Loss of DUX causes minor defects in zygotic genome activation and is compatible with mouse development

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How maternal factors in oocytes trigger zygotic genome activation (ZGA) is a long-standing question in developmental biology. Recent studies in 2-cell-like embryonic stem cells (2C-like cells) suggest that transcription factors of the DUX family are key regulators of ZGA in placental mammals. To characterize the role of DUX in ZGA, we generated Dux cluster knockout (KO) mouse lines. Unexpectedly, we found that both Dux zygotic KO (Z-KO) and maternal and zygotic KO (MZ-KO) embryos can survive to adulthood despite showing reduced developmental potential. Furthermore, transcriptome profiling of the MZ-KO embryos revealed that loss of DUX has minimal effects on ZGA and most DUX targets in 2C-like cells are normally activated in MZ-KO embryos. Thus, contrary to the key function of DUX in inducing 2C-like cells, our data indicate that DUX has only a minor role in ZGA and that loss of DUX is compatible with mouse development.

In mammals, early embryonic development is supported first by maternal factors in the egg and later by newly transcribed genes from the zygotic genome. Successful ZGA is essential for embryonic development. In mice, the major wave of ZGA takes place at the 2-cell stage with the activation of thousands of genes and transposable elements, including the ERVL-family retrotransposons. Interestingly, ERVL and ERVL-linked genes can also be activated spontaneously in rare and transient embryonic stem (ES) cells known as 2C-like cells. As 2C-like cells mimic 2-cell embryos in terms of the expression of 2-cell transient transcripts and have the capacity to contribute to both embryo and extra-embryonic tissues, 2C-like cells have been a useful model for understanding totipotency and early embryonic development. However, 2C-like cells are not equivalent to 2-cell embryos, as the genes induced in 2C-like cells only represent a subset of the mouse ZGA genes that are activated in 2-cell stage embryos.

Dux (also known as Duxf3) in mice and its human homolog DUX4 are double-homeodomain genes that are activated at the onset of ZGA in early embryos. In mice, the Dux cluster also includes a truncated variant named Gm4981 (also known as Duxf4), which lacks the first homeodomain and is transcribed as early as during oogenesis. In humans, incomplete silencing of DUX4 causes facioscapulohumeral muscular dystrophy (FSHD) characterized by de-repression of genes and repeats, such as ZSCAN4 and ERVL, that are only expressed during ZGA in muscle cells in patients with FSHD. In addition, in ES cells, mouse DUX can activate ERVL family repeats and ERVL-linked genes and is both necessary and sufficient for the ES to 2C-like cell transition.

To determine whether DUX deficiency causes developmental arrest, we genotyped 255 pups from 35 litters of Dux Het × Het (F1 × F1 and F1 × F2) mating pairs (Supplementary Fig. 2c). Contrary to the expectation that Dux Z-KO embryos arrest during pre-implantation development owing to ZGA defects, Dux Z-KO mice can survive to adulthood without obvious abnormalities, although they are born at a reduced frequency (a birth rate of 18% versus an expected birth rate of 25%, P = 0.005) (Fig. 1b,c and Supplementary Fig. 2c). To exclude the possibility that Dux copies outside of the deleted macrosatellite repeats may compensate for DUX deficiency, we determined Dux RNA levels in the testis, one of the few organs in which Dux is expressed in adults.

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As expected, the level of Dux transcript in the testis in Het mice is about half that in WT mice and is undetectable in the testis samples from KO mice (Fig. 1d). This result is consistent with the previous report that the Dux cluster on chromosome 10 is the only Dux locus in mice. Collectively, these data indicate that loss of zygotic DUX does not arrest mouse development.

To determine whether the truncated DUX variant Gm4981 in oocytes might compensate for the deficiency of DUX in Z-KO embryos, we assessed the development of Dux Z-KO × Z-KO offspring, which should lack both zygotic DUX and maternal Gm4981. Dux MZ-KO embryos did not show impaired preimplantation development (Supplementary Fig. 3) and also survived to adulthood without obvious abnormalities (Fig. 1e, f, and Supplementary Fig. 2c). Consistent with the reduced frequency of Z-KO pups in Het × Het crosses, the litter sizes of Z-KO × Z-KO mating pairs are also significantly smaller than those of controls (4.0 ± 1.2 versus 7.6 ± 1.8, P = 0.0003). Interestingly, Dux Z-KO females showed slightly reduced litter size (5.6 ± 1.5, P = 0.03).
when their fertility was tested using WT B6 male mice. The reduction in litter size of Dux Z-KO female progeny should occur after implantation as both Z-KO female ovulation and pre-implantation development of MZ-KO embryos appear normal compared to control WT or Het mice (Supplementary Fig. 3). Nevertheless, the fact that both Dux Z-KO and MZ-KO develop to adulthood indicates that DUX and its truncated variant Gm4981 are not essential for mouse development.

As lack of DUX does not arrest mouse development, DUX is unlikely to have a major role in ZGA. To investigate this, we generated late 1-cell and late 2-cell Dux MZ-KO embryos by fertilizing F2 Z-KO oocytes with F2 Z-KO sperm, then performed RNA-sequencing (RNA-seq). Embryos that were generated by fertilizing F2 WT oocytes with F2 WT sperm were used as controls. After confirming data reproducibility (Supplementary Fig. 4), we performed comparative analyses of late 1-cell RNA-seq data sets that revealed that, out of the 10,554 detectable genes (reads per kilobase of transcript per million mapped reads (RPKM) > 1 in either WT or KO), only 50 (0.47%) and 28 (0.26%) were significantly up- and downregulated, respectively, in Dux MZ-KO embryos (fold change (FC) > 2 and false discovery rate (FDR) < 0.05) (Fig. 1g and Supplementary Table 1), suggesting that DUX and Gm4981 deficiency has little effect on late 1-cell gene expression. Although it is not feasible to assess the expression level of each Dux repeat, owing to the assembly gap at the Dux cluster, we note that the annotated Dux and the other four known genes (that is, AW822073/Duxf1,
Overall, our results demonstrate that mouse ZGA genes, including many exogenous DUX targets identified in mouse ES cells, can be activated in Dux MZ-KO embryos and therefore that loss of DUX does not arrest mouse development. It is possible that other transcription factors and/or chromatin remodelers have a redundant role in 2-cell embryos for successful ZGA. Our results in mice...
seem to be in direct contrast to observations in ES cells, in which DUX is essential for the entry of ES cells into the 2C-like state12. Therefore, despite the simplicity of the 2C-like state, caution should be taken in using the ES cell system to study the totipotent state as there are fundamental differences between the in vitro 2C-like cell state and 2-cell embryos.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41588-019-0418-7.

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Author contributions
Z.C. and Y.Z. conceived the project. Z.C. designed and performed experiments. Z.C. analyzed sequencing datasets. Z.C. and Y.Z. interpreted the data and wrote the manuscript.

Competing interests
The authors declare no competing financial interests.

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Methods

Generation of Dux KO mice. All animal experiments were performed in accordance with the protocols of the Institutional Animal Care and Use Committee at Harvard Medical School. For superovulation, B6D2F1 (BDF1) female mice (6–8 weeks old) (Jackson Laboratory, 100006) were injected interperitoneally with 7.5 IU of pregnant mare serum gonadotropin (PMSG, Millipore) on day 1 and human chorionic gonadotropin (hCG, Millipore) on day 3 (44–48h after PMSG injection). For in vitro fertilization (IVF), the oocytes collected 12–16h after hCG injection were inseedated with the activated spermatoza collected from the caudal epidymidis of BDF1 males (9–10 weeks old) in human tubal fluid (HTF) medium supplemented with 10 mg ml⁻¹ bovine serum albumin (BSA, Sigma). The spermatoza were capacitated by pre-incubation in HTF medium for 1 h. At 2 h post IVF (hpi), Cas9 mRNA (100 ng μl⁻¹) and sgRNA (50 ng μl⁻¹ each) were injected into cytoplasm of fertilized eggs using a Picozoe impact-driven micromanipulator (Primer Tech). Following injection, zygotes were cultured in HTF medium for another 4 h and then cultured in KSOM (Millipore) at 37 °C under 5% CO₂ with air. At approximately 24 hpi, 2-cell embryos were transferred into oviducts of surrogate ICR strain mothers. The synthesis of Cas9 mRNA and sgRNA was carried out as described previously.

To genotype blastocysts, each embryo collected at 120 hpi was lysed in 8 μl lysis buffer (50 mM Tris-HCl (pH 8.0), 0.5% Triton, 400 μg ml⁻¹ Proteinase K (Sigma)) at 60 °C for 1 h. Following heat inactivation at 90 °C for 5 min, 2 μl of lysis buffer containing genomic DNA was used as template for nested PCR. The primers used for genotyping are included in Supplementary Table 6 (WT allele 268 bp and KO allele ~320 bp). For both rounds of PCR, the following program was used: initial denaturation, 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72°C; final extension, 5 min at 72 °C.

To genotype colonies, a mouse tail tip was lysed in the same lysis buffer (70 μl) at 60 °C overnight and the supernatants were used as template for PCR (only inner primers were used, WT allele 268 bp, KO allele 322 bp and 318 bp for lines 423 and 426, respectively).

RNA-seq. For embryos collected for RNA-seq (that is, Dux F×F), IVF was performed as described above, except that the micro-injection steps were omitted. Late 1-cell and late 2-cell were collected at approximately 12 and 30 hpi, respectively. For each biological replicate, 11–13 embryos were pooled for RNA-seq analyses. Specifically, the embryos were incubated brieﬂy in Acidic Tyrode’s Solution (Millipore) to remove zona pellucida and then washed three times in 0.2% BSA in PBS prior to library construction.

RNA-seq libraries were prepared as described previously. In brief, SMARTer Ultra Low Input RNA cDNA preparation kit (Clontech, 643890) was used for reverse transcription and cDNA amplification (11 cycles). cDNA was then fragmented, adapter-ligated and amplified using a Nextera XT DNA Library Preparation Kit (Illumina) according to the manufacturer’s instructions. Single-end 100-bp sequencing was performed on a HiSeq 2500 sequencer (Illumina). A summary of the generated data sets can be found in Supplementary Table 5.

RNA-seq analyses. RNA-seq reads were first trimmed to remove adaptor sequences and low-quality bases using Trimgalore (version 0.4.5). Reads (>35 bp) were aligned to mm9 reference genome using HISAT2 (version 2.1.0) with default parameters and RPKM values for each gene were computed using Cufflinks (version 2.2.1). For differential gene and repeat expression analyses, TExpranscripts (version 1.5.1) was used to generate read counts for genes (uniquely aligned reads only) and repeats (including both unique- and multi-aligned reads), and DESeq (ref. 23) was used to compute the FDR using the ‘nbinomTest’ function. For the comparative analyses of WT and MZ-KO late 1-cell and 2-cell embryos, only genes with both FC>2 and FDR<0.05 were considered as differentially expressed (Supplementary Tables 1,2). For the determination of whether known DUX targets or major ZGA genes were affected in KO embryos, a more relaxed criterion that only considers fold change (FC>2 and FDR<1) was used (Supplementary Tables 3,4).

Detection of RNA synthesis by EU incorporation. Early (~21 hpi) and late (~29 hpi) 2-cell embryos were incubated in KSOM supplemented with 500 μM EU (Invitrogen) for 1 h prior to fixation in 3.7% paraformaldehyde (Sigma). Following permeabilization in PBS containing 0.5% Triton X-100 (Sigma), embryos were stained using a Click-it RNA Alexa Fluor 488 Imaging Kit (Invitrogen). Fluorescence was detected using a laser scanning confocal microscope (Zeiss LSM800) and the images were acquired using Axiovision software (Carl Zeiss). Signal intensity of nuclei and cytoplasm of two blastomeres were acquired and the cytoplasmic signal was subtracted from the nuclei signal as background. The averaged signal intensity of the WT late 2-cell (~30hpi) was set as 1.0.

RNA isolation, reverse transcription and quantitative PCR. Total RNA was isolated from testis of adult mice (9–12 weeks old) using Trizol Reagent (Invitrogen) according to the manufacturer’s instructions. Following RQ1 DNase (Promega) treatment, RNA was used as template to synthesize cDNA with the use of SuperScriptIII First-Strand Synthesis System (Invitrogen). To ensure no genomic DNA contamination, a raccus reverse-transcriptase control were also included. A SYBR green gene expression assay (Invitrogen) was used to determine Dux transcript abundance in a Via7 Real-Time PCR System (ThermoFisher Scientific). The threshold cycles were normalized to the housekeeping gene Gapdh and the relative abundance in each sample was calculated using the comparative Ct method. The primers used are included in Supplementary Table 6.

Statistical analyses and data visualization. All statistical analyses were performed with R (http://www-r-project.org/). Pearson’s r co-efficient was computed using the ‘cor’ function. Figure 3c was generated using the R function ‘heatmap.2’.

Smoothed scatter plots (Supplementary Fig. 4) were generated with the R function ‘smoothScatter’ and all other plots were generated using the ggplot2 package. The RNA-seq and ChIP-seq bigwig tracks were generated with uniquely aligned reads using deeptools (version 3.0.2) with the following parameters ‘–skipNonCoveredRegions –binSize 10 —scaleFactor 1/DESeq sizeFactor’. The bigwig tracks were visualized in the Integrative Genomic Viewer (http://www.integrativegenomicsviewer.org) browser.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All RNA-seq data sets that were generated in this study have been deposited in the Gene Expression Omnibus under accession number GSE121746. Oocyte and 1-cell RNA-seq data were obtained from a previous publication. HA-DUX ChIP-seq data and Dux overexpression RNA-seq data in mouse ES cells were downloaded from a previous report.

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Software and code

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Data collection

RNA-sequencing reads were generated at Illumina HiSeq 2500 platform (illumina). Signal intensity of EU-staining were acquired using Axiovision software (Carl Zeiss). Quantitative PCR was performed in a ViiA 7 Real-Time PCR System (ThermoFisher Scientific).

Data analysis

RNA-seq analyses:

RNA-seq reads were first trimmed to remove adaptor sequences and low-quality bases using Trimgalore (version 0.4.5). Reads (>35bp) were aligned to mm9 reference genome using HiSAT2 (version 2.1.0) with default parameters and RPKM values for each gene were computed using Cufflinks (version 2.2.1). For differential gene/repeats expression analyses, TETranscripts (version 1.5.1) was used to generate read counts for genes (uniquely aligned reads only) and repeats (including both unique- and multi-aligned reads) and DESeq package was used to compute false discovery rate using the ‘nbinomTest’ function. Pearson’s \( r \) co-efficient was computed using ‘cor’ function. Heatmap was generated using the R function ‘heatmap.2’. Smoothed scatter plots were generated with the R function ‘smoothScatter’ and all other plots such as dot plots, bar plots, and box plots were generated using ggplot2 package. The RNA-seq bigwig tracks were generated with uniquely aligned reads using deeptools (version 3.0.2). The bigwig tracks were visualized in the Integrative Genomic Viewer genome (IGV) browser.

Other analyses:

Chi-square good of fitness test was performed for assessing whether mice with different genotypes fit the expected Mendelian ratio. Student t-test (two-sided) was used to assess whether the litter sizes, oocytes number, 1-cell embryos number, and EU signal intensity are significantly different between groups.
Fisher’s exact test (two-sided) was used to compare the blastocyst stage embryos between groups.

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Sample size
Sample sizes were determined without statistical measures, but based on widely accepted sample sizes in relevant publications within this field of research. See Figures legends for each experiment.

Data exclusions
No data were excluded for analyses.

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All attempts of replication were successful. RNA-seq experiments included two replicates for 1-cell embryos and three replicates for 2-cell embryos. The reproducibility between replicates were assessed by Pearson correlation.

Randomization
Selection of mice for collection of embryos and RNA-seq analyses within either WT or KO group was random.

Blinding
Blinding is not practicable in our study as mice need to be assigned to WT or KO groups based on their genotypes.

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Laboratory animals
Mouse, B6/DBA background, 6-10 weeks, females/males

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