Establishment of 3D chromatin structure after fertilization and the metabolic switch at the morulato-blastocyst transition require CTCF

Graphical abstract

Highlights
- Mouse embryos without CTCF do not progress beyond the late blastocyst stage
- Maternal CTCF is not necessary for normal development
- Loss of CTCF independently disrupts 3D chromatin organization and gene expression
- CTCF controls the metabolic switch occurring at the morulato-blastocyst transition

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In brief
The genome is organized in a precise 3D structure by chromatin-bound factors such as CTCF. However, its implication in gene expression is unclear. Andreu et al. describe mainly independent roles of CTCF in structuring the genome and in regulating the expression of key metabolic genes necessary for early mouse development.
Establishment of 3D chromatin structure after fertilization and the metabolic switch at the morula-to-blastocyst transition require CTCF

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SUMMARY

The eukaryotic genome is organized in 3D at different scales. This structure is driven and maintained by different chromatin states and by architectural factors, such as the zinc finger protein CTCF. Zygotic genome structure is established de novo after fertilization, but its impact during the first stages of mammalian development is unclear. We show that deletion of Ctcf in mouse embryos impairs the establishment of chromatin structure, but the first cell fate decision is unperturbed and embryos are viable until the late blastocyst. Furthermore, maternal CTCF is not necessary for development. Gene expression changes in metabolic and protein homeostasis programs that occur during the morula-to-blastocyst transition depend on CTCF. However, these changes do not correlate with disruption of chromatin but with binding of CTCF to the promoter of downregulated genes. Our results show that CTCF regulates both 3D genome organization and transcription during mouse preimplantation development, but as independent processes.

INTRODUCTION

Chromatin is not randomly organized inside the eukaryotic cell nuclei. At the nuclear scale, individual chromosomes occupy distinct spatial territories during interphase (Cremer et al., 2006; Cremer and Cremer, 2010). Within chromosomes, the cell genome is compartmentalized into regions with similar chromatin characteristics, termed A- and B-compartments. A-compartments are transcriptionally active, gene rich, and nuclease sensitive, whereas B-compartments are relatively gene poor, transcriptionally silent, and insensitive to nucleases (Lieberman-Aiden et al., 2009). At intermediate length scales, high-resolution chromatin interaction maps have revealed that the genome folds into distinct modules called topologically associating domains (TADs). TADs are sub-megabase-sized chromatin domains in which genomic interactions are strong but sharply depleted on crossing the boundary to the adjacent one (Dixon et al., 2012; Hou et al., 2012; Nora et al., 2012; Sexton et al., 2012). Finally, at a smaller scale, distal genomic regions can physically interact, forming DNA loop structures that bring distant regulatory elements in close proximity to their target promoters (Dowen et al., 2014; Rao et al., 2014).

During early embryogenesis, there is a massive reorganization of the chromatin. In mature mouse sperm, TADs and compartments are already established (Jung et al., 2017; Ke et al., 2017). However, mature oocytes arrested in metaphase II stage of meiosis are depleted of both structures (Du et al., 2017; Ke et al., 2017), as happens in mitotic cells when chromosomes are highly condensed (Naumova et al., 2013). After fertilization, chromatin adopts a more relaxed state and the strength of TADs and compartments is highly reduced, although some weak TADs and interaction domains are detected (Du et al., 2017; Ke et al., 2017; Flyamer et al., 2017; Collombet et al., 2020). During the first cleavage stages, a slow re-establishment of TADs and compartments starts but it is not completed until the eight-cell stage. Moreover, the progressive maturation of chromatin structure is conserved in early animal development, as has also been observed in Drosophila (Hug et al., 2017), zebrafish (Kaaaj et al., 2018), and humans (Chen et al., 2019).

A characteristic feature of more than 75% of the borders of TADs and 86% of anchoring points of smaller DNA loops in mammals is the presence of the CTCF architectural protein (Dixon et al., 2012; Rao et al., 2014; Phillips-Cremins et al., 2013). CTCF is a ubiquitously expressed and highly conserved 11-zinc-finger DNA-binding protein (Filippova et al., 1996). It has been shown to be involved in transcriptional regulation, chromatin insulation, genomic imprinting, X-chromosome inactivation, and higher-order chromatin organization (Klenova et al., 1993; Bell et al., 1999; Cuddapah et al., 2006; Bell and Felsenfeld, 2000; Hark et al., 2000; Chao et al., 2002; Xu et al., 2017). However, mature oocytes arrested in metaphase II stage of meiosis are depleted of both structures (Du et al., 2017; Ke et al., 2017), as happens in mitotic cells when chromosomes are highly condensed (Naumova et al., 2013). After fertilization, chromatin adopts a more relaxed state and the strength of TADs and compartments is highly reduced, although some weak TADs and interaction domains are detected (Du et al., 2017; Ke et al., 2017; Flyamer et al., 2017; Collombet et al., 2020). During the first cleavage stages, a slow re-establishment of TADs and compartments starts but it is not completed until the eight-cell stage. Moreover, the progressive maturation of chromatin structure is conserved in early animal development, as has also been observed in Drosophila (Hug et al., 2017), zebrafish (Kaaaj et al., 2018), and humans (Chen et al., 2019).
Figure 1. CTCF loss leads to embryonic lethality at the late blastocyst stage

(A–D) Confocal images of representative control (Ctcffl/fl) and different combinations of Ctcf mutant alleles at eight-cell, morula, and blastocyst stages immunostained with CTCF antibody (green). Nuclei were stained with DAPI (gray). Scale bar, 25 μm. Embryos were obtained from three and two independent litters from Ctcffl/fl/C0 or Zp3-Cre;Ctcffl/fl/C0 crosses, respectively, for all time points. The total number of embryos analyzed at each stage were, for eight-cell, nine control, four Z KO, six M KO, and five MZ KO; for morula, nine control, three Z KO, six M KO, and seven MZ KO; and for blastocysts, 10 control, three Z KO, seven M KO, and eight MZ KO. (B–D) Quantification of the total cell number per embryo of early (B; 24–42 cells), mid (C; 43–63 cells), and late (D; 64–80 cells) blastocyst stages for the different Ctcf genotypes. Each dot represents an individual embryo. Embryos were obtained from six and four (B), four and four (C) and three and two (D) independent litters from Ctcffl/fl×Ctcffl/fl or Zp3-Cre;Ctcffl/fl×Ctcffl/fl crosses, respectively. The reduced sample size of MZ embryos at the late blastocyst stage (D, n = 3) is due to reduced embryo viability. Horizontal bars represent means. ns, non-significant; ***p < 0.0005, **p < 0.005, and *p < 0.05 by Student’s t test.

(E) Confocal images of control (Ctcffl/fl), zygotic, maternal, and maternal-zygotic Ctcf mutants immunostained with anti-CDX2 (green) and anti-NANOG (cyan) antibodies. Nuclei were stained with DAPI (gray). Arrowheads indicate cells double positive for CDX2 and NANOG. Scale bar, 25 μm. Embryos were obtained from three and two independent litters from Ctcffl/fl/C0 or Zp3-Cre;Ctcffl/fl/C0 crosses, respectively.
et al., 2007; Dixon et al., 2012). Homozygous CTCF deletion results in early embryonic lethality (Fedoriw et al., 2004; Heath et al., 2008; Wan et al., 2008; Moore et al., 2012; Chen et al., 2019). In somatic cells, conditional knockouts (KOs) showed additional important roles for CTCF in cell-cycle progression, apoptosis, and differentiation (Heath et al., 2008; Soshnikova et al., 2010; Li and Lu, 2007; Arzate-Mejía et al., 2018). However, global depletion of CTCF in cell cultures leads to subtle and context-specific changes in gene expression (Zuin et al., 2014; Nora et al., 2017; Hyle et al., 2019; Kübo et al., 2021), although chromatin structure is affected with a reduction of TAD insulation and intra-TAD loop formation. However, we are still missing a clear understanding of the role of CTCF in the initial establishment of chromatin structure, and its impact on regulated gene expression.

Here, we have studied the requirement of CTCF protein during mouse preimplantation development, when 3D chromatin structure is radically reorganized after fertilization, by genetically eliminating the maternal, zygotic, or both contributions of CTCF. Our results show that CTCF is not necessary for the first phases of mouse preimplantation development, but, after blastocyst cavitation, it is absolutely required for developmental progression.

RESULTS

CTCF loss leads to embryonic lethality at the late blastocyst stage

To eliminate maternal contribution of CTCF, we used females carrying a CTCF floxed allele (Heath et al., 2008) and the Zp3-Cre driver. This Cre driver is expressed during the growing phase of oocytes prior to the completion of the first meiotic division, and, when combined with a floxed allele, it will result in no mRNA or protein generated by the mother to be deposited in the oocyte as it is formed (Lewandoski et al., 1997). Both maternal and maternal-zygotic mutant embryos (M KO and MZ KO) develop up to the blastocyst stage, and they proliferate and undergo cavitation just as zygotic mutant embryos (Z KO) and controls (Figure 1A). We confirmed the absence of CTCF protein by immunostaining (Figure 1A), and subsequent image quantification (Figure S1A). Quantitative differences at the eight-cell stage are subtle, although there is clear reduction when comparing M and MZ KOs. This may be due to the lower levels of CTCF protein we detect at the eight-cell stage that in-crease sharply at the morula stage, in agreement with previous levels of CTCF protein we detect at the eight-cell stage that increase sharply at the morula stage, in agreement with previous observations on CTCF expression during preimplantation development (Olbrich et al., 2021). On the other hand, M KO morula observations on CTCF expression during preimplantation development (Olbrich et al., 2021). On the other hand, M KO morula expression remained mostly unchanged and we do not observe differences in the number of cells among genotypes at the early (24–42 cells; Figure 1B) or mid-blastocyst stage (43–63 cells; Figure 1C). However, in the late blastocyst stage (64–80 cells) we observed a striking reduction in the number of cells in Z and MZ KO embryos compared with wild-type (WT) and heterozy-gous embryos, or M KO embryos, respectively (Figure 1D). While Z mutants show a similar median number of cells in late blastocysts compared with mid-blastocysts (40–45 cells; Figures 1C and 1D), MZ mutants have even fewer cells at this later stage (approximately 30 cells, compared with approximately 50 at the mid-blastocyst stage; Figures 1C and 1D). It is true that we have a reduced sample size of MZ KO at the late blastocyst stage (n = 3, Figure 1D), but this is due to embryo viability, as we consistently obtain fewer than expected embryos of this genotype at this late stage. Next, we examined whether this growth arrest is due to a decrease in proliferation or to increased apoptosis, as the number of cells in mutant embryos decreases.

We labeled proliferating cells in mid-blastocyst stage M (with a phenotype equivalent to WT embryos) or MZ KO embryos with phosphorlated histone H3, and apoptotic cells with TUNEL staining. However, MZ KO show no significant differences in proliferation or cell death compared with M KO (Figures S1C and S1D). As CTCF has been described to have a role in maintaining genome stability (Lang et al., 2017), we examined the degree of DNA damage in control and mutant blastocyst. Indeed, we found that there was an increase in γH2AX staining in nuclear foci in maternal-zygotic mutant compared with maternal mutants (Figures S1E and S1F). These results suggest that cells from the preimplantation embryo with no CTCF divide and grow normally but at a certain stage are unable to maintain cell viability, and show a moderate increase in DNA damage. Nevertheless, the exact reasons for embryo lethality remain unclear.

A possibility that could be occurring was that initial lineage specification in the mouse embryo was not taking place correctly, and that mis-specified cells were leading to embryo arrest. Therefore, we performed immunostainings to detect key factors involved in the commitment of the trophectoderm (CDX2) and the inner cell mass (SOX2 and NANOG) in mid-stage blastocysts. We observed no obvious change in the levels of CDX2 and SOX2 between genotypes, and these were correctly expressed in restricted patterns in the trophectoderm (TE) and the inner cell mass (ICM), respectively (Figures 1E and 1F). Although the general levels of NANOG expression remained mostly unchanged and we do not observe differences in the number of cells expressing both CDX2 and NANOG across genotypes (Figure S1G), there is a tendency for TE from mutants to express higher levels of NANOG (Figure S1H). Nevertheless, and despite these subtle changes in four and three independent litters from Ctcffloxed−/− × Ctcffloxed−/− or Zp3-Cre;Ctcffloxed−/− × Ctcffloxed−/− crosses, respectively. The total number of embryos analyzed at each stage were 19 controls, four Z KO, 10 M KO, and eight MZ KO.

(F) Confocal images of control (Ctcffloxed−/−), zygotic, maternal, and maternal-zygotic Ctcf mutants immunostained with anti-SOX2 antibody (red). Nuclei were stained with DAPI (gray). Scale bar, 25 μm. Embryos were obtained from four and two independent litters from Ctcffloxed−/− × Ctcffloxed−/− or Zp3-Cre;Ctcffloxed−/− × Ctcffloxed−/− crosses, respectively. The total number of embryos analyzed at each stage were 12 Controls, five Z KO, nine MKO, and five M ZKO. See also Figure S1.
the expression of NANOG, overall we can conclude that there is no gross mis-specification of the TE and ICM in CTCF depleted blastocysts and that the first cell fate decision is correctly specified.

We next attempted to see if the second lineage-specification event, that taking place in the ICM to produce the epiblast and the primitive endoderm, characterized by the segregated expression of NANOG and GATA6 respectively (Chazaud et al., 2006), occurs properly. However, this takes place in the late blastocyst, when Ctcf mutants are abnormal and a much-reduced cell number (Figures 1D and S1B), which could make interpretation of expression patterns of these two markers difficult. Therefore, we carried out staining for NANOG and GATA6 in mid to late blastocyst (64–80 cells), at a stage where there is still not a mutually exclusive pattern of expression of NANOG and GATA6, but rather a mix of single and double-positive cells for each of the markers (Plusa et al., 2008). We detect the presence of single- and double-positive cells in MZ KO embryos (Figure S1I), just as we do in M KO embryos. Our analysis suggests that the specification of primitive endoderm and epiblast initiates correctly in the absence of CTCF, but, due to embryo lethality, we do not know if full segregation of the two lineages is completed in the mutants.

Reorganization of early chromatin structure in Ctcf mutant embryos

We developed single-blastocyst Hi-C adapting the volumes and timings from the single-cell Hi-C protocol (Nagano et al., 2015). This was necessary, as at this stage (mid-blastocyst), mutant embryos are abnormal and a much-reduced cell number (Figures 1D and S1B), which could make interpretation of expression patterns of these two markers difficult. Therefore, we carried out staining for NANOG and GATA6 in mid to late blastocyst (64–80 cells), at a stage where there is still not a mutually exclusive pattern of expression of NANOG and GATA6, but rather a mix of single and double-positive cells for each of the markers (Plusa et al., 2008). We detect the presence of single- and double-positive cells in MZ KO embryos (Figure S1I), just as we do in M KO embryos. Our analysis suggests that the specification of primitive endoderm and epiblast initiates correctly in the absence of CTCF, but, due to embryo lethality, we do not know if full segregation of the two lineages is completed in the mutants.

TAD assembly in preimplantation development is partially dependent on CTCF

CTCF-depleted blastocysts showed reduction in the number of TADs (2,898 TADs detected in WT, 2,915 in M KO, and 2,497 in MZ KO), leading to an increase in median size (Figure 3A). We also observed a high degree of TAD reorganization, even in genomic loci that will not be active until later stages in development, such as the Wnt6-Epha4-Pax3 locus (Figure S3A; Lupiáñez et al., 2015), or the HoxD cluster (Figure S3B; Rodríguez-Carballo et al., 2017).

TADs also showed a decrease in their reproducibility score (a statistical measure of the robustness of a TAD; see STAR Methods) and in the density of intra-TAD contacts (number of interactions by TAD size) (Figure 3A). Changes in insulation score (IS), a measure of the interactions passing across a given genomic position, at the TAD boundaries are less pronounced in MZ KO blastocysts (Figure 3B), confirming a weaker insulation of these domains when CTCF is not present. Likewise, we detect a reduction in the difference of IS at CTCF peaks when using CTCF chromatin immunoprecipitation (ChIP) data from naive embryonic stem (ES) cells (Figure 3C). Consistent with these results, clear differences in TAD strength are appreciated when data from TADs are stacked in metaplots, showing a greatly diminished signal in MZ KO compared with WT or M KO embryos (Figure 3D).

Intriguingly, this decrease of insulation is independent of the presence of CTCF at the TAD borders (Figure S3C). This would suggest that other effects, different from direct binding of CTCF to chromatin but triggered by its absence, contribute to the global loss of chromatin structure at these stages. These could be changes in the expression of other factors that would also be involved in genome organization. Otherwise, the global depletion of CTCF before the structuring of the genome at these early developmental stages might lead to an overall weakening of TADs and chromatin structure. We also observe a much higher number/proportion of TADs where the position of their borders is not stable in the MZ KO compared with the control (Figure 3E), once more showing how 3D structure is disrupted in embryos that lack CTCF.

Interestingly, if we compare our results with those obtained in ES cells depleted of CTCF (Nora et al., 2017; Kubo et al., 2021), we observe similar loss of insulation and ectopic contacts across
TAD boundaries upon CTCF depletion (Figure 3F). However, as discussed above, in the embryo, this occurs independently of CTCF occupancy of TAD boundaries, which does not happen in ES cells. On the other hand, the minor but reproducible reduction in the strength of compartments we observe was also detected in ES and immune cells depleted of CTCF, where up to a 10% reduction in strength has been described (Nora et al., 2017; Stik et al., 2020).

Transcriptional changes in blastocyst-stage mutant embryos
We analyzed the transcriptional changes in single blastocysts when mutants for Ctcf still do not show any overt phenotype (Figures 1A and 1C), although there is a measurable effect on chromatin structure. We studied the expression of control (Ctcffl/fl and Ctcffl/fl), M KO and MZ KO embryos. Comparison of MZ KO and Ctcffl/fl embryos revealed approximately 1,000
differentially expressed genes (DEGs) with 574 genes downregulated and 464 of them upregulated (Figure 4A; Table S2). We did not find any differences when we compared M KO with control embryos that grouped together in principal-component analysis (Figures S5B and S5D; Table S2), in line with our previous observations on the lack of phenotype of the maternal Ctcf mutant. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (Kanehisa and Goto, 2000) of DEG in MZ

Figure 3. CTCF is required for proper TAD assembly in blastocysts
(A) TAD characterization. Violin plots of TAD size (left), TAD reproducibility score (center), and intra-TAD contact density (right). WT (green), M KO (blue), MZ KO (orange). ns, non-significant; ****p < 0.0001 by Wilcoxon test.
(B) Changes in insulation score (Delta IS) at TAD borders in WT (green line), M KO (blue line), and MZ KO (orange line) embryos. Plots are centered on boundary regions ±1,000 kb and normalized using a 100-kb window.
(C) Delta IS at CTCF peaks in WT (green line), M KO (blue line), and MZ KO (orange line) embryos. Plots are centered on peaks ±1,000 kb and normalized using a 100-kb window.
(D) TAD pile-up contact enrichment map representation in WT, M KO, and MZ KO embryos at 25-kb resolution.
(E) Barplot comparing TAD number with none, one, or both borders stable between genotypes. A border is considered stable compared with WT when its position in the other condition is located ±1 bin (40 kb) with respect to the WT. ****p < 0.0001 by chi-square test comparing the distribution of TAD categories.
(F) Violin plots of median values for inter-TAD/intra-TAD interactions by chromosomes in WT (green), M KO (blue), and MZ KO (orange) blastocysts. ns, non-significant; **p < 0.005 by Mann–Whitney U test. See also Figure S3.
mutants revealed an enrichment of genes involved in metabolic pathways (glycolysis, such as Gpi1 or Slca1; fatty acid metabolism, Acadsb and Acsl6; lysosomal enzymes for amino and nucleotide sugar metabolism, Neu1 and Hexa; oxidative phosphorylation, Ndufb7, Ndufb10, Ndufab1, Uqcrcl, or Ppa2) and DNA replication for downregulated genes, and protein homeostasis (the ribosome, various Rpl and Rps genes; the proteasome, Psmd12 or Seml; and the spliceosome, Hspa or Snrpb) and cell cycle for upregulated genes (Figure 4B; Table S3).

Interestingly, we did not observe changes in lineage determination genes, with the exception of Nanog, which matches our previous observations with whole-mount immunohistochemistry (Figures 1E, S1G, and S1H).

Binding to promoters of downregulated genes suggests that CTCF activates gene expression
To characterize the relationship of direct CTCF binding with changes in gene expression, we checked binding of CTCF, as identified by ChIP sequencing (ChIP-seq) in naive ES cells, along the loci of all genes expressed at the blastocyst stage. We used CTCF binding data from ES cells because of the lack of good-quality data in early mouse embryos. The observed conservation of CTCF binding in different cell types from the same organism and also across different species (Cuddapah et al., 2009; Martin et al., 2011) made us consider that the majority of CTCF peaks found in naive ES cells would also be present in the embryo. We first analyzed the distribution of CTCF in a 2-kb region

Figure 4. CTCF absence causes transcriptional deregulation at the blastocyst stage
(A) Volcano plot of DEGs between control (Ctcffl/fl) and MZ KO single blastocysts (n = 3 in both cases). Blue indicates downregulated genes in MZ KO (adjusted p value <0.05 and logFC < −1), orange indicates upregulated genes in MZ KO (adjusted p value <0.05 and logFC >1), and gray indicates non-DEGs. Representative genes are indicated.
(B) Significantly enriched KEGG pathways for genes downregulated (blue) and upregulated (orange) in MZ KO blastocysts.
(C) Read-density plot comparing the ChIP-seq read distribution of CTCF around the TSS (±1,000 bp) of downregulated genes (blue), upregulated genes (orange), non-DEGs (dark gray), and non-expressed genes (light gray) in MZ KO blastocysts.
(D) Proportion of downregulated genes (blue), upregulated genes (orange), and non-DEGs (gray) with CTCF binding in the promoter region (top), introns (middle), or intergenic regions (bottom). ns, non-significant; ****p < 0.0001 by chi-square test.
(E) Violin plots showing the distance to the immediately neighboring upstream and downstream genes of downregulated genes (blue), upregulated genes (orange), and non-DEGs (gray) in MZ KO blastocysts. Distances are measured from the transcriptional start or termination sites of each gene, whichever is nearest. ns, non-significant; ***p < 0.001 by Wilcoxon and Kruskal-Wallis test. See also Figure S4 and Tables S2 and S3.
surrounding the transcriptional start site (TSS), finding that downregulated genes have a much higher tendency to have CTCF bound at their promoter region than non-DEGs. We also observe this trend for upregulated genes, although to a lesser extent. Interestingly, there is hardly binding of CTCF to the TSS region of genes that are not expressed in the blastocyst (Figure 4C).

We confirmed these observations by quantifying the proportion of upregulated, downregulated, and non-DEGs with CTCF binding at their promoters that is enriched for both categories of DEGs (Figure 4D). Interestingly, if we extend our search to other regions, we find that downregulated genes are enriched for the binding of CTCF to at least one intron (Figure 4D). Overall, these results show that proximal binding of CTCF to promoters is associated with positive regulation of gene expression. The observation of enrichment for intron binding only in downregulated genes is intriguing and could indicate cooperative mechanisms involving short-range CTCF-mediated interactions.

If we examine intergenic regions, we find an opposite behavior: genes upregulated in mutant blastocyst are more likely to present binding of CTCF in upstream or downstream non-coding regions than downregulated or non-DEGs (Figure 4D). This is suggestive of CTCF acting upon cis-regulatory elements located in these regions. This could be explained by upregulated genes being localized at regions with lower gene density, and therefore with larger intergenic regions associated with each gene, with more complex and numerous intergenic regulatory elements. However, upregulated genes on average have a similar distribution of intergenic distances than non-DEGs; therefore, such bias cannot account for the observed enrichment of CTCF at these regions. On the other hand, we observed that downregulated genes tend to be nearer to neighboring genes, and therefore in more gene-dense regions than non-DEG or upregulated genes (Figure 4E).

Reorganization of chromatin structure has a limited impact on transcription
To assess the possible link between structural and transcriptional changes due to the lack of CTCF, we first analyzed changes in the IS surrounding the TSS of DEG. Genes that are downregulated in Ctfc mutant blastocysts show a trend for higher insulation at their TSS in WT conditions compared with those upregulated or with non-DEG (Figure 5A). In order to quantify the extent of the change in insulation, we have calculated the difference in Delta IS (Δ-Delta IS, Figure 5A; see STAR Methods) around the TSS of expressed genes for WT, M KO, and MZ KO embryos. While there are no differences between different group of genes when comparing M KO with WT embryos, when we compare MZ KO with WT blastocysts, the changes in Δ-Delta IS are significantly higher between both up- and downregulated genes (right and left panels, Figure 5A) and non-differentially expressed (middle panel, Figure 5A; Figure S3D); However, these differences can be explained by increased insulation surrounding CTCF bound regions (Figure 3C) as promoter regions of DEGs are more frequent bound by CTCF, most notably downregulated genes (Figure 4C). In fact, differences in Delta IS are higher in DEGs that show binding of CTCF to their promoters (Figures S4A and S4B).

We next examined the distribution of DEG in compartments, taking into account whether they are stable in MZ KO embryos compared with controls or change their identity from A to B and vice versa. We observe that genes that change their expression have a higher tendency to be localized in A-compartments and less in B-compartments compared with non-DEGs (Figure 5B). We hardly find DEGs located to compartments that change their identity from B to A. However, we see a slight enrichment of downregulated genes in compartments changing from A to B (Figure 5B). We next analyzed whether DEGs are related to TAD stability. For this, we assigned each expressed gene to a TAD and examined whether it was preserved, or one or both boundaries were lost, when comparing controls with mutants. In this case, we could not detect differences between DEGs and non-DEGs regarding the behavior of the TAD that contains them upon CTCF loss (Figure 5C).

These results are consistent with what we observe at specific loci. Slc2a1 gene, encoding the GLUT1 glucose transporter, is downregulated in MZ KO embryos (Figure 4A), and is localized in a TAD that shows a displacement of one of its borders without affecting the expression of any the immediately neighboring genes. However, the rewiring of contacts at the sub-TAD level results in loss of interactions near Slc2a1, as can be appreciated in the Hi-C matrix and the virtual 4C (Figure S4C). Also, this region contains DNAse I hypersensitive sites (DHSs) mapped in morula-stage embryos (Lu et al., 2016), suggestive of the presence of open chromatin and cis-regulatory elements. The adjacent TAD is split in two in MZ KO blastocysts, but only two genes inside them have their expression affected. For example, Ppcs, which encodes an enzyme involved in coenzyme A biosynthesis, is inside one of the split TADs and is the only gene dysregulated (in this case upregulated) inside the TAD, although an increase in contacts is detected in the region (Figure S4C). Similarly, if we focus on the Nanog locus, we find some pluripotency genes that, together with Nanog, are upregulated in MZ mutants (Dppa3, Gdf3; Figures 4A and S4D). They are in the same TADs that have their borders expanded and suffer a reshuffling of the local interactions in CTCF-depleted embryos (Figure S4D). Curiously, in the same TAD and neighboring Nanog sits Slc2a3, encoding GLUT3, another glucose transporter, which does not change its transcriptional activity. Again, this region contains DHSs suggestive of the presence of putative cis-regulatory elements. Moreover, by searching for the presence of DHSs within 10 kb upstream the TSS or 10 kb downstream the TTS of expressed genes, we observed that both up- and downregulated genes were enriched in open regions compared with non-DEGs irrespective of their location in A- or B-compartments (Figure S4E). Together, these results suggest that, although lack of CTCF affects 3D genome structure at different scales, only local reshuffling of the contacts at the sub-TAD level can partially explain the changes in expression we observe.

The transcriptional switch of metabolic programs from the morula to the blastocyst is impaired in Ctfc mutants
As CTCF has been suggested to have a role in the coordination of dynamic transitions in expression (Sams et al., 2016; Gomez-Velazquez et al., 2017), we investigated whether genes changing expression in the morula-to-blastocyst transition...
were more susceptible to being affected by the lack of CTCF. To do so, we complemented our transcriptomic analysis on blastocysts described above with single-embryo RNA sequencing (RNA-seq) at the early morula stage (16–20 cells; Figure 1A) of control, Z, and MZ mutants. Samples from different genotypes were not resolved by principal-component analysis (Figure S5A), and the comparison of MZ KO with control morulae showed only 55 DEGs, most of them being pseudogenes (Figure S5C; Table S2). Therefore, we observe that the loss of maternal and zygotic CTCF has little to no effect on transcription before the blastocyst stage, once more reinforcing the notion of its dispensability for the very first stages of mouse development.

We next used our single-embryo expression data from controls to examine the transcriptional changes occurring at the morula-to-blastocyst transition. We identified 114 downregulated genes and 702 upregulated genes in WT (green), M KO (blue), and MZ KO (orange) blastocysts. The graphical representation of the differences in Delta IS between conditions is indicated (Δ-Delta IS) on the left panel. The barplot (upper panel) showing the percentage of downregulated genes (DOWN), upregulated genes (UP), and non-DEGs located in genomic regions where A- and B-compartments are stable between controls and MZ KO (dark red and dark blue, respectively), or where the identity of the compartment changed from A to B (light red) or B to A (light blue). Below, statistical analysis of the enrichments of down- and upregulated genes of the different categories as shown above. *p < 0.05, **p < 0.005, and ****p < 0.0001 by Fisher exact test and corrected by Benjamin-Yekutieli procedure. (C) Barplot (upper panel) showing the percentage of downregulated genes (DOWN), upregulated genes (UP), and non-DEGs in each of the TAD groups defined by their boundary stability between WT and MZ KO datasets (both borders stable, light gray; one border stable, gray; no border stable, dark gray). Below, statistical analysis of the enrichments of down- and upregulated genes of the different categories as shown above. There are no significant differences by Fisher exact test and corrected by Benjamin-Yekutieli procedure. See also Figure S4.

Figure 5. Chromatin reorganization has a limited impact on gene expression

(A) Delta IS at the TSS (±1,000 kb) of downregulated (left), non-differentially expressed (center), and upregulated (right) genes in WT (green), M KO (blue), and MZ KO (orange) blastocysts. The graphical representation of the differences in Delta IS between conditions is indicated (Δ-Delta IS) on the left panel.

(B) Barplot (upper panel) showing the percentage of downregulated genes (DOWN), upregulated genes (UP), and non-DEGs located in genomic regions where A- and B-compartments are stable between controls and MZ KO (dark red and dark blue, respectively), or where the identity of the compartment changed from A to B (light red) or B to A (light blue). Below, statistical analysis of the enrichments of down- and upregulated genes of the different categories as shown above. *p < 0.05, **p < 0.005, and ****p < 0.0001 by Fisher exact test and corrected by Benjamin-Yekutieli procedure.

(C) Barplot (upper panel) showing the percentage of downregulated genes (DOWN), upregulated genes (UP), and non-DEGs in each of the TAD groups defined by their boundary stability between WT and MZ KO datasets (both borders stable, light gray; one border stable, gray; no border stable, dark gray). Below, statistical analysis of the enrichments of down- and upregulated genes of the different categories as shown above. There are no significant differences by Fisher exact test and corrected by Benjamin-Yekutieli procedure. See also Figure S4.
phosphorylation, amino and nucleotide sugar metabolism, or lysosome pathways are upregulated from morula to blastocyst (Figure 6B), while they are downregulated in MZ KO blastocyst compared with controls (Figure 4B). Equally, genes related to the ribosome are downregulated in the blastocyst, but upregulated in Ctcf mutants (Figures 4A and 4B; 6A and 6B; Table S3).

If we perform a genome-wide comparison of the changes in gene expression of MZ KO blastocysts with the changes that occur during the morula-to-blastocyst transition, we observe an inverse correlation for both sets of DEGs (Figure 6C). In fact, while we identify 127 genes that are downregulated in MZ KO compared with controls and also upregulated in the

Figure 6. Transcriptional changes occurring from morula to blastocyst are impaired in Ctcf mutants
(A) Volcano plot of DEGs between control morulae (Ctcffl/fl; C0) and control blastocysts (Ctcffl/fl; C0). Blue indicates downregulated genes in blastocysts compared with morulas (adjusted p value <0.05 and logFC < −1), orange indicates upregulated genes in blastocysts compared with morulas (adjusted p value <0.05 and logFC > 1), and gray indicates non-DEGs. Representative genes are indicated.
(B) Significantly enriched KEGG pathways for downregulated (blue) and upregulated (orange) genes in control blastocysts compared with morulas.
(C) Correlation of the changes in expression between control morulae (Ctcffl/fl; C0) compared with control blastocysts (Ctcffl/fl; C0) in the y axis, and control blastocysts (Ctcffl/fl; C0) compared with MZ KO blastocysts in the x axis. Purple indicates DEG in MZ KO blastocysts only, green indicates DEG in morula-to-blastocyst transition only, and orange indicates common DEG for both conditions. Regressions are shown on top of the plot, and the correlation values are shown on the bottom left-hand corner.
(D) Heatmap showing the expression (Z scores) of metabolic genes that are downregulated in Ctcf mutants, in control morulae (E2.5, purple), control blastocysts (E3.5, green), and MZ KO blastocysts (orange). Clustering of the conditions is shown on the top. See also Figure S5 and Tables S2 and S3.
(E) Heatmap showing the expression (Z scores) of ribosomal genes that are upregulated in Ctcf mutants, in control morulae (E2.5, purple), control blastocysts (E3.5, green), and MZ KO blastocysts (orange). On the right, color code shown on the left side of the heatmaps for KEGG pathways categories. Clustering of the conditions is shown on top. See also Figure S5 and Tables S2 and S3.
morula-to-blastocyst transition, only 19 genes show the opposite trend (upregulated in MZ KO and downregulated in the morula-to-blastocyst transition; Figure S5E; Table S3). Moreover, clustering of metabolism and ribosomal genes previously identified as DEGs grouped together E2.5 controls and E3.5 MZKO, with both these showing a similar signature of expression (Figures 6D and 6E). Overall, these results suggest that loss of CTCF leads to a failure to correctly implement the changes in gene expression, mainly related to metabolism and protein homeostasis, which would normally occur when the morula progresses to the blastocyst stage.

**DISCUSSION**

The role of CTCF in structuring the genome in 3D is undisputed (Ong and Corces, 2014). Equally, the relationship between between chromatin structure and the regulation of gene expression, although subject to debate, is also clear (Ibrahim and Mundlos, 2020). The limited transcriptional effect of CTCF deletion, as observed for example in ES cells (Nora et al., 2017; Kubo et al., 2021), is therefore surprising. This could be the result of CTCF being necessary for the initial establishment of regulatory interactions along chromatin but not for the posterior maintenance of these contacts. Therefore, depletion of CTCF would not affect established transcriptional programs but could be needed for changes in gene expression associated with differentiation events (Gomez-Velazquez et al., 2017; Arzate-Mejia et al., 2018). In this view, 3D chromatin structure mediated by CTCF is not required for transcriptional homeostasis but is essential for cell state transitions that involve deployment of different expression programs.

We have explored this issue by taking advantage of the first stages of mouse development, starting from fertilization, when the maternal and paternal haploid genomes come together to establish the embryonic diploid genome. We find that maternal CTCF is not required for development or adult life, nor for the establishment of 3D chromatin structure or transcriptional programs in the preimplantation mouse embryo. Our results are in contradiction with previous studies (Wan et al., 2008; Moore et al., 2012), surely due to the different approaches used to deplete CTCF (microinjection of RNAi versus genetic deletion). Furthermore, we observe that CTCF is dispensable for the first stages of development, as the block we observe in zygotic and maternal-zygotic mutants only occurs after blastocyst formation. This lack of phenotype suggests that CTCF is not required in the first hours of development before the zygotic gene is expressed and protein produced. Another possibility is that maternal CTCF is rescued by the paternal contribution, as it has been shown that CTCF protein is present on the mature sperm genome, bound to a small number of specific sites (Jung et al., 2017). Nevertheless, this low amount of protein is not sufficient to establish correct chromatin structure in embryos lacking maternal and zygotic CTCF.

Despite limitations in resolution of the contact maps, we observe that complete depletion of CTCF in blastocyst nuclei generates a less organized chromatin structure with an overall reduction in the isolation of interaction domains. Compartment identity remains mainly unaffected, but we have found higher frequency of inter-compartment interactions that translates into a reduction of compartment strength. TADs do not completely disappear in mutant embryos, but again their strength is much reduced and there is a reorganization of domains due to more promiscuous genomic contacts. Indeed, the increase in the number of long-range contacts (200 kb to 1 Mb) suggests the formation of larger DNA loops, probably as a consequence of cohesin not encountering obstacles at CTCF binding sites during loop extrusion (Wutz et al., 2020; Hansen 2020).

The fact that interaction domains are established de novo during preimplantation development in the absence of CTCF, albeit with a strong reduction in domain isolation, argues that other complementary factors or processes must be taking place to initially structure the genome. Similar conclusions have been reached in other systems, as for example the human embryo (Chen et al., 2019). In this scenario, CTCF could be acting as a scaffolding factor that stabilizes and maintains 3D organization generated by other means. Another possibility is that 3D structure is inherited from pre-patterned chromatin present in the separate gamete haploid genomes. However, recent work has shown that the majority of chromatin domains present in the maternal or paternal genomes prior to fertilization are not preserved in the embryo (Collombet et al., 2020). At present, there are no clear candidates for this triggering factor of genome structure, and at this point we cannot rule out that it appears as a side effect of other processes involving genome dynamics, such as DNA replication, transcription (Pope et al., 2014; Du et al., 2017; Ke et al., 2017), or lamina-chromatin interactions (Borsos et al., 2019).

The absence of CTCF triggers changes in expression related to metabolism and protein homeostasis in the blastocyst, while, surprisingly, there are no changes yet at the morula stage. Interestingly, in the morula-to-blastocyst transition, there is a metabolic switch in the use of energy (Kaneko, 2016; Zhang et al., 2018) and a downregulation of ribosomal components (Gao et al., 2017). Therefore, changes in the expression of metabolic pathways and structural components of the ribosomes in this transition do not take place in the CTCF mutants, which we believe is the cause of the developmental block of late blastocysts. It is interesting to note that metabolic transitions are important in other developmental transitions in the early mouse embryos, as is the case of the 2C state (Rodriguez-Terrones et al., 2020).

On the one hand, we have not found a clear relationship between the transcriptional changes we observe in mutant embryos and the structural reorganization caused by CTCF absence. Changes in insulation or TAD organization are not translated into global changes of gene expression in those regions, suggesting that transcription is resilient to alterations in TADs. Although mutant blastocysts exhibit a rewiring of interactions, these local changes do not alter all the genes in a given region in the same way, indicating that there are other properties that influence the response of genes to modifications of the 3D structure of the genome. The different susceptibility of genes could depend on the proximity between promoters and their enhancers, enhancer-promoter intrinsic preferences (Zabidi et al., 2015; Yokoshi et al., 2020), or whether they are genes with dynamic or continuous and stable expression (Sams et al., 2016; Gomez-Velazquez et al., 2017; Stik et al., 2020).
On the other hand, we see a link between CTCF binding at promoters and downregulation of the expression, suggesting another role of CTCF protein different from insulation. In fact, it has been shown that promoter-proximal CTCF can promote long-range enhancer-promoter contacts and facilitate gene activation (Kubo et al., 2021). It is noteworthy that, in the case of genes downregulated in mutants, we see enrichment of CTCF bound at introns together with promoters, suggestive of a more complex mechanism than transcriptional activation by binding to promoter regions.

In summary, we find that CTCF is necessary for a proper 3D organization of the chromatin after fertilization and for transcriptional regulation of the metabolic switch in the morula-to-blastocyst transition required for developmental progression beyond the blastocyst stage. A rather unexpected observation was that we did not see major disruption of lineage-specification events or the early expression of key factors, such as Sox2, Oct4, Cdx2, or Gata6, when comparing Ctf mutant blastocysts with controls. Therefore, our mutant embryos, which show an impaired 3D genome structure, were able to properly progress through the first cell fate decision. This argues that the gene regulatory networks responsible for these early events are largely independent of chromatin organization and must rely on short-range regulatory mechanism.

Limitations of the study
A limitation of our study is the small amount of starting material we can obtain from the Ctf mutants. The necessity to determine the genotype of individual embryos on sequence data from Hi-C or RNA-seq libraries generated from single embryos prevents us from being able to pool embryos of the same genotype to obtain a larger sample. A further limitation of our genetic models is the need to maintain and generate embryos from two separate breeding schemes, to obtain on one hand homozygous zygotic mutants and heterozygotes, and, on the other, maternal and maternal-zygotic mutants. Finally, our analyses come from a heterogeneous population, as at the blastocyst stage there are two distinct cell types, trophectoderm and ICM. Therefore, we have not been able to distinguish changes specific to one or another of these cell types caused by the loss of CTCF.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**
Supplemental information can be found online at [https://doi.org/10.1016/j.celrep.2022.111501](https://doi.org/10.1016/j.celrep.2022.111501).

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**AUTHOR CONTRIBUTIONS**
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**DECLARATION OF INTERESTS**
The authors declare no competing interests.

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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| **Antibodies**       |        |            |
| mouse monoclonal anti-CTCF (IF) | Santa Cruz Biotechnology | Cat# sc-271474; RRID:AB_10649800 |
| rabbit polyclonal anti-CTCF (ChIP) | Millipore | Cat# 07-729, RRID:AB_441965 |
| rabbit monoclonal anti-CDX2 | Abcam | Cat# ab76541, RRID:AB_1523334 |
| rat monoclonal anti-NANOG | Thermo Fisher Scientific | Cat# 14-5761-80, RRID:AB_763613 |
| goat polyclonal anti-SOX2 | R and D Systems | Cat# AF2018, RRID:AB_355110 |
| goat polyclonal anti-GATA6 | R and D Systems | Cat# AF1700, RRID:AB_2108901 |
| rabbit polyclonal anti-PH3Ser10 | Millipore | Cat# 06-570, RRID:AB_310177 |
| mouse monoclonal anti-gamma H2AX | Millipore | Cat# 05-636, RRID:AB_309864 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Biotin-16-dUTP | Roche | Cat# 11093070910 |
| PD0325901 (MEK inhibitor) | Sigma-Aldrich | Cat# PZ0162 |
| CHIR99021 (GSK3 inhibitor) | Sigma-Aldrich | Cat# SML1046 |
| acid Tyrode’s solution | Sigma-Aldrich | Cat# T-1788 |
| MboI | New United Kingdom Biolabs | Cat# R0147M |
| Biotin-14-dATP | Thermofisher | Cat# 19524-016 |
| DNA Polymerase I, large (Klenow) | New United Kingdom Biolabs | Cat# M0210L |
| T4 DNA Ligase (2000U/mL) | New United Kingdom Biolabs | Cat# M0202M |
| Dynabeads MyOne Streptavidin C1 | Thermofisher | Cat# 65001 |
| Alul | New United Kingdom Biolabs | Cat# R0137S |
| T4 Polynucleotides Kinase | New United Kingdom Biolabs | Cat# M0201L |
| T4 DNA Polymerase | New United Kingdom Biolabs | Cat# M0203L |
| Klenow Fragment 3'->5' exo- | New United Kingdom Biolabs | Cat# M0212S |
| Proteinase K | Roche | Cat# 10910000 |
| Agencourt AMPure XP Beads | Beckman-Coulter | Cat# A63880 |
| NEBNext® High-Fidelity 2X PCR Master Mix | New United Kingdom Biolabs | Cat# M0541S |
| **Critical commercial assays** | | |
| Terminal Transferase recombinant kit | Roche | Cat# 03333574001 |
| NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1) | New United Kingdom Biolabs | Cat# E7355S |
| NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 2) | New United Kingdom Biolabs | Cat# E7500S |
| **Deposited data** | | |
| RNA-seq, ChIP-seq and Hi-C datasets | This paper | GSE180307 |
| **Experimental models: Cell lines** | | |
| R1 129 murine male embryonic stem cells with 129/Sv background | CNIO Transgenic mice Unit | NA |
| **Experimental models: Organisms/strains** | | |
| CD-1 mice | Charles Rivers | Crl:CD1(ICR) |
| Ctcf floxed allele mice | Heath et al., 2008 | N/A |
| Zp3-CreERT2 mice | Lewandoski et al., 1997 | N/A |
| **Oligonucleotides** | | |
| Forward common primer for Ctcf 5’ CTAGGAGTGTTAGTTAGTGGA GCCC-3’ | Heath et al., 2008 | N/A |

(Continued on next page)
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Miguel Manzanares (mmanzanares@cbm.csic.es).

Materials availability
The study did not generate new unique reagents.

Data and code availability
- RNA-seq, ChIP-seq and Hi-C data have been deposited at the Gene Expression Omnibus GEO database and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
The following mouse lines were used in this work: CD-1 (Charles Rivers), Ctcf floxed allele (Heath et al., 2008), Zp3-Cre<sup>tg/tg</sup> (Lewandoski et al., 1997). All mouse lines were maintained on an outbred background using CD-1 mice. To obtain controls and zygotic mutant
embryos, Ctcf<sup>fl/fl</sup> individuals were inter-crossed. To obtain maternal and maternal-zygotic mutant embryos Zp3-Cre<sup>tp+; Ctcf<sup>fl/fl</sup></sup> females were crossed with Ctcf<sup>fl/fl</sup> males. In this way, all embryos will lack maternal contribution of Ctcf. As for the zygotic Ctcf deletion, 50% will be heterozygous (Ctcf<sup>fl/fl</sup>) and 50% homozygous (Ctcf<sup>-/-</sup>). Adults were genotyped by PCR of tail-tip DNA using primers detailed in Table S4. For preimplantation embryos, genotyping was performed directly on individually isolated embryos after antibody staining. The stage of embryos used is indicated in the text. This ranges from morula used for RNA-seq (Figure S5) to blastocyst in all other experiments. No differences related to the sex of the embryos was expected, and therefore this was not determined.

Mice were housed and maintained in the animal facility at the Centro Nacional de Investigaciones Cardiovasculares (Madrid, Spain) in accordance with national and European legislation. Procedures were approved by the CNIC Animal Welfare Ethics Committee and by the Area of Animal Protection of the Regional Government of Madrid (ref. PROEX 196/14).

**METHOD DETAILS**

**Embryo collection and immunofluorescence**

Embryos were collected at morula or blastocyst stage by flushing the oviduct or the uterus with M2 medium (Sigma) and fixed for 10 min in 4% PFA in PBS. After permeabilization with 0.5% Triton X-100 in PBS (PBST) for 20 min, embryos were blocked for 1h with 10% FBS in PBST at room temperature. Incubation with primary antibodies was done overnight at 4°C in blocking solution and incubation with secondary antibodies was performed for 1h at room temperature in blocking solution. The following antibodies and dilutions were used: mouse monoclonal anti-CTCF (sc-271474, Santa Cruz Biotechnology) 1:200, rabbit monoclonal anti-CDX2 (ab76541, Abcam) 1:300, rat monoclonal anti-NANOG (14-5761, eBioscience) 1:200, goat polyclonal anti-BOX2 (AF2018, R&D systems) 1:100, goat polyclonal anti-GATA6 (AF1700, R&D systems) 1:200, rabbit polyclonal anti-PH3Ser10 (06-570, Millipore) 1:200, mouse monoclonal anti-gamma H2AX (05-636, Millipore) 1:200. Secondary Alexa Fluor conjugated antibodies (Life Technologies) were used at 1:1000. In addition, nuclei were visualized by incubating embryos in DAPI at 1 µg/mL for 15 min. For TUNEL assay, the Terminal Transferase recombinant kit (Roche 03 333 574 001) and biotin-16-DUTP (Roche 11 093 070 910) were used.

Embryos where staged by counting the number of cells using DAPI staining, and subdivided at blastocyst stage in early (24-42 cells), mid (43-63 cells) and late (64-80 cells). Embryos where imaged on glass-bottomed dishes (Ibidi or MatTek) with a Leica SP5 or Leica SP8 confocal microscope.

**Immunofluorescence quantification**

For quantification of CTCF intensity, DAPI-stained nuclei were segmented using the StarDist deep-learning image segmentation method (Weigert et al., 2020) through ImageJ software (Schindelin et al., 2012). The mean CTCF was quantified within the segmented nuclei and averaged per each embryo.

For NANOG intensity quantification, IMARIS imaging software (v. 7.6.3) was used. Images were smoothed with Gaussian filter 3 x 3 x 1 and then nuclei were segmented in 3D reconstructions with the tool Spots detection based on DAPI, CDX2 and NANOG staining with a 7 µm isosurface. Spots were visually evaluated and corrected if necessary. Using spot-to-spot colocalization analysis, NANOG-CDX2 double-positive cells were selected, and mean fluorescence intensity was measured within the spots. NANOG intensity values for each blastomere were normalized to the brightest blastomere of each litter of embryos to minimize staining and confocal imaging variability. Statistical analyses were performed with GraphPad Prism v.8.4.3 (GraphPad Software, USA).

**ChiP-seq**

4x10<sup>7</sup> mESCs growing at 70% of confluence were washed with PBS, trypsinized, resuspended in 20 mL of growing media and cross-linked with 1% formaldehyde for 15 min at RT. After quenching with 0.125 M Glycine, fixed cells were washed twice with PBS containing 1 µM PMSF and protease inhibitors, pelleted and lysed in lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1) at 2x10<sup>7</sup> cells/ml. 10<sup>7</sup> cells equivalent to 40-50 µg of chromatin were used per immunoprecipitation reaction with 10 µg of antibody. Sonication was performed with a Covaris system (shearing time 20 min, 20% duty cycle, intensity 6, 200 cycles per burst and 30 s per cycle) in a minimum volume of 2 mL. From 6 to 15 ng of immunoprecipitated chromatin (as quantitated by fluorometry) were electrophoresed on an agarose gel and independent sample-specific fractions of 100–200 bp were taken. Adaptor-ligated library was completed by limited-cycle PCR with Illumina PE primers (10-12 cycles). DNA libraries were applied to an Illumina flow cell for cluster generation and sequenced on the Illumina HiSeq2500.

Alignment of sequences to the reference mouse genome (mm10) was performed using ‘Bowtie2’ (version 2.3.3.1) under default settings (Langmead and Salzberg, 2012). Duplicates were removed using Picardtools (version 2.13.2) and peak calling was carried out using MACS2 (version 2.1.1.20160309) after setting the qvalue (FDR) to 0.05 and using the ‘–extsize’ argument with the values obtained in the ‘macs2 predictd’ step (Zhang et al., 2008).
CTCF presence was studied in a 2 kb region around the TSS of DEG and non-DEG genes. To avoid the differences in number of genes between groups, we shuffled 571 genes from the non-DEG group and averaged their signal, repeating the process 1000 times, and finally 571 were randomly selected from this processed list.

Mean read-density profile was performed with deepTools 2.5.4 (Ramirez et al., 2016) and plotted with R v3.6.3 (R Core Team, 2020).

**RNA-seq**

RNA-seq was performed on single embryos at the morulae and blastocyst stages. cDNA synthesis was performed using SMART-Seq Ultra Low Input RNA Kit (Clontech). Library preparation and sequencing was performed by the CNIC Genomics Unit using the Illumina HiSeq 2500 sequencer.

Adapters were removed with Cutadapt v1.14 and sequences were mapped with Bowtie2 2.2.9 (Langmead and Salzberg, 2012) and quantified using RSEM v1.3.1 (Li and Dewey, 2011) to the transcriptome set from Mouse Genome Reference GRCm38 and Ensembl Gene Build version 98. We manually add the corresponding genomic sequences of LacZ, Puromycin and Neomycin to our reference genome, fasta and gtf files, for in silico genotyping. Differentially expressed genes between groups were normalized and identified using the limma (Ritchie et al., 2015) biocca package. We considered as significant, only p-values < 0.05 adjusted through Benjamini–Hochberg procedure. To study morula-to-blastocyst transition, RNA-seq libraries of morula (16-20 cells) and mid-blastocyst (43-63 cells) stage, were analyzed together by including a common condition in the sequenced libraries and batch as a covariate in the linear model. Molecular Signatures Database (MSigDB) was used for gene enrichment analysis (Liberzon et al., 2011).

**Gene density calculation**

Intergenic distances were obtained from Mouse Genome Reference GRCm38 and Ensembl Gene Build version 98. For proper calculation and to avoid artifacts, genes overlapping their neighbors were excluded from the analysis. Pairwise comparison was performed with the use of Wilcoxon test, and multiple groups’ comparison via Kruskal-Wallis test analysis.

**Single embryo Hi-C**

We adapted the protocol for single cell Hi-C described in Nagano et al. (2015), optimizing it for single embryo by scaling the reaction volumes and reducing the experimental procedures to avoid sample loss. Briefly, blastocysts were treated with Tyrode acidic solution to remove the zona pellucida, fixed with 2% formaldehyde at room temperature for 10 min and quenched with glycine for 10 min on ice. Blastocysts were then washed with PBS and incubated in 100 μL lysis buffer supplemented with protease inhibitors (10 mM Tris-HCl pH8.0, 10 mM NaCl, 0.2% NP40) on ice for 30 min with occasional mixing. After spinning at 3,000 rpm at 4°C for 5 min, the pellet was washed with 1X Cutsmart Buffer, gently resuspended in 20 μL of 0.5% SDS in 1 x Cutsmart Buffer and incubated at 65°C for 10 min. SDS was quenched with 40 μL of 1 x Cutsmart Buffer and 12 μL of 10% Triton X-100 at 37°C for 15 min. Then the chromatin was digested by adding 125 U of MboI and incubated at 37°C overnight with constant agitation. To label with biotin the digested DNA ends, 0.3 μL of 10 mM dCTP, 0.3 μL of 10 mM dGTP, 0.3 μL of 10 mM dTTP, 7.5 μL of 0.4 mM biotin-14-dATP and 1.6 μL of 5 U/μL DNA Polymerase I Large (Klenow) were added to the solution and the reaction was carried out at 37°C for 90 min with occasional mixing. Samples were incubated for 15 min at 65°C to inactivate the Klenow enzyme. After spinning at 3,000 rpm at 4°C for 5 min, supernatant was discarded and 95.8 μL of water, 12 μL of 10 x NEB T4 DNA ligase buffer, 10 μL 10% Triton X-100, 1.2 μL 10mg/ml BSA and 2000 cohesive end units of T4 DNA ligase (New England Biolabs) were added to ligate the biotin-labelled DNA ends and the reaction was incubated at 16°C overnight. This was followed by spinning the samples at 3,000 rpm at 4°C for 5 min and addition of 50 μL of 1 x Cutsmart Buffer and 1 μL of 20 mg/ml Proteinase K for overnight incubation at 65°C to reverse the cross-link. The biotin-labelled DNA was bound to Dynabeads MyOne Streptavidin T1 (Life Technology) magnetic beads, the washed beads were suspended in 60 μL of 2 x BB buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 M NaCl) and added to the single blastocyst sample for 60 min incubation at RT in a rotating wheel. After washing the beads with 1 x BB buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1 M NaCl) and 10 mM Tris-HCl (pH 7.5), the beads were incubated at 37°C with 10 U of Alu I in 1 x Cutsmart Buffer for 60 min with constant agitation for DNA fragmentation. Sequencing library preparation was performed on beads: with 5 U of Klenow Fragment (3′-5′ exo-) in 1 x NEBuffer 2 and 0.2 mM dATP at 37°C for 30 min for the A-tailing reaction, and 800 cohesive end units of T4 DNA ligase (New England Biolabs) in 1 x T4 DNA ligase buffer and 2.5 μL NEBNext adaptor for Illumina (diluted 1/30 from stock) at RT for 60 min, each step with the same washes before and after each reaction. Then the beads were re-suspended in amplification mixture containing 25 μL of water, 25 μL NEBNext High-fidelity PCR Master Mix 2X, 2.5 μL universal primer and 2.5 μL indexed primer and amplified with the program: 95°C for 2 min, 18 cycles of (95°C 10 s, 55°C 30 s, 72°C 30 s) and 72°C for 5 min. After amplification, magnetic beads were removed, and the single embryo Hi-C library was purified with Agencourt AMPure XP magnetic beads (Beckman Coulter) and eluted in 12 μL of 10 mM Tris-HCl (pH 8.5). Libraries were sequenced with Illumina HiSeq2500 (50bp, paired end) at the CNAG-CRG Sequencing Unit (Barcelona, Spain), obtaining around 5 million reads each (Table S1).

**Single embryo Hi-C data processing and analysis**

Data were processed using TADbit (Serra et al., 2017) for read quality control, read mapping, interaction detection, interaction filtering, and matrix normalization. After a FastQC protocol to discard artifacts, the remaining reads were mapped to the reference mouse genome (mm10) using a fragment-based strategy in TADbit. After discarding non-informative contacts including self-circles,
dangling-ends, errors, random breaks or duplicates, those experiments with more than 800,000 valid-pairs were kept. Then, the experiments with the same genotype were merged and normalized. Due to WT and MZ KO merged experiments had similar number of reads, the M KO merged experiment was subsampled for a better comparison among samples. For genotyping on sequence, we considered as MZ KO those embryos whose libraries contained no reads in the 22 kb of the deleted region chr8:105662421-105684451 at Ctcf locus (Heath et al., 2008), using as a control other two random regions of the same size chr8:122710142-122732172 and chr8:122710142-122732172. Contact probability as a function of genomic distance was calculated as an intra-chromosomal contact frequency distribution, using logarithmically increasing genomic distance bins.

TADs were identified by using 40 and 100 kb resolution vanilla-normalized and decay-corrected matrices as input to the TAD detection algorithm implemented in TADbit, with identical results. A 25 kb resolution was used for the TAD pile-up contact enrichment maps generated with the tool coolpup (Flyamer et al., 2020). A–B compartments were detected by calculating the first component of a principal-component analysis (PCA) of chromosome-wide matrices (100 kb) and assigning A compartments to the genomic bin with positive PCA1 values and high GC content. Conversely, B compartments were assigned to the genomic bin with negative PCA1 values and low GC content. The same results were obtained using 200 and 500 kb windows. The insulation score and Delta signal were computed using a custom python script following the same methodology as Crane et al., 2015, extracting 10 kb resolution raw matrices and using a sliding window of 100 kb x 100 kb. The Observed/Expected metaplots were created using the tool coolpup (Flyamer et al., 2020) and unbalanced matrices. The TAD reproducibility score is the number of times a TAD is called by the algorithm. As the algorithm of TAD calling is carried out 10 times, TAD reproducibility values range from 1 to 10. TADs with a score lower than 5 will be considered not robust and are likely to be undetectable in replicates or at different resolutions (Serra et al., 2017). In the case of TAD analysis, 25 kb resolution matrices were used with the rescale option to scale all the TADs to the same size. For TAD classification a border is considered stable if the bin border in one condition is one bin more or one bin less of the other. In TSS analysis, the resolution used was 10 kb and they are centered in the TSS of the genes. Juicebox software was used for Hi-C data visualization and virtual 4C (Robinson et al., 2018). In order to quantify the differences in Delta IS between upregulated, downregulated and no-differentially expressed genes, we have extracted for each gene and condition the change in Delta IS (\( D \)-Delta IS). This value is defined as the difference between the Delta IS 80 kb upstream from the TSS and 80 kb downstream (which are the minimum and maximum values in all Delta IS metaplots). Then we have measured the differences of this variable between MZ KO and WT and between M KO and WT datasets for the three group of genes to analyze if there are significant differences between them.

**Compartment dynamics and strength**

Using 100 kb resolution matrices normalized by coverage, we have considered a bin as A or B if the eigenvector is greater than 0.008 or less than −0.008 respectively. Then, for each chromosome, we have added the interactions between A-A, B-B or A-B bins only if the distance of the two bins is greater than 2 Mb. Finally, the compartmentalization strength is defined by the following formula (AA + BB)/AB. Lower values of this metric are related with more AB contacts or less AA and BB contacts, meaning a lack of proper compartmentalization.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analyses were performed with GraphPad Prism v.8.4.3 (GraphPad Software, USA). All details regarding statistical analysis can be found in figure legends for both main and supplementary figures. All data show means. Differences were considered statistically significant when \( p < 0.05 \).