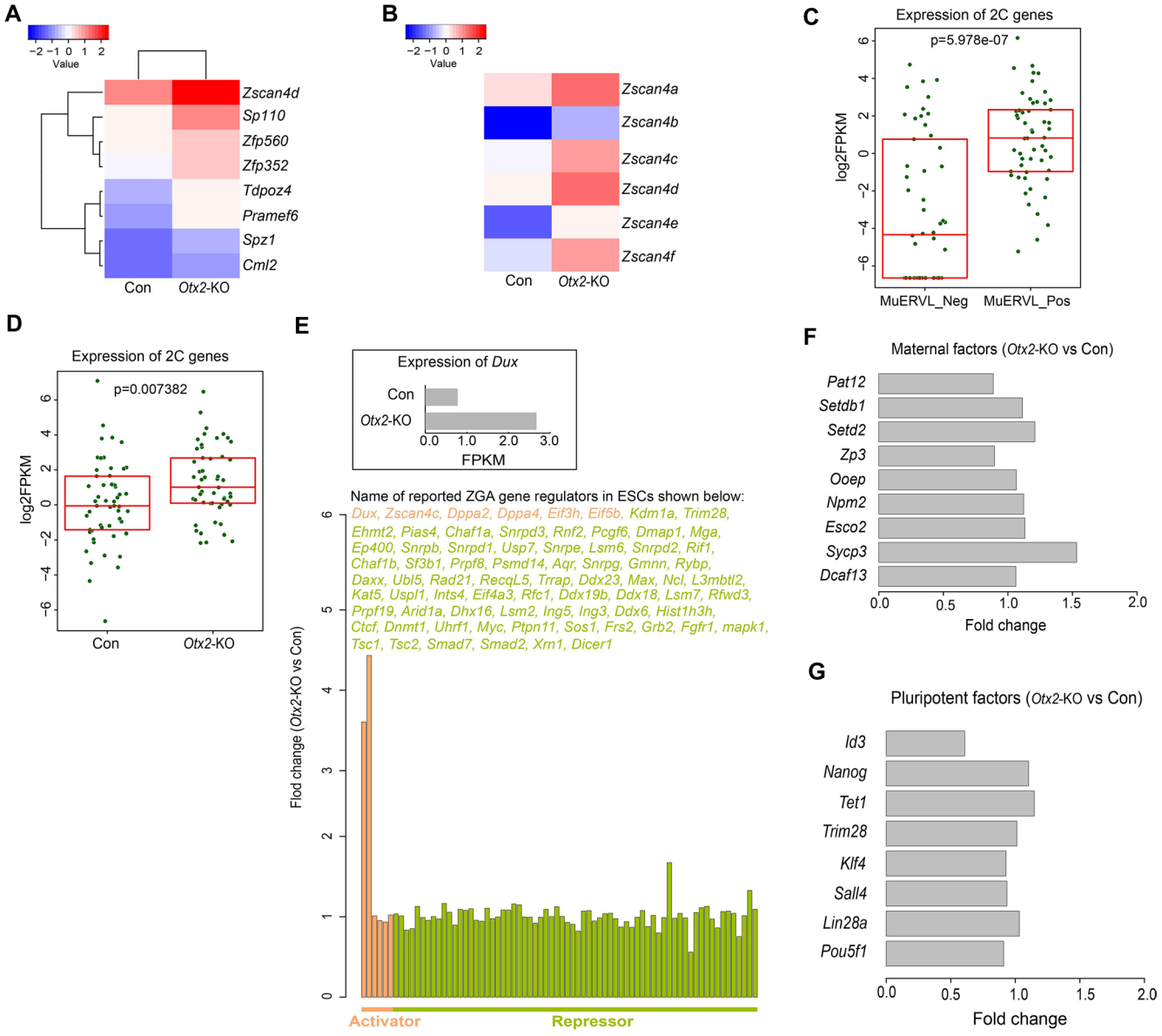

Fig. 1. *Otx2* depletion leads to upregulation of cleavage embryo genes in mouse embryonic stem cells (mESCs). A: Heatmap showing the abundance of representative ZGA genes in control and *Otx2* knockout (*Otx2*-KO) mESCs. B: Heatmap of abundance showing the upregulation of *Zscan4* family genes after *Otx2* knockout (*Otx2*-KO) in mESCs. C: Dot-and-box plot depicting the abundance of ZGA genes in MuERVL^{-} and MuERVL^{+} populations of mESCs. D: Dot-and-box plot depicting the abundance of ZGA genes in control and Otx2 KO mESCs. E: Barplot indicating the log-ratio RNA abundance of reported ZGA genes in *Otx2* knockout (*Otx2*-KO) versus control mESCs. Orange indicates ZGA activators and aqua indicates ZGA repressors. Note that *Dux* is significantly upregulated after *Otx2* knockout in mESCs. F: Barplot indicating the log-ratio RNA abundance of representative maternal factors in *Otx2* knockout (*Otx2*-KO) versus control mESCs. G: Barplot indicating the log-ratio RNA abundance of representative pluripotent factors in *Otx2* knockout (*Otx2*-KO) versus control mESCs.

OTX2 is regarded as a factor that facilitates ESC differentiation. Our results suggest that OTX2 may participate in ESC differentiation by increasing the transient totipotency state of stem cells, which were reported to have higher chromatin opening. During the transition of stem cell conditions, the appearance of transient totipotency status with activated ZGA genes and overall enhanced chromatin accessibility across the genome [21] may be important regulatory factors.

The OTX family of homeobox genes is a vertebrate ortholog of the Drosophila orthodenticle homeobox gene [41]. It is expressed in both early embryos and ESCs in mice (Supplementary Fig. 1: online only) [22, 29, 37]. *Otx2* is first detected in the murine blastula [42] and is then restricted to ICM with Nanog expression [37]. *Otx2* expression is gradually downregulated in the epiblast, where it is virtually absent from E3.7 to E4.5 [37]. The RNA-seq data showed

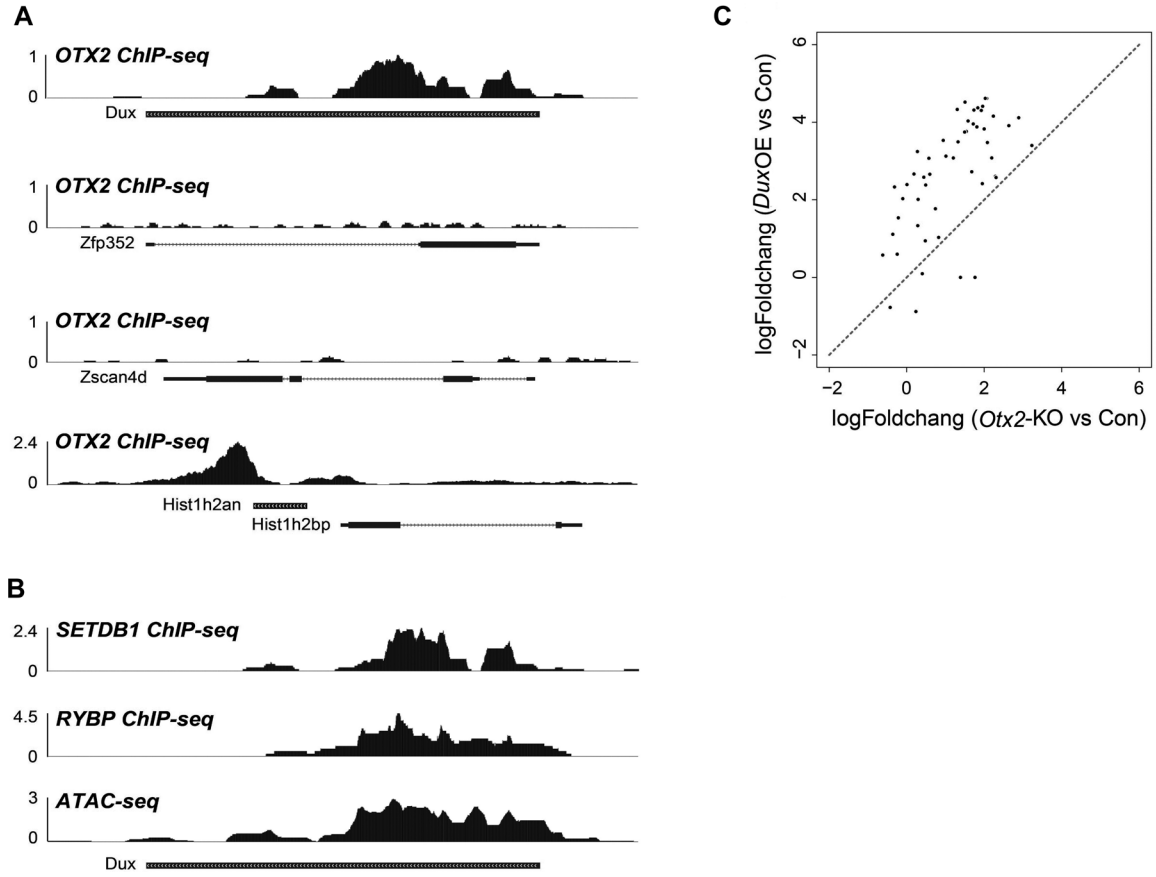

Fig. 2. OTX2 associates with *Dux* locus. A: Genome browser screenshot of OTX2 ChIP-seq signals at *Dux*, *Zscan4*, *Zfp352*, *Zscan4d*, and histone loci in mESCs. B: Genome browser screenshot of ChIP-seq and ATAC-seq signals of corepressors at *Dux* locus in mESCs. C: Scatterplot depicting the co-relationship of *Dux* overexpression (OE) and *Otx2* knockout (*Otx2*-KO)-mediated changes in ZGA gene expression.

that *Otx2* was undetectable in mouse 2-cell embryos, whereas *Otx1* could be detected at this stage. However, *Otx2* was detected in ESCs instead of *Otx1*. This indicates that other OTX family proteins may be essential during development. At late embryonic stages, the expression of *Otx1*, although more restricted, is detected in the same areas as those of *Otx2* [42]. Based on the different expression patterns of OTX family genes, we propose that orthologous genes *Otx1* and *Otx2* regulate ZGA and TE in early mouse embryos and mESCs, respectively.

Until now, the roles of OTX-family genes in early mouse embryos have been poorly understood. Our finding that the transcription factor *Otx2* regulates *Dux* and TE expression in mESCs provides new clues for the developmental functions of OTX family genes.

Collectively, our analysis shows that *Otx2* represses the activation of ZGA genes and subfamilies of TE expression by directly binding to genomic loci of the *Dux* gene and TE elements (Fig. 4).

Conflict of interests: The authors declare no conflict of interest.

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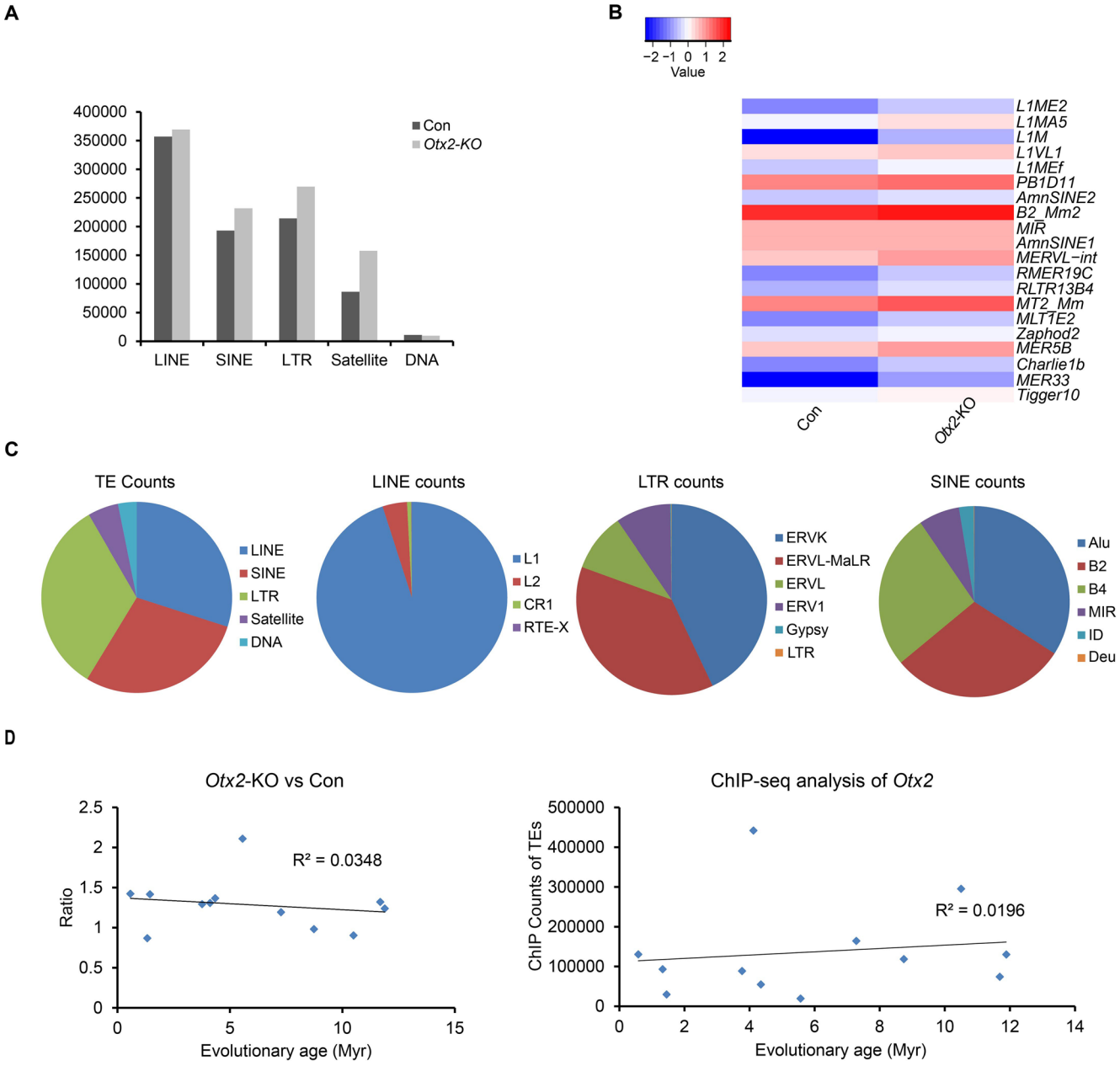

Fig. 3. OTX2 regulates the expression of transposable elements (TEs). A: Total reads of TE expression in control and *Otx2* knockout (*Otx2*-KO) mouse embryonic stem cells (mESCs). B: Heatmap of abundance depicting the differentially expressed genes of TE subtypes after *Otx2* knockout (*Otx2*-KO) in mESCs. C: Pie charts depicting the distribution of OTX2 at TEs, including the long interspersed nuclear elements (LINEs), long terminal repeat (LTR) elements, and short interspersed nuclear elements (SINEs) in mESCs. D: Relative expression ratio of *Otx2* knockout (*Otx2*-KO) to control mESCs versus evolutionary ages of L1 subfamilies (left). Enrichment of OTX2 at L1 subfamilies versus evolutionary ages of L1 subfamilies (right).

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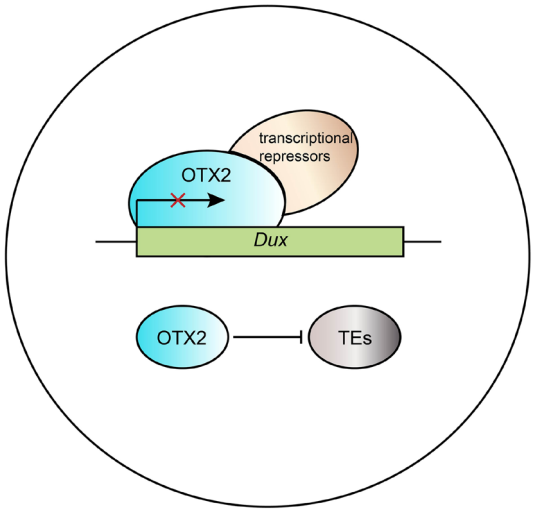

Fig. 4. Scheme of how *Otx2* regulates cleavage embryo genes and TE in mESC.

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