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Exploring trophoblast-specific Tead4 enhancers through chromatin conformation capture assays followed by functional screening

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ABSTRACT

***Tead4* is critical for blastocyst development and trophoblast differentiation. We assayed long-range chromosomal interactions on the *Tead4* promoter in mouse embryonic stem (ES) cells and trophoblast stem (TS) cells. Using luciferase reporter assays with ES and TS cells for 34 candidate enhancer regions, we identified five genomic fragments that increased *Tead4* promoter activity in a TS-specific manner. The five loci consisted of three intra- and two inter-chromosomal loci relative to *Tead4* on chromosome 6. We established five mouse lines with one of the five enhancer elements deleted and evaluated the effect of each deletion on *Tead4* expression in blastocysts. By quantitative RT-PCR, we measured a 42% decrease in *Tead4* expression in the blastocysts with a homozygous deletion with a 1.5 kb genomic interval on chromosome 19 ($n = 14$) than in wild-type blastocysts. By conducting RNA-seq analysis, we confirmed the *trans* effect of this enhancer deletion on *Tead4* without significant *cis* effects on its neighbor genes at least within a 1.7 Mb distance. Our results demonstrated that the genomic interval on chromosome 19 is required for the appropriate level of *Tead4* expression in blastocysts and suggested that an inter-chromosomal enhancer-promoter interaction may be the underlying mechanism.**

INTRODUCTION

Mammalian genomes are characterized by higher-order chromatin organization orchestrating spatial and temporal gene regulation. Genomic distribution of *cis*-regulatory elements can be predicted by histone modification and transcription factor (TF) binding patterns. Active enhancer elements are predominantly marked with H3K4me1 and H3K27ac modifications and p300 histone acetyltransferase binding (1,2). Although it is widely recognized that not only proximal but also distal enhancers play important roles in gene expression regulation (3,4), our knowledge of long-range enhancer-promoter interactions is still limited. Chromosomal DNA looping is one of the major mechanisms, through which a distal enhancer physically interacts with a target gene promoter. Such long-range regulatory interactions are emerging as important determinants of tissue-specific expression (5–7) and of regulatory variations (4,8,9). Chromosome conformation capture (3C) and 3C-related technologies are powerful methods for studying three-dimensional nuclear organization (10–14). These methods generate chimeric DNA products by the ligation of two restriction enzyme-digested fragments that are located one-dimensionally far away (i.e. >100 kb) from each other on the same chromosome or on different chromosomes but in spatial proximity due to DNA looping.

Tead4 encodes a member of TEAD family transcription factors, which contain the TEA domain as an evolutionarily conserved DNA binding domain (15). Mouse embryos deficient in *Tead4* did not express genes characteristic of trophectoderm (TE) such as *Cdx2* and *Gata3*, failed to form blastocoels and died during early development (16,17). These findings suggest that *Tead4* is critical for first cell lineage specification, the segregation of TE and the inner cell mass (ICM), in mammalian embryogenesis. Although *Tead4* is expressed in all blastomeres during early development, *Tead4* knockout embryos showed defects only in the TE but not in the ICM lineage (17). Although differ-

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ences in the subcellular localization of TEAD4 in TE and ICM are thought to be involved in cell lineage commitment (18,19), little is known about the higher order chromatin architectures involved in the transcriptional regulation of *Tead4* during early development.

In the present study, as a means of identifying potential regulatory elements associated with the *Tead4* promoter, we applied circular chromosome conformation capture followed by the high-throughput sequencing (4C-seq) (20) to the *Tead4* gene promoter. We successfully detected long-range chromatin interactions involving the *Tead4* promoter in trophoblast stem (TS) cells and embryonic stem (ES) cells, which represent the TE and ICM models, respectively. Consistent with the critical role of *Tead4* in the TE lineage, in TS cells, we observed frequent overlaps between the *Tead4* promoter interactomes, the genomic regions interacting with the *Tead4* promoter, and the open chromatin regions identified by the formaldehyde-assisted isolation of regulatory elements combined with high-throughput sequencing (FAIRE-seq) (21). Through functional screening, we identified five genomic regions that enhanced *Tead4* promoter activity in a TS-specific manner *in vitro* and characterized one of them as an inter-chromosomally located enhancer *in vivo*. The *Tead4* promoter interactomes possibly play an important role in defining the spatiotemporal patterns and the quantitative levels of *Tead4* gene expression during development.

MATERIALS AND METHODS

Detailed descriptions of all procedures, including 4C-seq, FAIRE-seq, ChIP-seq, reporter assays, RNA-seq and generation of deletion mutant mouse lines can be found in the SI Appendix (**Supplementary Materials and Methods**). The accession numbers of the ChIP-seq data retrieved from the NCBI Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) are GSM918749 (ES_Pol II), GSM769009 (ES_H3K4me1), GSM1000099 (ES_H3K27ac) and GSM967644 (TS_Pol II).

RESULTS

Identification of *Tead4* promoter interactomes

We used TS and ES cell lines established from C57BL/6 male embryos. *Tead4* expression was higher (6.2-fold) in TS cells than in ES cells (Figure 1A). Consistently, in the luciferase reporter assays for the *Tead4* promoter, pGL4prom containing a 1411 bp fragment from the *Tead4* promoter region showed a significant increase in luciferase activity compared to the empty vector (pGL4) in TS cells but not in ES cells (Figure 1B). We performed 4C-seq analyses to delineate long-range interactions involving the *Tead4* promoter in undifferentiated ES and TS cells (Figure 1C, B6ES_4C_T1_Rep1 and B6TS_4C_T1_Rep1 in Supplementary Table S1). Using in-house data analysis pipelines similar to those published previously (20), we identified 105 and 64 putative distal regions interacting with the *Tead4* promoter (the HindIII fragment H641132 in Supplementary Tables S2a and S3a) intra- or inter-chromosomally in ES (Figure 1D) and TS (Figure 1E) cells, respectively, and defined these long-range interactions as the *Tead4* promoter interactomes (Figure 1F, Supplementary Tables S2a and S3a). We validated a subset of long-range interactions by 3C-qPCR assays using 3C libraries that were prepared independently from the same batch of fixed chromatin samples used for the 4C-seq analysis. We subjected seven intra-chromosomally interacting regions to the 3C-qPCR analysis (**a** to **g** in Figure 2A) using the primer *Tead4*-bait_H641132_F (shown by the grey arrow in Figure 1C) as the common forward primer and a specific primer for each locus (Supplementary Table S4) as a reverse primer to amplify ligation junctions. We observed similar interaction patterns in the 4C-seq data and 3C-qPCR assays for five (**a**, **b**, **e**, **f** and **g**) out of seven regions examined but failed to detect interactions in the two regions (**c** and **d**) (Figure 2A).

We subsequently examined genomic features of the *Tead4* promoter interactomes (B6ES_4C_T1_Rep1 and B6TS_4C_T1_Rep1 in Supplementary Table S1, Figure 2B). It was previously shown in a capture Hi-C study that intra-chromosomal regions interacting with active promoters tend to be located more distantly than those with inactive promoters (22) and that regions interacting with active promoters are enriched with intragenic regions whereas regions interacting with inactive promoters are not (22). Consistent with the high and low expression levels of *Tead4* in TS and ES cells, the *Tead4* promoter was found to interact with intragenic regions more frequently in TS cells (29/64, 43.0%) than in ES cells (31/105, 29.5%) (Figure 2B). Likewise, whereas the majority (99.8% in read numbers) of the intra-chromosomal interactions with the *Tead4* promoter were identified within an interval of 115 kb (from H641101 to H641133 in Supplementary Table S2a) in ES cells, the intra-chromosomal interactions were spread over a 3 Mb interval (from H638133 to H647670 in Supplementary Table S3a) in TS cells.

We examined repeat sequence contents among the 67 ES and 28 TS regions categorized to "intergenic". When the frequencies of the "intergenic" regions containing one or more repeat copies were compared for SINE, LINE, LTR, simple repeats, and others, the frequency of the presence of the LTR sequences was most strikingly higher in "intergenic" regions in ES cells than in TS cells (Figure 2C). We also counted the base-pair ratios of each of these repeat sequence subclasses in the "intergenic" regions as well as in the entire interactomes and found that the ratios of LTRs and LINEs were higher, whereas the ratio of SINEs was lower in the ES interactome than in the TS interactome (Supplementary Table S5a and S5b). The ratios of repeats in the ES and the TS interactomes (and the ratio in the entire genome) were 17.1% versus 11.1% (19.7%) for LINEs, 12.7% versus 6.5% (10.6%) for LTRs, and 7.9% versus 11.5% (7.6%) for SINEs. LINEs/LTRs and SINEs were found to be enriched in heterochromatic and euchromatic regions, respectively (23). Therefore, our results indicated that the *Tead4* promoter was surrounded by active and inactive chromatin environments in TS and ES cells, respectively, consistent with the expression patterns of *Tead4* in these cells (Figure 1A).

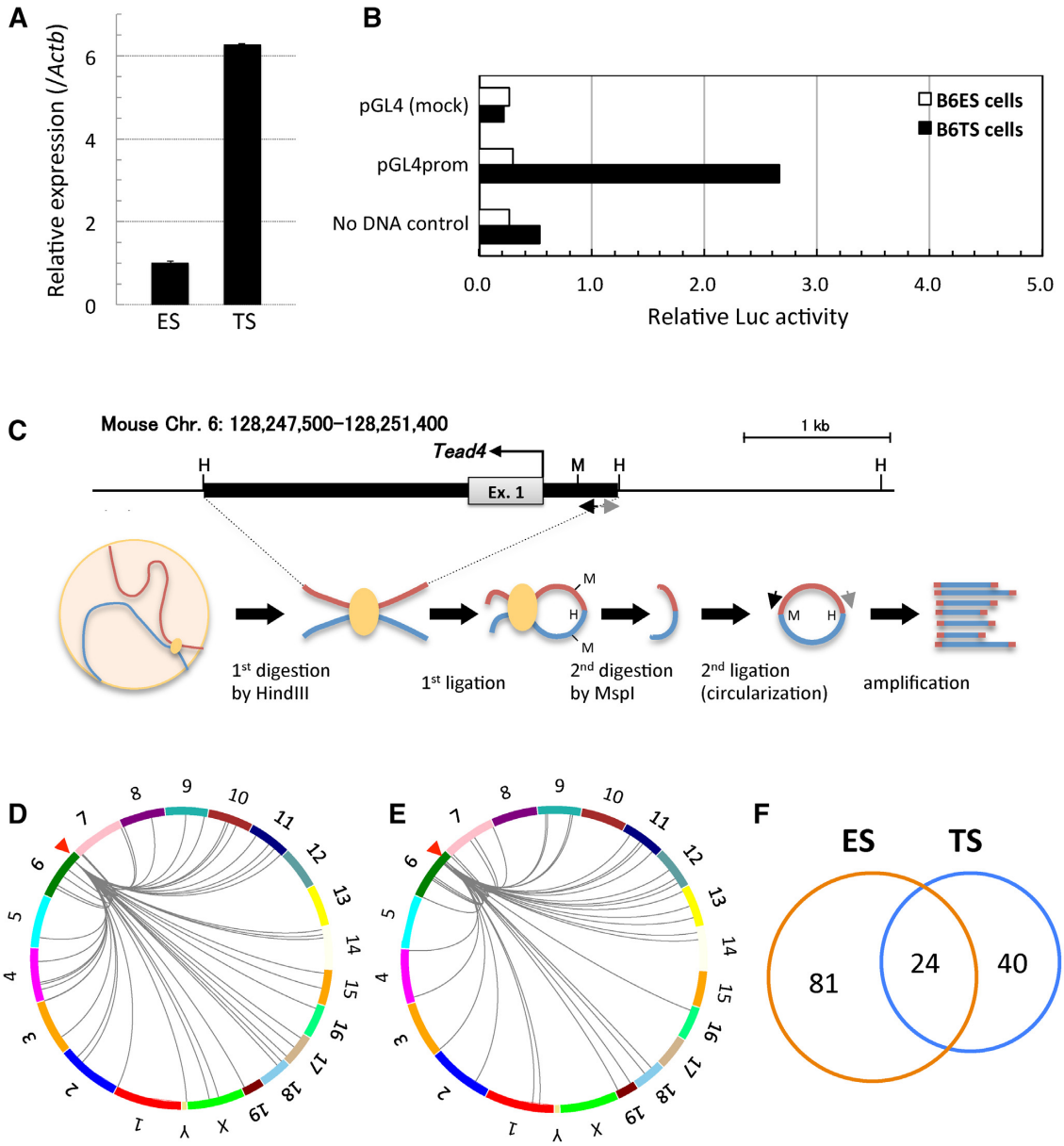

Figure 1. Identification of intra- and inter-chromosomal interactions with the *Tead4* promoter by 4C-seq (**A**) Quantitative RT-PCR analysis of *Tead4* expression in undifferentiated ES and TS cells. (**B**) Luciferase reporter assays for *Tead4* promoter activity. pGL4 (empty vector) or pGL4prom carrying a 1411 bp fragment (chr6:128249989–128251399 [mm9]) from the *Tead4* promoter region was transfected into ES and TS cells. Luciferase activity of pGL4prom relative to that of GL4 is shown. (**C**) The genomic region, chr6:128247500–128251400 (mm9), containing the transcription start site of *Tead4* (NM_011567) is schematically shown. H and M indicate the position of the HindIII and MspI sites within the region. The HindIII fragment containing *Tead4* exon 1 is shown in bold. Arrows in the MspI-HindIII fragment indicate the positions of PCR primers used for 4C-seq library amplification (grey and black arrows correspond to the primers Tead4_4C-Hind3F(+) and Tead4_4C-Msp1R(–), respectively). Procedures for 4C-seq library preparation are shown underneath. HindIII and MspI were used in the first and second restriction enzyme digestion procedures, respectively. (**D** and **E**) Circular plots showing interactions between the *Tead4* promoter locus (bait) on chromosome 6 and interacting regions identified in ES (D) and TS (E) cells. Chromosomes are shown in a circular orientation. (**F**) Venn diagram showing the numbers of overlapping and cell type-specific interactions.

Genome-wide profiling of chromatin states of ES and TS cells

Although 4C-seq analysis identifies genomic regions spatially located near the *Tead4* promoter, it alone cannot pinpoint the precise location of the regulatory elements responsible for long-range interactions. Whereas the average sizes of the HindIII fragments identified as ES and TS interactomes were 5.0 kb and 6.2 kb, respectively, distal enhancer regions within them could be much smaller in size. We collected chromatin state profiles of ES and TS cells as information resources to narrow down the location of regulatory elements within the *Tead4* promoter interactomes. The datasets (Supplementary Table S1) which we obtained in this study or retrieved from the NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/) included open chromatin profiles by FAIRE-seq (Supplementary Figure S1), histone modification profiles for H3K4me1 and H3K27ac

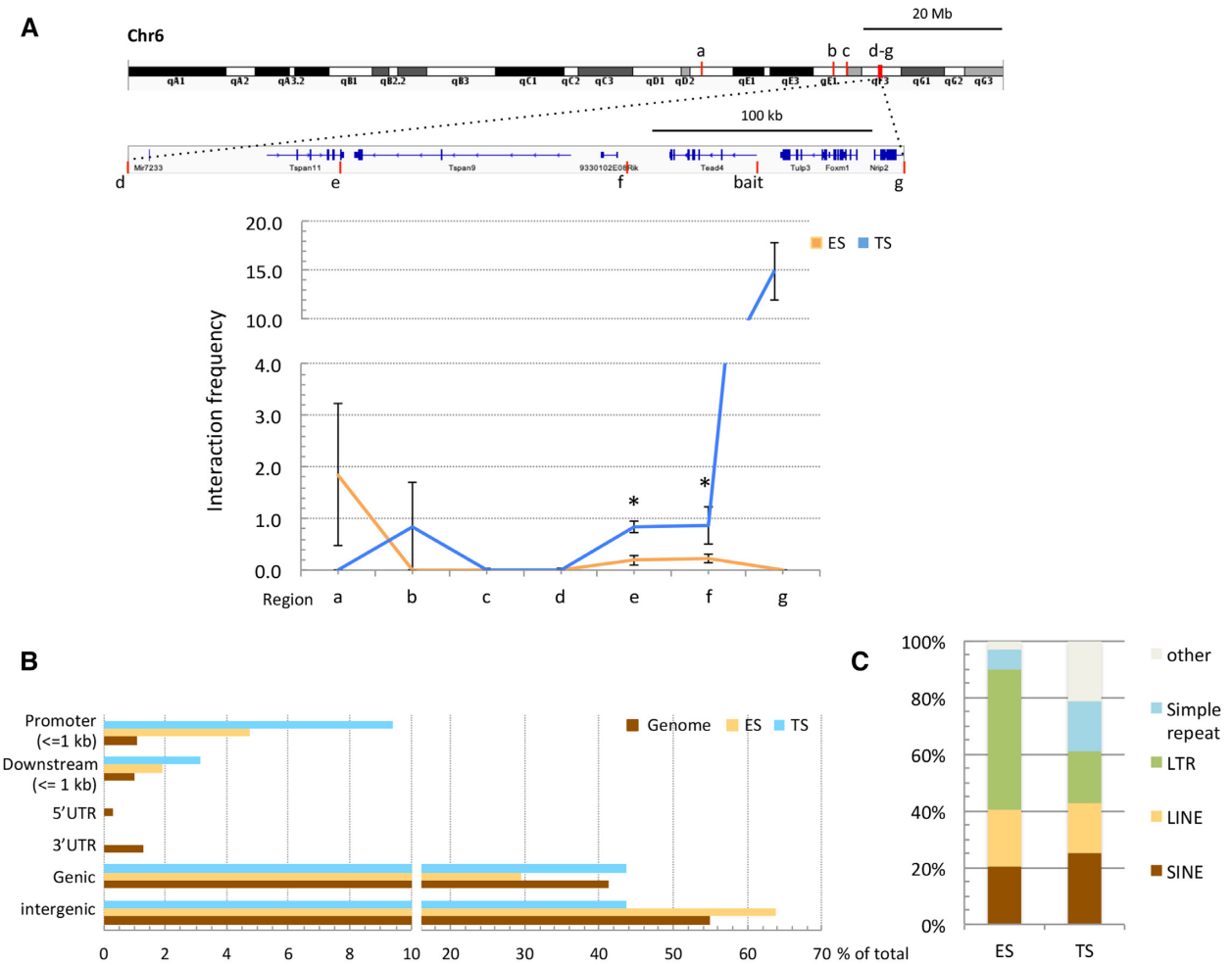

Figure 2. Comparison of the *Tead4* promoter interactomes in ES and TS cells (**A**) 3C-qPCR validation of seven intra-chromosomal interactions. (Top) The entire chromosome 6 and an enlarged view of the target regions **d** to **g**. The locations of the bait region and the validation targets (**a** to **g**) are shown by red, vertical bars. (Bottom) Normalized, relative interaction frequencies between candidate distal regulatory elements (**a** to **g**) and the *Tead4* promoter in ES and TS cells (shown in orange and blue, respectively). Interaction frequencies were calculated using the standard curve data obtained for each target using a serial dilution of the junction amplicon (1 to 10^5 copies equivalent) as template DNA and normalized by the quantitative values of the *Gapdh* locus. Vertical bars represent the mean ± standard deviation (SD) values determined by triplicate measurements (* Mann–Whitney U test *P*-value < 0.05). (**B**) Enrichment analysis for the *Tead4* promoter interactomes in six genomic features. Each of the HindIII fragments constituting the interactomes was annotated for its feature relative to genes using the "RefSeq Genes" track data in the UCSC genome browser (mm9). (**C**) Percentage representation of the presence of repeat sequence subclasses within the intergenic regions interacting with the *Tead4* promoter.

(enhancer-associated histone marks) (1,2,24), and binding profiles for RNA polymerase II (Pol II), TEAD4 and KLF5 by ChIP sequencing (ChIP-seq). TEAD4 and KLF5 are known as master regulators of trophoblast lineage specification (16,25). These profiles in ES and TS cells are given for six gene loci as examples (Figure 3A).

We identified 6410 and 293 TEAD4 binding sites, and 175 and 119 KLF5 binding sites in TS and ES cells, respectively (B6TS_TEAD4_Rep1, B6ES_TEAD4, B6TS_KLF5, and B6ES_KLF5 in Supplementary Table S1, Supplementary Figure S2). The majority of the 6383 TS-specific TEAD4 binding sites were associated with enhancer marks (H3K4me1-positive, 96.2%; H3K4me1/H3K27ac-positive, 67.8%) (Supplementary Figure S2). We also identified 12100 and 33817 FAIRE peaks in ES and TS cells, of which 7613 peaks were common to both cell types (Figure 3B, B6ES_FAIRE_Rep1 and B6TS_FAIRE_Rep1 in Supplementary Table S1), and compared the functional features of common and cell-type specific peaks by assessing the extent of enrichment of Pol II (an active promoter signature), H3K27ac (an active promoter/enhancer signature), and H3K4me1 (a enhancer signature) in these FAIRE peak sub-groups. When 1 kb intervals from transcription start sites (TSSs) on RefSeq genes to 1 kb upstream were regarded as promoter regions, the percentage of the promoter-type FAIRE peaks was 33.7% and 20.0% in ES and TS cells, respectively (Figure 3C), which was similar to previously reported findings for other cell types (26). We confirmed that the promoter-type FAIRE peaks were enriched with Pol ll and H3K27ac in both cell-types, indicating a high proportion of active promoters (Supplementary Figure S3). The 7613 common FAIRE peaks were also enriched with Pol II and H3K27ac peaks in both cell types (Figure 3D), suggesting a high proportion of active promoters among the com-

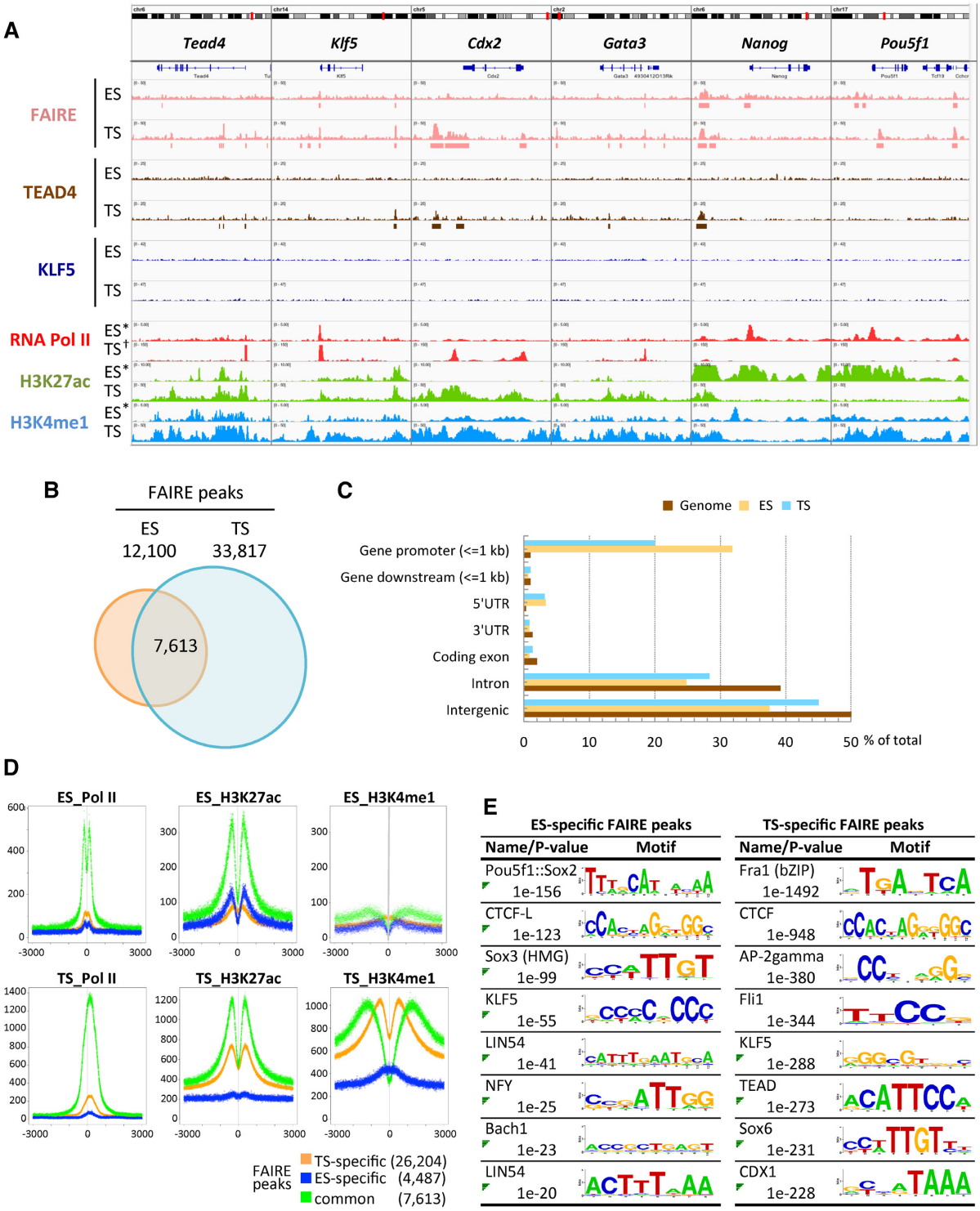

Figure 3. Genome-wide profiling of open chromatin regions by FAIRE-seq in ES and TS cells (**A**) Epigenetic and TF-binding profiles of *Tead4, Klf5, Cdx2, Gata3, Nanog,* and *Pou5f1* loci in ES and TS cells. Data marked with asterisk (*) and dagger (†) were obtained from the ENCODE Project Consortium (www.encodeproject.org) and GEO GSM967644 (48), respectively. *Tead4, Klf5, Cdx2,* and *Gata3* loci represent examples of genes expressed in TS cells. *Cdx2* and *Gata3* are known to be regulated by TEAD4 and to control trophoblast development (42,43). *Nanog* and *Pou5f1* (*Oct4*), involved in ICM development, are shown as examples of genes expressed in ES cells. FAIRE peaks at the promoters of actively transcribed genes coincided with the Pol II and H3K27ac peaks. (**B**) Venn diagram showing the numbers of total and overlapping FAIRE peaks in ES and TS cells. (**C**) Enrichment analysis for the FAIRE peaks in seven genomic features using CEAS (49). (**D**) The extent of enrichment of Pol II (left), H3K27ac (middle), and H3K4me1 (right) among TS-specific (orange), ES-specific (blue), and common (green) FAIRE peaks in ES (upper panels) and TS (lower panels) cells. In each panel, the x-axis (ranging −3000 bp to 3000 bp) represents the relative distance from the center of the FAIRE peaks (TS-specific, ES-specific, or common peak groups), and the y-axis represents the total mapped read counts from ChIP-seq reads (Pol II, H3K27ac, or H3K4me1) at each relative base position. (**E**) Motif enrichment analysis for ES- or TS-specific FAIRE peaks using HOMER (50).

mon FAIRE peaks. On the other hand, ES- and TS-specific FAIRE peaks were devoid of Pol II peaks and were enriched with H3K27ac in a cell-type specific manner (Figure 3D), suggesting a high proportion of cell-type specific enhancers among these FAIRE peaks. The remarkable enrichment of H3K4me1 in the TS-specific FAIRE peaks observed in TS cells (Figure 3D) also suggested the presence of TS-specific enhancers among the TS-specific FAIRE peaks.

Motif enrichment analysis by HOMER revealed that cell type-specific FAIRE peaks were enriched with binding motifs of TFs critical for cell lineage specification and development, such as the Pou5f1::Sox2 motif among ES-specific peaks and the KLF5 and TEAD motifs among TS-specific peaks (Figure 3E). Binding motifs of the insulator DNA-binding proteins, CTCF and CTCF-L (BORIS) (27,28), were also enriched among the TS- and ES-specific FAIRE peaks. Taken together, these results demonstrated that the open chromatin regions identified by FAIRE-seq frequently contained regulatory elements such as promoters, enhancers, and insulators. Among 64 and 105 regions interacting with the *Tead4* promoter in TS and ES cells, we identified ten (15.6%) and three (2.8%) regions, respectively, that contained one or more FAIRE peaks within the region (the HindIII-digested interval) or its neighboring intervals (Figure 4A). The total number of FAIRE peaks included in these ten and three regions was 22 and three peaks, respectively, in TS and ES cells.

Identification of the loci possessing *Tead4* enhancer activity

We selected 34 regions (1.1–2.2 kb in size) as candidate regulatory elements for the *Tead4* promoter (Supplementary Table S6) among the regions with enhancer signatures in the vicinity of the *Tead4* promoter and among the *Tead4* interactomes identified by 4C-seq as described in the SI Appendix (**Supplementary Materials and Methods**). The selected regions consisted of 29 intra-chromosomal and five inter-chromosomal candidates, including 26 candidates with enhancer signatures in either or both ES and TS cells and eight candidates without any enhancer signatures. As a first screening for their enhancer (or silencer) activities, we carried out luciferase reporter assays. Each DNA fragment was cloned into the plasmid PGL4prom harboring the *Tead4* promoter and was assessed for its enhancer/silencer activity in undifferentiated ES and TS cells (Figure 4B). Among 18 DNA fragments for which luciferase assays were conducted at least twice for both ES and TS cells, DNA fragments A, D, E1, L2 and M reproducibly increased luciferase activities in TS cells but not in ES cells (Figure 4B). Regions A, E1 and D are located intra-chromosomally relative to the *Tead4* promoter at a position 350 kb downstream, 15 kb downstream, and −124 kb upstream, respectively. Regions L2 and M are located inter-chromosomally relative to the *Tead4* promoter (L2 on chromosome 11, and M on chromosome 19) (Supplementary Table S6). The inter-chromosomally interacting regions L2 and M showed higher luciferase activity than intra-chromosomally interacting regions (Figure 4B). Interaction with the *Tead4* promoter was detected specifically or predominantly in TS cells for Regions A, D, and M, predominantly in ES cells for Region E1, and commonly in both cell types for Region L2. ChIP-seq data for TEAD4 indicated that Regions E1 and M contained a TEAD4 binding site bound by the protein only in TS cells but not in ES cells (Supplementary Figure S4).

Region M on chromosome 19 is required for the proper expression level of *Tead4* at the blastocyst stage

Tead4 is first expressed at the two- to four-cell stage, and embryos with *Tead4* homozygously deleted reportedly stop their development at the morula stage (16,17). To assess the function of the five candidate enhancers for the *Tead4* promoter during preimplantation development, we generated mouse lines with one of the five candidate enhancer regions (A, D, E1, L2 and M) deleted using the CRISPR/cas9 system (Supplementary Figure S4). We observed normal phenotypes in knockout homozygotes of all five lines at the blastocyst state and after birth (data not shown). We quantitatively measured the *Tead4* expression levels in the blastocysts by real-time PCR and consistently found that it was significantly decreased in the line lacking Region M in two independent experiments (Figure 4C). Region M, a 1479 bp interval corresponding to chr19:10635700−10637178, is intergenically located between the *Tmem216* and *Tmem138* genes on chromosome 19, enriched with active enhancer marks (H3K4me1 and H3K27ac), and bound by TEAD4 only in TS cells but not in ES cells (Figure 4D).

To examine the effect of the 2042 bp deletion at chr19:10635587–10637628 (mm9) encompassing Region M (Supplementary Figure S4, Supplementary Table S6) in a transcriptome-wide manner, we conducted RNA-sequencing (RNA-seq) analysis for blastocysts with Region M heterozygously or homozygously deleted and for wild-type controls. We obtained 27 blastocysts by mating mice heterozygous for Region M deletion, genotyped them by PCR, and obtained nine homozygotes of the wild-type allele (wt), 14 heterozygotes (ht) and four homozygotes of the deletion allele (hm). We subjected four blastocysts from each of the three genotypes (twelve in total) to RNA-seq analysis. In line with the qRT-PCR results (Figure 4C), *Tead4* expression was reduced significantly in both heterozygotes and homozygotes (ANOVA P-values 0.031 and 0.0036, respectively) than in wild type blastocysts (Figure 5A). We also confirmed the rank correlation of *Tead4* expression in wt, ht, and hm samples by Jonckheere's trend test ($P = 0.0022$). Among the 11952 genes that were found to be expressed (FPKM value > 1) in at least one of the twelve blastocyst samples, we selected 471 differentially expressed genes (DEGs) after Region M deletion in the blastocysts which fulfilled two criteria: minimum $P < 0.05$ in the ANOVA and $P < 0.05$ in Jonckheere's trend test for the FPKM values of the three groups (wt, ht and hm) (Supplementary Table S7). When ranked by the statistical significance of differential expression and its correlation with the copy number of Region M using ANOVA and the trend test P-values, *Tead4* ranked at 28th and 29th, respectively, and 14th in the additive mean of the ranks, among the 471 genes (Supplementary Table S7). Importantly, none of the genes located near Region M (such as its neighboring genes *Tmem263* and *Tmem138*) were found to be differentially expressed. Among the 16 genes on chromosome 19 in the

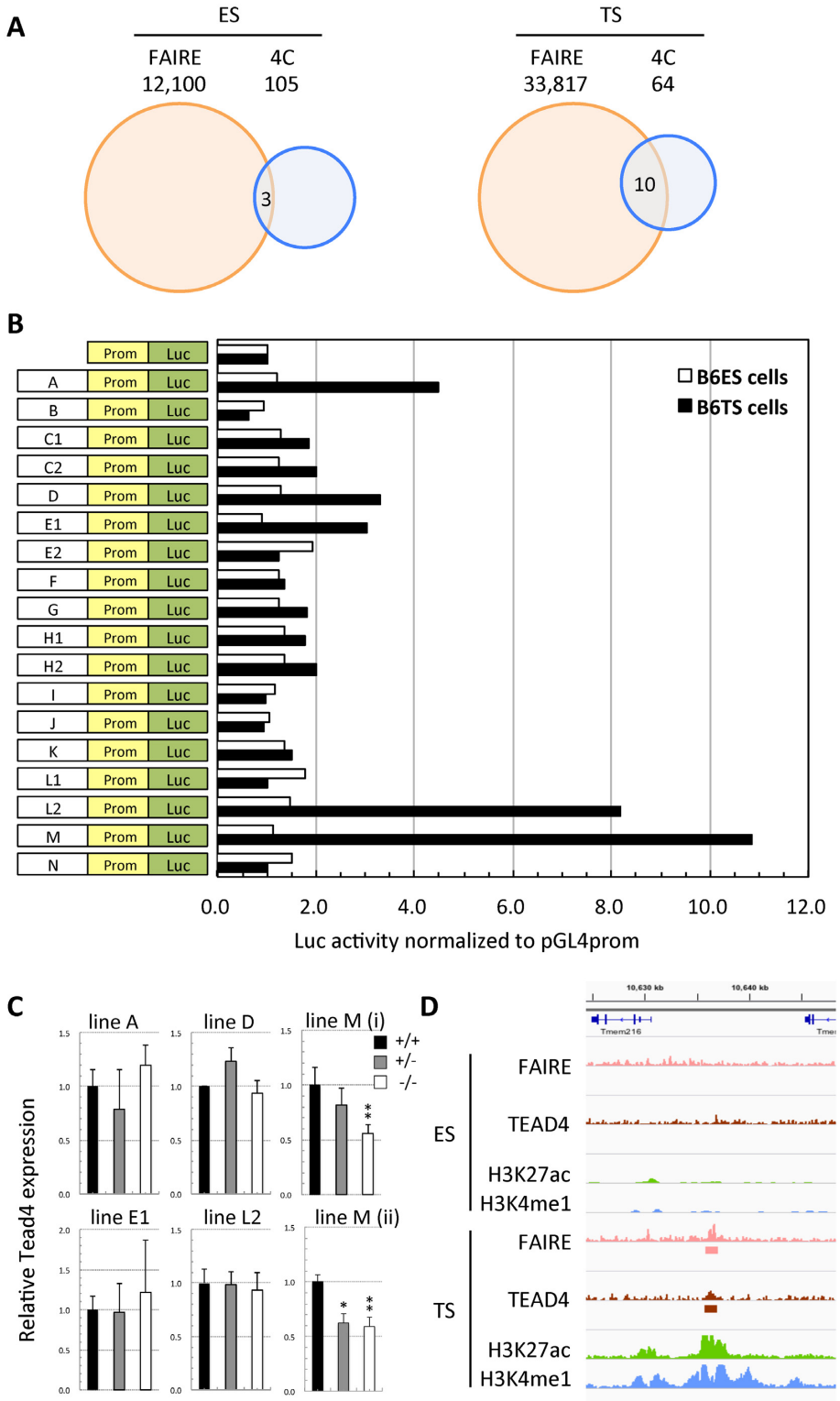

Figure 4. Identification of enhancers for the *Tead4* gene promoter through *in vitro* and *in vivo* assays (**A**) Venn diagrams showing the numbers of overlapping regions between the 4C interactomes and the FAIRE peaks in ES and TS cells. (**B**) Luciferase reporter assays assessing the enhancer activity of the genomic intervals containing a candidate *cis*/*trans*-regulatory element in ES and TS cells. The candidate DNA fragments were amplified by PCR and cloned into the upstream region of the *Tead4* promoter in the pGL4prom reporter vector. Open and closed bars represent luciferase activity in ES and TS cells relative to those of the control pGL4prom (promoter-luc only) vector. Data from one of the triplicate or quadruplicate experiments are shown for 18 candidate regions. (**C**) Quantitative RT-PCR analysis of *Tead4* expression at blastocyst stage embryos. * and ** indicate statistical significance at $P < 0.05$ and $P < 0.01$, respectively. The numbers of the blastocysts analyzed for three genotypes (+/+, +/−, −/−) were: 2, 6, 7 for line A; 2, 3, 4 for line D; 6, 3, 3 for line E1; 4, 14, 10 for line L2 (from two litters); 6, 7, 7 for line M (i); and 5, 4, 7 for line M (ii). (**D**) FAIRE-seq and ChIP-seq profiles for Region M in ES and TS cells.

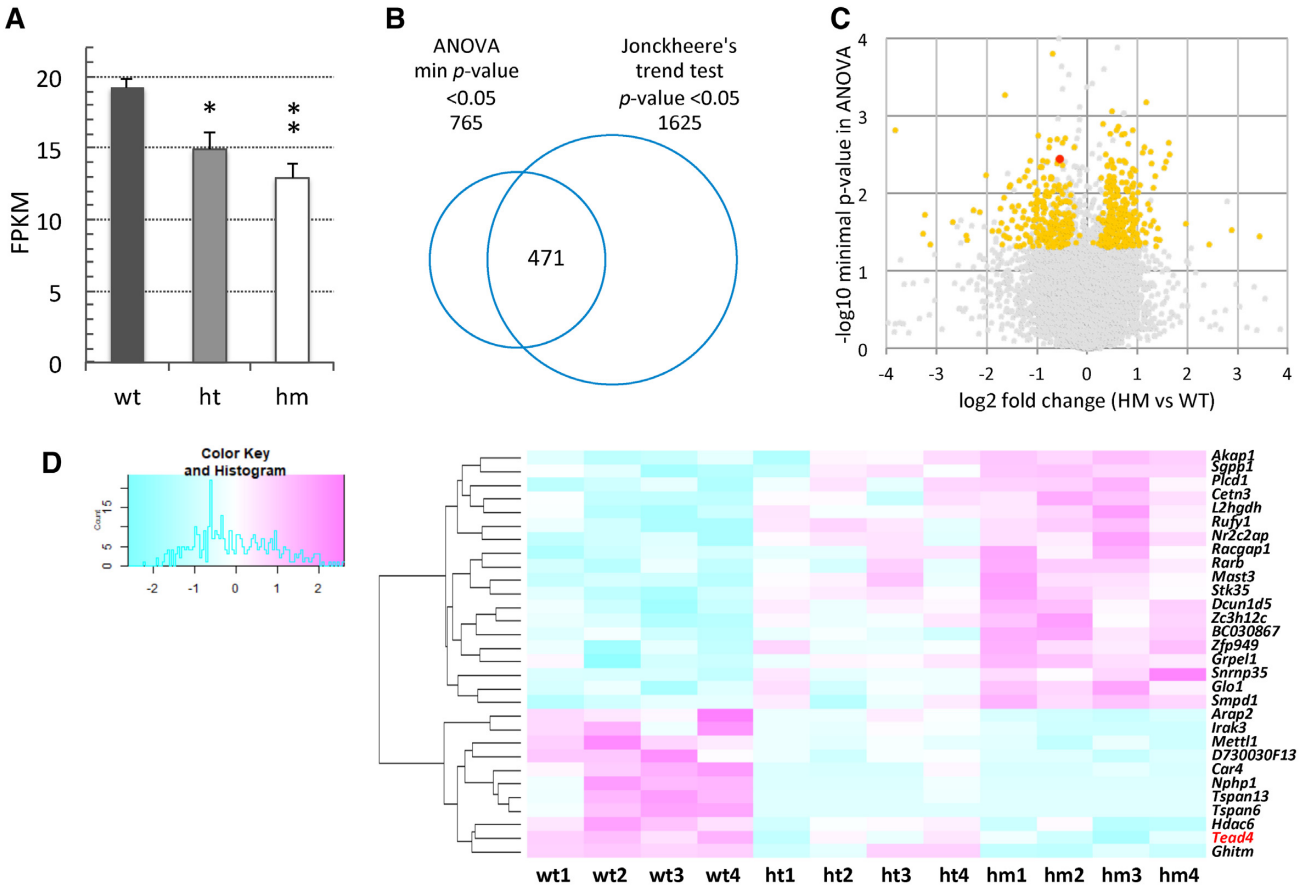

Figure 5. Effects of the targeted deletion of the Region M on the blastocyst transcriptome (**A**) The expression levels of *Tead4* in wild-type (wt) and knockout blastocysts (ht and hm). "ht" and "hm" represent the blastocysts with heterozygously and homozygously deletion of Region M, respectively. The bars show the mean and SD of FPKM values of "wt", "ht", and "hm" groups (n = 4 for each group). * and ** indicate the following respective levels of statistical significance in ANOVA: $P < 0.05$ and $P < 0.01$. (**B**) Venn diagram showing the numbers of genes meeting the statistical significance thresholds of the minimum *P*-value for ANOVA < 0.05 and *P*-value for the Jonckheere's trend test < 0.05. Among the 11952 genes subjected to statistical analyses, 471 genes meeting both thresholds were selected as DEGs. (**C**) Volcano plot showing the log2-fold changes in gene expression levels (FPKM values) in the "hm" group relative to the "wt" group on the x-axis and the $-\log_{10}$ values of the minimum *P*-value in the ANOVA for the three groups on the y-axis. Orange and red dots indicate 471 DEGs, and the red dot indicates *Tead4*. (**D**) Heatmap representation of the expression levels (z-scores) of top 30 genes ranked by the additive mean of two ranks, the ANOVA *P*-value and the Jonckheere's trend test *P*-value (Supplementary Table S7). Only genes but not samples were subjected to hierarchical clustering.

471 DEGs, the gene most closely located to Region M was *Ttc9c,* which is located 1.7 Mb distant. The additive mean of the ranks of these chromosome 19 gene was lower than that of *Tead4* and ranged from 55th to 446th. The deletion of Region M on chromosome 19 affected the gene expression of *Tead4* on chromosome 6 significantly, but not the expression of the neighboring genes at least within a 1.7 Mb distance on chromosome 19. These results demonstrated that Region M is required for the proper expression level of *Tead4* at the blastocyst stage and suggested the possibility that it interacts inter-chromosomally with the *Tead4* promoter as an enhancer.

Evaluation of the first ChIP-seq and 4C-seq datasets by obtaining their replicate datasets

In this study, we used only one of the two FAIRE-seq datasets ("Rep1" in Supplementary Table S1) and a single set of ChIP-seq and 4C-seq data ("Rep1" in Supplementary Table S1) to choose 34 candidate regulatory elements for the *Tead4* promoter and subjected them to cellular luciferase assays and in *vivo* deletion assays. To confirm their reproducibility afterwards, we obtained the second ChIP-seq datasets for TEAD4 binding patterns and histone modifications (H3K27ac and H3K4me1) in TS cells ("Rep2" in Supplementary Table S1) and the second 4C-seq datasets for ES and TS cells using the *Tead4* promoter region (H641132) as bait ("Rep2" in Supplementary Tables S1, S2b, S3b, S5c, S5d and S6). We observed high or satisfactory concordances between the first and the second ChIP-seq datasets. The numbers (and the ratios) of the peaks in the first dataset (Rep1) also detected in the second dataset (Rep2) were: TEAD4, 6308 (98.4%); H3K27ac, 24005 (83.4%); and H3Kme1 57791 (65.2%) (Supplementary Figure S5). In contrast, in the comparison of two 4C-seq datasets ("Rep1" and "Rep2" in Supplementary Table

S1), only a few regions were commonly detected: seven of 105 regions (corresponding to 1.59 million reads [29.9%]) in ES cells, and five of 64 regions (0.38 million reads [52.4%]) in TS cells (Supplementary Figure S5). The interaction of Region M with the *Tead4* promoter region in TS cells was detected only in the "Rep1" dataset of the 4C-seq analysis but not in the "Rep2" dataset.

DISCUSSION

Using the 4C-seq technique, we identified the interactomes for the *Tead4* promoter in mouse ES and TS cells. We utilized open chromatin profiles obtained by FAIRE-seq and histone modification profiles of enhancer marks to narrow down candidate regulatory elements among the interactomes. Through the *in vitro* enhancer/silencer assays for the 29 intra-chromosomal and five inter-chromosomal regions relative to the *Tead4* promoter on chromosome 6, we identified five genomic regions as enhancers that increased *Tead4* promoter activity in TS cells but not in ES cells. Intriguingly, whereas only three of the 29 intra-chromosomal regions showed enhancer activity (10.3%), two of five inter-chromosomal regions showed enhancer activity (40%). In addition, the inter-chromosomal enhancers showed higher activity (8.2- and 10.8-fold) than the three intra-chromosomal enhancers (3.0- to 4.5-fold). To assess the functions of these genomic regions *in vivo* directly, we established five mouse lines with one of the five enhancers deleted using CRISPR-Cas9 mediated genome editing, and measured the expression levels of *Tead4* at the blastocyst stage. Interestingly, the blastocysts with Region M heterozygously and homozygously deleted on chromosome 19 showed decreased levels of *Tead4* expression whereas the blastocysts with one of the four other regions deleted did not (Figure 4C). Whereas these results of *in vivo* deletion assays indicated the possibility that Region M functions as an inter-chromosomal enhancer acting on the *Tead4* promoter at the blastocyst stage, an alternative explanation of this result is that Region M regulates a gene (or genes) in closer proximity in the linear genome on chromosome 19 and that the product of this gene acts as a trans-regulator for the *Tead4* promoter region. The RNA-seq data for the blastocysts, in which *Tead4* was ranked 14th among 471 DEGs after deletion of region M (Figure 5, Supplementary Table S7), provided genetic evidence demonstrating the genomic function of Region M as a regulatory element and statistical evidence suggesting the critical role of Region M in the transcriptional regulation of *Tead4* at the blastocyst stage. However, since the data did not provide solid evidence to exclude indirect, alternative mechanisms, there may be multiple, alternate mechanisms, through which Region M affects the *Tead4* expression level. Although located >1.7 Mb away from Region M and ranked lower than *Tead4*, 16 genes on chromosome 19 were included among the 471 DEGs (highlighted in gray in Supplementary Table S7). Long-range *cis* effects of the deletion of Region M on these genes and the resultant changes in the amounts of their gene products may be responsible for the differential expression of *Tead4* and other genes. On the other hand, when Region M is considered as an inter-chromosomal enhancer to the *Tead4* promoter, the expression changes observed in genes other than *Tead4* can be explained either as a direct effect or a consequence of the deletion of Region M. It is plausible that Region M directly acts as an enhancer for multiple gene promoters including *Tead4* in the blastocyst. A lower *Tead4* expression level due to the deletion of Regions M could lead to a decreased TEAD4 protein production, which might then affect the expression levels of multiple genes. It should be also noted that *Hdac6* (encoding a histone deacetylase) showed a similar expression pattern with that of *Tead4* in the blastocysts subjected to the RNA-seq analysis (Figure 5D). The decreased expression levels of *Hdac6* associated with Region M deletion could lead to insufficient deacetylation at the *cis*-regulatory elements in multiple genes and result in elevated expression levels of these genes. Region M showed the highest enhancer activity un relation to the *Tead4* promoter in a TS-specific manner among the enhancer candidates examined by reporter assays (Figure 4B) and was found to contain a TEAD4 binding site (Figure 4D). Collectively, through a combination of chromatin state and interaction analyses followed by functional assays for enhancer candidates, we identified a novel trophoblast lineage-specific, regulatory element on chromosome 19 controlling *Tead4* gene expression at the blastocyst stage possibly through an inter-chromosomal enhancer-promoter interaction.

Although deletion of a single enhancer element could result in the complete loss of the target gene expression associated with developmental abnormality as shown by the case of a limb-specific distant enhancer for *Shh* (29), it could also result in weaker or no effects. For example, deletion of a craniofacial enhancer for *Snail2*, *Msx1*, and *Isl1* resulted in various degrees of gene expression reduction ranging from 0% to 75% depending on the developmental stage and craniofacial sites examined (30). Multiple enhancers are often involved in defining the spatiotemporal patterns and the quantitative levels of one gene, and some of the modular enhancers may be functionally redundant (31). A recent large scale enhancer deletion study, in which 23 mouse deletion lines were created and assessed for their abnormalities in limb development, also demonstrated enhancer redundancy (32). The same study also revealed that mammalian genes are very commonly associated with multiple enhancers that have similar spatiotemporal activity (32). It should be noted that, in these enhancer studies (29–32), only *cis*-acting (intra-chromosomal) enhancers but not *trans*-acting (inter-chromosomal) enhancers were characterized. The functional redundancy of the *cis*-acting enhancers observed in the previous studies (30,32) accords with our own observation that the deletion of the regions intra-chromosomally interacting with the *Tead4* promoter (Regions A, D, and E1) did not decrease the gene expression level of *Tead4* (Figure 4C).

Whereas inter-chromosomal interactions in mammalian genomes have been reported by many studies (33–35), recent Hi-C and capture Hi-C studies have identified intra-chromosomal interactions as the major long-range chromatin interactions and inter-chromosomal interactions as the minor or rare ones (22,36). It was recently shown by a CRISPR-based live cell imaging method (named

4D-CLING) that inter-chromosomal interactions also occur frequently but are more difficult to detect by Hi-C type methods (37). This 4D-CLING study revealed that inter-chromosomal interactions are as frequently observed as intra-chromosomal interactions and occur at larger distances. The latter finding may explain why inter-chromosomal interactions are less frequently detected than intra-chromosomal interactions in Hi-C type methods. It was demonstrated that inter-chromosomal interactions predominate over intra-chromosomal ones at the promoter of several developmental genes using 4C-based techniques, which are based on the proximity ligation principle and were originally developed in 2006 as a method of systematically identifying long-range chromosomal interactions at a specific locus without a preconceived idea of the interacting partners (14). Using the *H19* imprinting control region (ICR) on chromosome 7 as a model, 114 unique sequences consisting of 22 intra- and 92 inter-chromosomal sequences were identified as interacting partners of H19 ICR. Subsequent studies using 4C-seq (38,39) or enhanced ChIP-4C (e4C) (40) also identified inter-chromosomal interactions as the major interactions for mouse globin genes (*Hba* and *Hbb*) in erythroid cells (40), for the *Nanog* promoter in the mouse ES, iPS and embryonic fibroblast cells (38), and for *POU5F1* and *SOX2* promoters in human ES cells (39). Our 4C-seq results for the *Tead4* promoter are consistent with these previous studies (38–40) in detecting the presence of inter- and intra-chromosomal interaction partners at multiple loci.

In this study, we regarded our first 4C-seq dataset for ES and TS cells as just one of the data sources for selecting candidate genomic intervals that may act as enhancers for the *Tead4* promoter, and placed more importance on the subsequent functional enhancer assays. This decision was largely based on our consideration that the 4C-seq protocol we adopted involved a potential amplification bias during library preparation, PCR duplicates can not be removed due to having identical sequences at the ends of all amplicons in the libraries. In our *ex-post* evaluation, we observed limited reproducibility between the first and the second 4C-seq datasets (Supplementary Figure S5). The low read-count rate (29.9%) in commonly detected regions in ES cells is potentially due to the *Tead4* promoter not requiring any distal enhancers for its basal level expression in ES cells. Although the read-count rate of commonly detected regions in TS cells was moderate (52.4%), the number of regions commonly detected was no more than five (Supplementary Figure S5). The majority of the 34 genomic intervals selected as candidate regulatory elements for the *Tead4* promoter (Supplementary Table S6), including Region M, were not detected in the second 4C-seq dataset for TS cells. This inconsistency between two 4C-seq datasets for TS cells is likely explained by library preparation conditions using 30 cycles of PCR amplification. Interacting regions with minor frequencies in a 4C-seq dataset (such as Region M with 1.56% [Supplementary Table S6]) may not be detectable in another dataset due to stochasticity and biases in PCR amplification. Experimental batch differences in cell culture, chromatin, and library preparations may have also affected the results. Nonetheless, in the functional enhancer assays, the results for Region M consistently indicated its enhancer feature for the *Tead4* promoter (Figures 3-5).

Recent promoter-centered chromatin interaction analyses such as ChIA-PET with Pol II ChIP and capture Hi-C have revealed genome-wide patterns of enhancer-promoter interactions as well as promoter-promoter interactions (22,36). Some genes with promoter-promoter interactions were found to be actively co-transcribed, and to affect each other (36). Although seven out the 64 regions interacting with the *Tead4* promoter in TS cells contained gene promoter(s) within the HindIII fragments, these genes were not highly expressed in TE cells nor known to play a critical role in trophoblast lineage specification or placental development. *Tead4*$^{-/-}$ embryos formed blastocysts when cultured under conditions alleviating oxidative stress, demonstrating the possibility that *Tead4* is not essential for TE specification (41). This finding led to the hypothesis that *Tead4* is essential for blastocyst formation because of its critical function in establishing energy homeostasis for blastocoel formation and expansion *in vivo* (41). Such multiple roles of TEAD4 may be linked to lack of the co-regulation of the *Tead4* gene with other genes encoding critical regulators for trophoblast development such as *Cdx2* (42) and *Gata3* (42,43) through promoter-promoter interactions in TS cells. Regardless of the absence of the co-regulated promoter feature, our ChIP-seq data for TEAD4 warrant its critical role in trophoblast development. TEAD4 binding sites were enriched with binding motifs of other trophoblast-specific TFs such as TFAP2C and EOMES (Supplementary Figure S2), which are both required for placental development and trophoblast stem cell maintenance (44,45). TEAD4 binding sites were also found in the *Cdx2* and *Gata3* loci. We also detected a TEAD4 site in the *Klf5* locus (Figure 3A) in line with the finding of a previous study (19). Targeted deletion of *Klf5* is known to be defective in blastocoel expansion (25), which is also a characteristic of *Tead4*$^{-/-}$ embryos (16). *Klf5* is possibly another downstream factor of *Tead4* in trophoblast development.

We detected the binding of TEAD4 at the promoter region of the *Tead4* gene itself in TS cells (Figure 3A), in line with a previous ChIP-seq study of TEAD4 (19). Importantly, the inter-chromosomally located enhancer on chromosome 19 (Region M) identified in our study was also bound by TEAD4 in TS cells but not in ES cells (Figure 4D). Interestingly, TEAD4 reportedly forms a domain-swapped homodimer (46). It has been suggested that the domain-swapped homodimer of FOXP3 mediates long-range chromatin interactions (47). Likewise, TEAD4 may form a domain-swapped homodimer and tether the *Tead4* promoter on chromosome 6 and Region M on chromosome 19 in the blastocyst.

DATA AVAILABILITY

The accession numbers of the data obtained in this study have been deposited with GSE109249 (4C-seq), GSE109250 (ChIP-seq), GSE109251 (FAIRE-seq), and GSE134991 (RNA-seq).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Creyghton,M., Cheng,A., Welstead,G., Kooistra,T., Carey,B., Steine,E., Hanna,J., Lodato,M., Frampton,G., Sharp,P. *et al.* (2011) Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 21931–21936.
2. Rada-Iglesias,A., Bajpai,R., Swigut,T., Brugmann,S., Flynn,R. and Wysocka,J. (2011) A unique chromatin signature uncovers early developmental enhancers in humans. *Nature*, **470**, 279–283.
3. Handoko,L., Xu,H., Li,G., Ngan,C., Chew,E., Schnapp,M., Lee,C., Ye,C., Ping,J., Mulawadi,F. *et al.* (2011) CTCF-mediated functional chromatin interactome in pluripotent cells. *Nat. Genet.*, **43**, 630–638.
4. Li,G., Ruan,X., Auerbach,R., Sandhu,K., Zheng,M., Wang,P., Poh,H., Goh,Y., Lim,J., Zhang,J. *et al.* (2012) Extensive promoter-centered chromatin interactions provide a topological basis for transcription regulation. *Cell*, **148**, 84–98.
5. Gheldof,N., Smith,E., Tabuchi,T., Koch,C., Dunham,I., Stamatoyannopoulos,J. and Dekker,J. (2010) Cell-type-specific long-range looping interactions identify distant regulatory elements of the CFTR gene. *Nucleic Acids Res.*, **38**, 4325–4336.
6. Ott,C., Blackledge,N., Kerschner,J., Leir,S., Crawford,G., Cotton,C. and Harris,A. (2009) Intronic enhancers coordinate epithelial-specific looping of the active CFTR locus. *Proc. Natl. Acad. Sci. U.S.A.*, **106**, 19934–19939.
7. Tomikawa,J., Uenoyama,Y., Ozawa,M., Fukanuma,T., Takase,K., Goto,T., Abe,H., Ieda,N., Minabe,S., Deura,C. *et al.* (2012) Epigenetic regulation of Kiss1 gene expression mediating estrogen-positive feedback action in the mouse brain. *Proc. Natl. Acad. Sci. U.S.A.*, **109**, E1294–E1301.
8. Goto,T., Tomikawa,J., Ikegami,K., Minabe,S., Abe,H., Fukanuma,T., Imamura,T., Takase,K., Sanbo,M., Tomita,K. *et al.* (2016) Identification of hypothalamic arcuate nucleus-specific enhancer region of Kiss1 gene in mice. *Mol. Endocrinol.*, **29**, 121–129.
9. Maurano,M., Humbert,R., Rynes,E., Thurman,R., Haugen,E., Wang,H., Reynolds,A., Sandstrom,R., Qu,H., Brody,J. *et al.* (2012) Systematic localization of common disease-associated variation in regulatory DNA. *Science*, **337**, 1190–1195.
10. de Wit,E. and de Laat,W. (2012) A decade of 3C technologies: insights into nuclear organization. *Genes Dev.*, **26**, 11–24.
11. Dekker,J., Rippe,K., Dekker,M. and Kleckner,N. (2002) Capturing chromosome conformation. *Science*, **295**, 1306–1311.
12. Lieberman-Aiden,E., van Berkum,N., Williams,L., Imakaev,M., Ragoczy,T., Telling,A., Amit,I., Lajoie,B., Sabo,P., Dorschner,M. *et al.* (2009) Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science*, **326**, 289–293.
13. Simonis,M., Klous,P., Splinter,E., Moshkin,Y., Willemsen,R., de Wit,E., van Steensel,B. and de Laat,W. (2006) Nuclear organization of active and inactive chromatin domains uncovered by chromosome conformation capture-on-chip (4C). *Nat. Genet.*, **38**, 1348–1354.
14. Zhao,Z., Tavoosidana,G., Sjölinder,M., Göndör,A., Mariano,P., Wang,S., Kanduri,C., Lezcano,M., Sandhu,K., Singh,U. *et al.* (2006) Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. *Nat. Genet.*, **38**, 1341–1347.
15. Anbanandam,A., Albarado,D., Nguyen,C., Halder,G., Gao,X. and Veeraraghavan,S. (2006) Insights into transcription enhancer factor 1 (TEF-1) activity from the solution structure of the TEA domain. *Proc. Natl. Acad. Sci. U.S.A.*, **103**, 17225–17230.
16. Yagi,R., Kohn,M., Karavanova,I., Kaneko,K., Vullhorst,D., DePamphilis,M. and Buonanno,A. (2007) Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development. *Development*, **134**, 3827–3836.
17. Nishioka,N., Yamamoto,S., Kiyonari,H., Sato,H., Sawada,A., Ota,M., Nakao,K. and Sasaki,H. (2007) Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. *Mech. Dev.*, **125**, 270–283.
18. Hirate,Y., Cockburn,K., Rossant,J. and Sasaki,H. (2013) Tead4 is constitutively nuclear, while nuclear vs. cytoplasmic Yap distribution is regulated in preimplantation mouse embryos. *Proc. Natl. Acad. Sci. U.S.A.*, **109**, E3389–E3390.
19. Home,P., Saha,B., Ray,S., Dutta,D., Gunewardena,S., Yoo,B., Pal,A., Vivian,J., Larson,M., Petroff,M. *et al.* (2012) Altered subcellular localization of transcription factor TEAD4 regulates first mammalian cell lineage commitment. *Proc. Natl. Acad. Sci. U.S.A.*, **109**, 7362–7367.
20. Gheldof,N., Leleu,M., Noordermeer,D., Rougemont,J. and Reymond,A. (2012) Detecting long-range chromatin interactions using the chromosome conformation capture sequencing (4C-seq) method. *Methods Mol. Biol.*, **786**, 211–225.
21. Simon,J., Giresi,P., Davis,I. and Lieb,J. (2012) Using formaldehyde-assisted isolation of regulatory elements (FAIRE) to isolate active regulatory DNA. *Nat. Protoc.*, **7**, 256–267.
22. Schoenfelder,S., Furlan-Magaril,M., Mifsud,B., Tavares-Cadete,F., Sugar,R., Javierre,B.M., Nagano,T., Katsman,Y., Sakthidevi,M., Wingett,S. W. *et al.* (2015) The pluripotent regulatory circuitry connecting promoters to their long-range interacting elements. *Genome Res.*, **25**, 582–597.
23. van de Werken,H. J. G., Haan,J. C., Feodorova,Y., Bijos,D., Weuts,A., Theunis,K., Holwerda,S. J. B., Meuleman,W., Pagie,L., Thanisch,K. *et al.* (2017) Small chromosomal regions position themselves autonomously according to their chromatin class. *Genome Res.*, **27**, 922–933.
24. Kundaje,A., Meuleman,W., Ernst,J., Bilenky,M., Yen,A., Heravi-Moussavi,A., Kheradpour,P., Zhang,Z., Wang,J., Ziller,M. J. *et al.* (2015) Integrative analysis of 111 reference human epigenomes. *Nature*, **518**, 317–330.
25. Lin,S., Wani,M., Whitsett,J. and Wells,J. (2010) Klf5 regulates lineage formation in the pre-implantation mouse embryo. *Development*, **137**, 3953–3963.
26. Waki,H., Nakamura,M., Yamauchi,T., Wakabayashi,K., Yu,J., Hirose-Yotsuya,L., Take,K., Sun,W., Iwabu,M., Okada-Iwabu,M. *et al.* (2011) Global mapping of cell type-specific open chromatin by FAIRE-seq reveals the regulatory role of the NFI family in adipocyte differentiation. *PLos Genet.*, **7**, e1002311.
27. Phillips,J. and Corces,V. (2009) CTCF: master weaver of the genome. *Cell*, **137**, 1194–1211.
28. Pugacheva,E., Rivero-Hinojosa,S., Espinoza,C., Méndez-Catalá,C.F., Kang,S., Suzuki,T., Kosaka-Suzuki,N., Robinson,S., Nagarajan,V., Ye,Z. *et al.* (2016) Comparative analyses of CTCF and BORIS occupancies uncover two distinct classes of CTCF binding genomic regions. *Genome Biol.*, **16**, 161.
29. Sagai,T., Hosoya,M., Mizushina,Y., Tamura,M. and Shiroishi,T. (2005) Elimination of a long-range cis-regulatory module causes complete loss of limb-specific Shh expression and truncation of the mouse limb. *Development*, **132**, 797–803.
30. Attanasio,C., Nord,A.S., Zhu,Y., Blow,M.J., Li,Z., Liberton,D.K., Morrison,H., Plajzer-Frick,I., Holt,A., Hosseini,R. *et al.* (2013) Fine tuning of craniofacial morphology by distant-acting enhancers. *Science*, **342**, 1241006.
31. Spilianakis,C.G., Lalioti,M.D., Town,T., Lee,G.R. and Flavell,R.A. (2005) Interchromosomal associations between alternatively expressed loci. *Nature*, **435**, 637–645.
32. Ling,J.Q., Li,T., Hu,J.F., Vu,T.H., Chen,H.L., Qiu,X.W., Cherry,A.M. and Hoffman,A.R. (2006) CTCF mediates interchromosomal colocalization between Igf2/H19 and Wsb1/Nf1. *Science*, **312**, 269–272.

33. Barolo,S. (2012) Shadow enhancers: frequently asked questions about distributed cis-regulatory information and enhancer redundancy. *Bioessays*, **34**, 135–141.
34. Ito,K., Noguchi,A., Uosaki,Y., Taga,T., Arakawa,H. and Takizawa,T. (2018) Gfap and Osmr regulation by BRG1 and STAT3 via interchromosomal gene clustering in astrocytes. *Mol. Biol. Cell*, **29**, 209–219.
35. Osterwalder,M., Barozzi,I., Tissières,V., Fukuda-Yuzawa,Y., Mannion,B.J., Afzal,S.Y., Lee,E.A., Zhu,Y., Plajzer-Frick,I., Pickle,C.S. *et al.* (2018) Enhancer redundancy provides phenotypic robustness in mammalian development. *Nature*, **554**, 239–243.
36. Li,G., Ruan,X., Auerbach,R.K., Sandhu,K.S., Zheng,M., Wang,P., Poh,H.M., Goh,Y., Lim,J., Zhang,J. *et al.* (2012) Extensive promoter-centered chromatin interactions provide a topological basis for transcription regulation. *Cell*, **148**, 84–98.
37. Maass,P.G., Barutcu,A.R., Weiner,C.L. and Rinn,J.L. (2018) Inter-chromosomal contact properties in Live-Cell imaging and in Hi-C. *Mol. Cell*, **70**, 188–189.
38. Apostolou,E., Ferrari,F., Walsh,R.M., Bar-Nur,O., Stadtfeld,M., Cheloufi,S., Stuart,H.T., Polo,J.M., Ohsumi,T.K., Borowsky,M.L. *et al.* (2013) Genome-wide chromatin interactions of the Nanog locus in pluripotency, differentiation, and reprogramming. *Cell Stem Cell*, **12**, 699–712.
39. Gao,F., Wei,Z., An,W., Wang,K. and Lu,W. (2013) The interactomes of POU5F1 and SOX2 enhancers in human embryonic stem cells. *Sci. Rep.*, **3**, 1588.
40. Schoenfelder,S., Sexton,T., Chakalova,L., Cope,N.F., Horton,A., Andrews,S., Kurukuti,S., Mitchell,J.A., Umlauf,D., Dimitrova,D.S. *et al.* (2010) Preferential associations between co-regulated genes reveal a transcriptional interactome in erythroid cells. *Nat. Genet.*, **42**, 53–61.
41. Kaneko,K.J. and DePamphilis,M.L. (2013) TEAD4 establishes the energy homeostasis essential for blastocoel formation. *Development*, **140**, 3680–3690.
42. Pfeffer,P. and Pearton,D. (2012) Trophoblast development. *Reproduction*, **143**, 231–246.
43. Ralston,A., Cox,B., Nishioka,N., Sasaki,H., Chea,E., Rugg-Gunn,P., Guo,G., Robson,P., Draper,J. and Rossant,J. (2010) Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2. *Development*, **137**, 395–403.
44. Kaiser,S., Koch,Y., Kühnel,E., Sharma,N., Gellhaus,A., Kuckenberg,P., Schorle,H. and Winterhager,E. (2016) Reduced gene dosage of Tfap2c impairs trophoblast lineage differentiation and alters maternal blood spaces in the mouse placenta. *Biol. Reprod.*, **93**, 31.
45. Russ,A., Wattler,S., Colledge,W.H., Aparicio,S.A., Carlton,M.B., Pearce,J.J., Barton,S.C., Surani,M.A., Ryan,K., Nehls,M.C. *et al.* (2000) Eomesodermin is required for mouse trophoblast development and mesoderm formation. *Nature*, **404**, 95–99.
46. Lee,D.S., Vonrhein,C., Albarado,D., Raman,C.S. and Veeraraghavan,S. (2016) A potential structural switch for regulating DNA-Binding by TEAD transcription factors. *J. Mol. Biol.*, **428**, 2557–2568.
47. Chen,Y., Chen,C., Zhang,Z., Liu,C.C., Johnson,M.E., Espinoza,C.A., Edsall,L.E., Ren,B., Zhou,X.J., Grant,S.F. *et al.* (2015) DNA binding by FOXP3 domain-swapped dimer suggests mechanisms of long-range chromosomal interactions. *Nucleic Acids Res.*, **43**, 1268–1282.
48. Calabrese,J., Sun,W., Song,L., Mugford,J., Williams,L., Yee,D., Starmer,J., Mieczkowski,P., Crawford,G. and Magnuson,T. (2013) Site-specific silencing of regulatory elements as a mechanism of X inactivation. *Cell*, **151**, 951–963.
49. Shin,H., Liu,T., Manrai,A. and Liu,X. (2010) CEAS: cis-regulatory element annotation system. *Bioinformatics*, **25**, 2605–2606.
50. Heinz,S., Benner,C., Spann,N., Bertolino,E., Lin,Y.C., Laslo,P., Cheng,J.X., Murre,C., Singh,H. and Glass,C.K. (2010) Simple combinations of lineage-determining transcription factors prime cis-Regulatory elements required for macrophage and B cell identities. *Mol. Cell*, **38**, 576–589.