Loss of Maternal CTCF Is Associated with Peri-Implantation Lethality of Ctcf Null Embryos

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Abstract

CTCF is a highly conserved, multifunctional zinc finger protein involved in critical aspects of gene regulation including transcription regulation, chromatin insulation, genomic imprinting, X-chromosome inactivation, and higher order chromatin organization. Such multifunctional properties of CTCF suggest an essential role in development. Indeed, a previous report on maternal depletion of CTCF suggested that CTCF is essential for pre-implantation development. To distinguish between the effects of maternal and zygotic expression of CTCF, we studied pre-implantation development in mice harboring a complete loss of function Ctcf knockout allele. Although we demonstrated that homozygous deletion of Ctf is early embryonically lethal, in contrast to previous observations, we showed that the Ctf nullizygous embryos developed up to the blastocyst stage (E3.5) followed by peri-implantation lethality (E4.5–E5.5). Moreover, one-cell stage Ctf nullizygous embryos cultured ex vivo developed to the 16–32 cell stage with no obvious abnormalities. Using a single embryo assay that allowed both genotype and mRNA expression analyses of the same embryo, we demonstrated that pre-implantation development of the Ctf nullizygous embryos was associated with the retention of the maternal wild type Ctf mRNA. Loss of this stable maternal transcript was temporally associated with loss of CTCF protein expression, apoptosis of the developing embryo, and failure to further develop an inner cell mass and trophectoderm ex vivo. This indicates that CTCF expression is critical to early embryogenesis and loss of its expression rapidly leads to apoptosis at a very early developmental stage. This is the first study documenting the presence of the stable maternal Ctf transcript in the blastocyst stage embryos. Furthermore, in the presence of maternal CTCF, zygotic CTCF expression does not seem to be required for pre-implantation development.

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Introduction

CTCF (CCCTC-binding factor) is a highly conserved, ubiquitously expressed 11 Zn finger DNA binding protein that was originally identified as a factor interacting with specific sequences in the c-myc gene promoters [1,2,3]. CTCF utilizes different combinations of its Zn-fingers to bind a relatively large number of highly divergent target sequences throughout genome [3,4,5,6,7]. CTCF was originally characterized as a transcription factor involved in repression [2,3,8,9] or activation of transcription [10,11], but its activity has now been extended to include a variety of other roles in gene regulation, including chromatin insulation [4,12,13]. Indeed, genomic regions displaying chromatin insulator activity harbor CTCF binding sites, and CTCF binding is required for their insulator activity [6,14,15,16]. Moreover, CTCF binding has been shown to mark boundaries between distinct chromatin domains in the genome [6,17,18]. Methylation-sensitive CTCF binding plays a critical role in regulating genomic imprinting at several genomic loci, including the Igf2/H19 locus [19,20,21,22]. In addition CTCF plays an important role in X-chromosome inactivation [23,24], and genes which escape X-inactivation are separated from stably inactivated X-chromosome genes by CTCF binding sites [25]. CTCF has also been shown to be involved in organizing higher order chromatin structure including the interchromosomal association of transcriptionally active genes [12,13,26].

Given the multifunctional nature of CTCF in regulating gene expression and a recent report on the essential function of CTCF in pre-implantation development using the oocyte-specific RNAi approach [27], we wished to determine what effect a complete loss of zygotic CTCF expression would have on embryogenesis. Accordingly, we engineered mice harboring a complete loss of function Ctf allele. While mice heterozygous for this allele appeared phenotypically normal, nullizygous mice displayed early embryonic lethality at the peri-implantation stage. Interestingly, Ctf nullizygous embryos appeared morphologically normal up to the blastocyst stage of development, which was associated with expression of the maternal wild type Ctf transcript and protein.
Subsequent loss of this stable maternal transcript was temporally associated with loss of CTCF protein expression, apoptosis and embryonic lethality.

**Results**

**Generation of Ctf (+/−) mice**

We previously observed that the coding region of CTCF exhibits marked (>93%) evolutionary conservation in chickens, mice and humans [3], and we therefore utilized a human CTCF cDNA probe to clone the mouse Ctf genomic locus. Utilizing the Jackson Laboratory Interspecific Backcross DNA Panels [28] together with a polymorphic CAA repeat marker that we identified in the second intron of the mouse Ctf locus, we mapped Ctf to a single locus in distal mouse chromosome 8 (Figure 1). Subsequently we observed that the Ctf locus in mouse (Figure 2A) and human [5,29] has a virtually identical exon/intron structure consisting of two non-coding and ten coding exons.

Our efforts to engineer Ctf knockout mice via homologous recombination in mouse embryonic stem (ES) cells are detailed in Materials and Methods. We first isolated the mouse Ctf genomic locus from a 129sv mouse genomic library, engineered a targeting vector from the Ctf genomic clones and used this vector to generate a null allele (Figure 2A). We chose to generate an allele that deleted all coding exons of Ctf since only partial deletion may result in production of an aberrant CTCF protein arising from internal cryptic translation initiation sites and alternative splicing.

The linearized targeting vector was electroporated into ES cells, and positive recombination was scored by Southern blots with the designated 5-prime and 3-prime probes located outside of the targeting cassette (Figure 2A, B). Seven positive ES clones were selected to produce chimeric mice and two clones of Ctf (+/−) heterozygous knockout mice were established, as confirmed by the presence of both the wild type and deleted Ctf alleles documented by Southern blot hybridization of EcoRV-digested tail genomic DNA (Figure 2B). We excise the neo cassette, one clone (5-3) of Ctf (+/−) heterozygous knockout mice was crossed to the MORE (Mox2Crc) mice, which expresses the Cre recombinase from the Mox2 locus [30]. The resulting neo-excised Ctf allele was documented by PCR using primers flanking the remaining LOX site shown in Figure 2A.

Deletion of the Ctf locus in the Ctf knockout allele was also confirmed utilizing a FISH assay on metaphase chromosomes from lymphocyte cultures derived from Ctf(+/−) and Ctf(+/+) mice (Figure 2C). As a Ctf probe we utilized a 17 kb lambda Ctf genomic clone (19.1) containing all of the coding exons that were deleted in the Ctf/knockout allele (Figure 2A). As a positive control we used a centromeric probe from mouse chromosome 8 where Ctf maps (170 kb BAC clone MGB 11301). The Ctf probe was visualized with fluorescein (green) and centromeric probe for chromosome 8 was visualized with Cy5 (red). Only a single chromosome from the Ctf(+/−) metaphase spreads displayed a Ctf signal, while two signals were clearly seen in similar spreads from the Ctf(+/+) mice (Figure 2C).

**Ctf knockout mice exhibit embryonic lethality**

To study the effect of CTFK knockout on embryonic development, we generated the Ctf (+/−) heterozygous mice by crossing C57/BL6 Ctf (+/+) mice to 129sv Ctf (+/−) mice. The C57BL/6/129sv F1 Ctf (+/−) mice were viable and displayed no apparent phenotypic abnormalities. However, when intercrossing heterozygotes we observed a selective absence of Ctf (−/−) pups in the newborn offspring (Table 1, top row) indicating that the absence of functional CTCF is lethal to the developing embryo. To determine at what stage of embryonic development this lethality occurs we genotyped embryos obtained at different stages of development following the breeding of Ctf (+/−) heterozygotes. For this genotyping we utilized a multiplex PCR-based assay that distinguishes the wild type from mutant Ctf alleles (Figure 3B). We observed a Mendelian ratio of genotypes at embryonic day 3.5 (E3.5) (Table 1). Ctf (−/−) E3.5 blastocysts had a distinct blastocoeal and intact zona pellucida, displaying no phenotypic abnormalities compared with their wild type or heterozygous littermates (Figure 4A). However, no Ctf (−/−) embryos were observed at embryonic day 5.5 (E5.5) and beyond (Table 1). Notably, the number of empty deciduae in E5.5-6.5 heterozygous intercrosses was markedly increased in comparison to control crosses (Table 2). Taken together our data indicate that the Ctf (−/−) embryos fail to implant and become non-viable between E4.5 and E5.5.

**Ctf (−/−) embryos reach the blastocyst stage but fail to develop further, which is associated with the loss of the maternal Ctf mRNA and CTCF protein**

To gain further insight into the embryonic defects resulting from the absence of CTFK we isolated newly fertilized eggs from Ctf heterozygous crosses, cultured these embryos ex ovo and then genotyped them. Cultures of explants from E0.5 one cell stage Ctf
Figure 2. Generation of Ctfc knockout mice. (A) A schematic diagram of the mouse Ctfc locus and the targeting vector derived from the wild type Ctfc allele as described in Materials and Methods are shown. E₁–E₁₂ denote Ctfc exons 1 through 12. Restriction enzyme sites shown on map are as follows: E denotes EcoRI, X denotes XbaI, and S denotes SpeI. The locations of both the 5-prime and 3-prime Ctfc genomic probes for the Southern blot analysis are indicated. Red arrows show the locations of genotyping primers. A schematic diagram of genomic digest with EcoRV of the Ctfc knock-out and wild type alleles is shown below. (B) A Southern blot analysis of EcoRV digested genomic DNA utilizing the 5-prime and 3-prime Ctfc genomic probes distinguishes the wild type (14 kb or 25 kb) from the mutated (8 kb or 13 kb) Ctfc alleles. (C) FISH analysis of chromosome spreads from lymphocyte cultures derived from Ctfc heterozygous (+/−) and wild type (+/+ ) mice. The probe for Ctfc, a lambda phage clone 19.1 containing a
17 kb Ctcf genomic DNA insert that includes all coding Ctcf exons that were deleted in the Ctcf knockout allele (Figure 2A), was visualized with fluorescein (green fluorescence). The probe for chromosome 8 identification, a BAC clone (MBI1301, Research Genetics), was visualized with Cy3 (red fluorescence). Examples of single metaphase cells from the Ctcf (+/−) and Ctcf (+/+) mice after hybridization to the Ctcf and chromosome 8 probes are shown. Both homologues of chromosome 8 are labeled with a red and a green signal in the wild type mouse, whereas absence of green signals on one homologue of chromosome 8 confirmed the presence of a deletion of Ctcf in the Ctcf (+/−) mouse.

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(+/−), (+/−), and (−/−) embryos all underwent cell division with many embryos successfully reaching the 32 cell blastocyst stage within four days (Figure 3A and Table 3). We observed no gross phenotypic abnormalities in the cultured pre-implantation stage Ctcf (−/−) embryos compared with their wild type or heterozygous littersmates.

To confirm the absence of CTCF expression in the Ctcf (−/−) E3.5 blastocyst stage embryos, we developed a single embryo assay that allowed both genotyping and mRNA expression analysis of the same embryo as detailed in Materials and Methods. For both genotyping and RT-PCR, we utilized multiplex PCR conditions to control for quality of genomic DNA and cDNA, respectively. Unexpectedly, RT-PCR analysis of mRNA extracted from E3.5 blastocyst stage embryos isolated from Ctcf (+/−) intercrosses revealed Ctcf transcripts not only in the Ctcf (+/+) and (+/−) embryos but also in the Ctcf (−/−) embryos (Figure 3B, left panel). In contrast, we could not detect any Ctcf transcripts in the freshly isolated E4.5 stage Ctcf (−/−) embryos (Figure 3B, right panel). These observation were confirmed utilizing a quantitative real-time RT-PCR assay of the Ctcf (+/+), (+/−), and (−/−) embryos (Figure 3C). The presence of the Ctcf mRNA in the Ctcf (−/−) blastocyst stage embryos likely represents the retention in the developing embryo of the maternal oocyte Ctcf transcripts that appear to remain up to E3.5 stage of development but are lost by E4.5. Although we found it technically difficult to perform both Ctcf mRNA quantitation and anti-CTCF immunohistochemistry on the same embryo, we observed that all (16/16) E3.5 embryos from Ctcf heterozygous intercrosses were positive by immunohistochemistry for CTCF protein expression (Figure 4B). The chance that none of these 16 embryos were Ctcf (−/−) according to the expected Mendelian ratio of genotypes is less than 0.05. This strongly suggests that the Ctcf mRNA present in the E3.5 Ctcf (−/−) embryos is indeed associated with CTCF protein expression.

To gain further insight into the mechanism of early embryonic lethality of the Ctcf (−/−) embryos, we isolated E3.5 blastocyst stage embryos from Ctcf heterozygous intercrosses and cultured them ex vivo. The cultured embryos were then PCR genotyped and assessed for Ctcf transcript expression utilizing RT-PCR. We observed that while the ex vivo cultures of the Ctcf (+/+) and (+/−) E3.5 stage embryos actively proliferated over 2–4 days to form an inner cell mass and trophectoderm (Figure 4A, top panel), the Ctcf (−/−) E3.5 embryos explants failed to outgrow and the inner cell mass and trophectoderm did not develop (Figure 4A, bottom panel). While both Ctcf transcripts and CTCF protein were observed in the Ctcf (−/−) E3.5 blastocyst stage embryos (Figure 3B, C and Figure 4B), following 2 days of ex vivo culture these embryos no longer expressed Ctcf mRNA or protein (data not shown and Figure 4C). Apoptosis as assessed by TUNEL assay was much more pronounced in the cultured Ctcf (−/−) blastocysts compared with the cultured Ctcf (+/+) blastocysts (Figure 4C).

**Discussion**

The ubiquitous expression of CTCF, its marked evolutionary conservation and its central role in controlling gene expression by regulating DNA methylation, genomic imprinting, insulator activity, and interchromosomal associations [4,5,12,13] suggests that it may be a crucial regulator of cell viability, proliferation, and differentiation. This is indeed confirmed in the present study in which we observe that mouse embryos nullizygous for Ctcf become arrested in development very early in embryogenesis. Embryos undergo initial cell division and reach the blastocyst stage (E3.5). However, these Ctcf nullizygous embryos appear incapable of uterine implantation and invariably undergo extensive apoptosis between E4.5 and E5.5.

Given the recently documented essential role for CTCF in pre-implantation development [27,31], together with the previous observation that conditional loss of CTCF expression in cell culture is associated with the rapid onset of apoptosis [32,33], it is perhaps surprising that the Ctcf nullizygous embryos are even capable of repeated cell division to reach the 32 cell blastocyst stage. We clearly demonstrate here that this initial cell prolifer-

| Cross | Stage | Average number of pups or embryos per cross | Ctcf genotype |
|-------|-------|---------------------------------------------|---------------|
|       |       |                                             | +/+ | +/− | −/− |
| (+/−) × (+/−) | Postnatal | 7.2 | 39 | 84 | 0 |
| (+/−) × (+/+)* | Postnatal | 7.1 | 29 | 27 | NA |
| (+/+) × (+/−)* | Postnatal | 7.4 | 18 | 19 | NA |
| (+/−) × (+/−) | E12.5 | 7.5 | 4 | 11 | 0 |
| (+/−) × (+/+−) | E10.5 | 8.1 | 8 | 16 | 0 |
| (+/−) × (+/−) | E8.5 | 7.6 | 13 | 26 | 0 |
| (+/−) × (+/−) | E6.5 | 8 | 10 | 24 | 0 |
| (+/−) × (+/−) | E5.5 | 7 | 6 | 14 | 0 |
| (+/−) × (+/−) | E3.5 | 8.5 | 18 | 34 | 14 |

*Control crosses.

NA = not applicable.

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Table 1. Ctcf nullizygous mice display early embryonic lethality.
ation/viability exhibited by the Ctcf (−/−) embryos is associated with the retention of the wild type maternally-derived Ctcf transcript at the preimplantation stage. Maternal transcripts in general are well documented to be present in the embryo up to the two-cell stage, when the zygotic transcription is activated, however recent evidence suggests that certain maternal transcripts are still retained up to the blastocyst stage [34,35,36]. In fact, many embryonically lethal null mutations are believed to be masked by prolonged maternal expression of the genes and therefore rarely result in early cleavage-stage pre-implantation lethality [37,38,39]. In our study, we reproducibly detected Ctcf maternal transcripts in the Ctcf nullizygous blastocysts corresponding to E3.5 (Figure 3B,C), suggesting that the presence of this wild type transcript may be involved in maintaining the viability of the Ctcf (−/−) embryo during pre-implantation development. Consistent with this hypothesis, we observed that by E4.5 stage of development, the Ctcf maternal transcript is no longer detected in the Ctcf nullizygous embryos, and this is temporally associated with the onset of apoptosis.

In mammals, the transition between oocyte and embryo development occurs in the absence of active transcription and depends on the presence and coordinated translation of stored mRNAs transcribed in the growing oocyte [40,41,42]. The stability and translation of such maternal transcripts was found to correlate with the presence of cytoplasmic polyadenylation elements (CPE) within about 90–120 nucleotides 5-prime of the nuclear polyadenylation signal, AAUAAA [43]. Consistently, cytoplasmic polyadenylation of some maternal transcripts was shown to be necessary for oocyte maturation and initiation of pre-implantation development in the mouse [44]. Several RNA-binding proteins including the CPE-binding protein (CPEB) were shown to bind CPEs to form a translation-repressing complex that upon activation would release the mRNA allowing formation of the initiation complex [41,45,46]. The CPE was originally identified as an UUUUAU sequence in 3-prime UTRs of maternal transcripts. Survey of 3-prime UTRs of a series of maternal mRNAs that are relatively abundant in the mouse oocyte expanded this consensus to (A)UUUU(UU)A(UAA) [43]. In our study, we identified a stable maternal Ctcf mRNA that...
Table 2. Analysis of E5.5 and E6.5 Ctcf embryos.

| Cross            | Female | Male | Total # of deciduae | Ctcf genotype of embryos | # of empty deciduae |
|------------------|--------|------|---------------------|--------------------------|---------------------|
|                  |        |      |                     | +/-                      | +/-                 | +/-                 |
| Heterozygous     |        |      |                     |                          |                     |                     |
| +/- x +/-        |        |      | 72                  | 16                       | 38                  | 0                   |
| +/- x +/-        |        |      |                      |                          |                     |                     |
| Control crosses  |        |      |                     |                          |                     |                     |
| +/- x +/-        |        |      | 15                  | 6                        | 9                   | NA                  |
| +/- x +/-        |        |      | 23                  | 7                        | 15                  | NA                  |

NA = not applicable.

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Figure 4. Ctcf nullizygous embryos fail to develop beyond the E3.5 stage and undergo apoptosis. (A) Ex vivo outgrowth of E3.5 embryos. Blastocysts (E3.5) were isolated from Ctcf heterozygous (+/-) intercrosses and cultured ex vivo for the indicated number of days. At the end of the culture period the embryos were harvested and PCR genotyping and RT-PCR performed. The cultured Ctcf (+/+) and (+/-) embryos outgrew to form an inner cell mass (ICM) and trophectoderm (TE) while little proliferation was noted in the cultured Ctcf (-/-) embryos (40%). (B) E3.5 blastocyst stage embryos isolated from the indicated crosses were subjected to DAPI staining and anti-CTCF immunochemistry (40%). All (16/16) embryos analyzed from the Ctcf (+/-) heterozygous intercrosses were positive for CTCF protein expression, displaying staining patterns similar to the two representative embryos shown here. The expected Mendelian ratio of genotypes in such crosses indicates that the chance that none of these 16 embryos were Ctcf (-/-) is less than 0.05 (i.e. (3/4)^16=0.01). (C) Day two outgrowths of E3.5 embryos of the indicated genotype. Blastocysts (E3.5) were cultured ex vivo for two days and then subjected to DAPI staining, anti-CTCF immunohistochemistry and TUNEL assay (40%). Ctcf (+/+ and (+/-) embryos outgrew and expressed CTCF, while cultured Ctcf (-/-) embryos failed to outgrow and exhibited loss of CTCF protein expression and increased apoptosis. The observations displayed here involve one Ctcf (+/+ and two Ctcf (-/-) embryos cultured ex vivo and are representative of over 60 embryos that were analyzed from Ctcf (+/-) heterozygous intercrosses.

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is efficiently translated and present in the embryo up to the implantation stage, suggesting that Ctcf transcripts might have a potential CPE element in its 3-prime UTR. Indeed, a CPE-like sequence, AUUAUUUA, is located ~80 nt upstream from a classic nuclear polyadenylation signal AUAAAA in the mouse Ctcf mRNA. Moreover, this potential CPE in Ctcf 3-prime UTR is 100% conserved between mouse (NCBI Accession Number: NM_181322) and human (NCBI Accession Number: NM_006565).

Maternal-effect genes are usually transcribed in oocytes and essential for early embryonic development [47,48,49,50]. Chromatin structure has been shown to be important for reprogramming gene expression during zygotic genome activation (ZGA) in pre-implantation development [38,51,52,53]. Perhaps it is not surprising that CTCF that is involved in chromatin insulation and organization of higher order chromatin structure was found to be highly expressed in both oocytes and pre-implantation embryos [21,27,54]. Moreover, a recent transgenic study using the oocyte-specific Ctcf/RNAi approach [27] demonstrated its essential role in pre-implantation development suggesting that Ctcf may also be a maternal-effect gene. The authors reported that maternal deletion of CTCF resulted in both meiotic defects in oocyte development and mitotic defects in the embryo and that mitotic defects and apoptosis occurred at an earlier embryonic developmental stage (morula) than we observed (peri-implantation). This apparent discrepancy between the previous and our present work can be readily explained by the completely different technical approaches utilized in these studies. The previous RNAi-based approach would likely have efficiently depleted both maternal and zygotic Ctcf mRNA, likely resulting in earlier apoptosis. Thus these previous studies did not clearly distinguish between the effects of maternal and zygotic expression of CTCF on pre-implantation development. Our present study, which utilizes a genetic knockout of the Ctcf locus, would not have affected the maternal Ctcf mRNA, which likely prolonged the viability of the embryo beyond the morula to the blastocyst stage. Thus our present observations clearly demonstrate that in the absence of the zygotic expression of CTCF, stable maternal Ctcf transcripts are sufficient to maintain pre-implantation development of the embryo, establishing Ctcf as a maternal-effect gene.

Materials and Methods

Ethics Statement

This study involved mice that were bred and housed in the centralized AAALAC accredited Fred Hutchinson Cancer Research Center Animal Facilities. All experimental procedures were performed in compliance with and approved by the Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee (IACUC) protocol # 1271. Animals were euthanized humanely by carbon dioxide overdose as recommended by the Panel of Euthanasia of the American Veterinary Medical Association (AVMA).

Chromosome mapping of the mouse Ctcf gene

The chromosomal location of Ctcf was determined by using the Jackson Laboratory interspecific backcross panels: C57BL/6J × M. spreitus F1 × C57BL/6J, called Jackson BSB, and (C57BL/6J × SPRET/Ei)F1 × SPRET/Ei, called Jackson BSS [28]. A polymorphic region encompassing a simple sequence repeat located in the second Ctcf intron was used to distinguish between the C57BL/6J and the M. spreitus Ctcf alleles. PCR primers used to amplify this region include: Forward: 5’ CCA GGA GAG CCA AGG ATA TAT AGT AGG ACC 3’ and Reverse: 5’ GGT TAG GAT TAC AGT GTA CAT CAT CAC CAT ACC 3’ with a product size of 320 bp for the C57BL/6J Ctcf allele and 340 bp for the M. spreitus allele.

Targeted disruption of the Ctcf locus

The mouse Ctcf genomic locus was isolated from a 129sv mouse genomic library and overlapping lambda genomic clones encompassing all coding exons of Ctcf were mapped and partially sequenced. To make the targeting vector (Figure 2A) we employed a three-step ligation strategy. First the "left arm", a 3.5 kb EcoR1 fragment from the lambda clone 4.12 was cloned into pZeoS (Invitrogen), then the Not1-Apa1 fragment from the pPGKneo-2pgKDTA plasmid (from Phil Soriano) was cloned into pZero3.5EL, resulting in plFLArm plasmid. Finally the "right arm", 2.6 kb SpeI fragment from the lambda clone 13.2 was ligated into a compatible and unique Nhe1 site of the plFLArm plasmid. This targeting construct harbors the PGK-neo cassette for positive selection and a PGK-DTA toxin expression cassette for negative selection. This linearized vector was electroporated into ES cells, and positive recombination was scored by Southern blots with the designated 5-prime and 3-prime probes located inside of the targeting cassette (Figure 2A, B). ES clones, positive for the wild type and mutated Ctcf EcoRV fragments, were selected to produce chimeric mice (Figure 2B). To excise the neo cassette, the Ctcf (+/−) heterozygous knockout mice were crossed to the MORE (Mox2Cre) mice, which express Cre recombinase from Mox2 locus [30]. The resulting neo-excised Ctcf allele was documented by PCR using primers flanking the remaining LOX site as described under Genotyping. The heterozygous Ctcf (+/−) mice were backcrossed to 129sv genetic background. For embryonic lethality studies, the C57BL6/129sv F1 Ctcf (+/−) mice were generated by crossing C57/BL6 Ctcf (+/+) mice to 129sv Ctcf (+/+) mice.

FISH analysis

Metaphase chromosomes preparations were obtained from mice heterozygous for the Ctcf deletion and from control wild type mice. Spleens were dissected from the mice and short-term lymphocyte cultures were stimulated with lipopolysaccharide. Chromosome preparations were harvested using 0.75 M KCl and methanol: acetic acid (3:1). Slides were denatured as described previously [55]. A 17 kb Ctcf genomic DNA fragment cloned in lambda phage (lambda 19.1, Figure 2A) was labeled with biotin by nick-transcription using a Bionick kit from Invitrogen. A 170 kb lambda phage (lambda 19.1, Figure 2A) was labeled with biotin by nick-transcription using a Bionick kit from Invitrogen. A 170 kb lambda phage (lambda 19.1, Figure 2A) was labeled with biotin by nick-transcription using a Bionick kit from Invitrogen. A 170 kb lambda phage (lambda 19.1, Figure 2A) was labeled with biotin by nick-transcription using a Bionick kit from Invitrogen.
with fluorescein. A Cy3-labeled anti-digoxigenin antibody revealed the digoxigenin-labeled M11301 probe. Slides were stained with Hoechst and actinomycin D and signals examined by fluorescence microscopy.

**Ex vivo culture of pre-implantation stage embryos**

All mouse studies were approved by the local Institutional Animal Care and Use Committee (IACUC) and all efforts were made to minimize suffering. *Ctf* heterozygous intercrosses produced an average of 8–9 pre-implantation stage embryos per mating. All embryos collected were from natural matings without the use of hormone-stimulated super-ovulation. To isolate E0.5 cell-stage embryos, mice on the day of cervical plug formation were sacrificed, and under an inverted microscope the egg sac was dissected from the oviduct and incubated briefly in M2 media (Speciality Media) plus hyaluronidase (100 ug/ml) to remove the cumulus layer as previously described [56]. The fertilized eggs were then isolated and cultured individually in 100 ul droplets of M16 media (Speciality Media) in 35 mm Petri dishes flooded with mineral oil. For the culture of E3.5 blastocyst stage embryos, mice were sacrificed three days after cervical plug formation and the uterus flushed with DMEM supplemented with 10% FBS. Under the inverted microscope, individual blastocysts were isolated and then cultured on pre-coated 0.1% gelatinized 6-well plates in DMEM, 10% FBS, and LIF (Leukaemia Inhibitory Factor, ESGRO, 1000 U/ml, Millipore) in a 37°C incubator with 5% CO₂. DNA and RNA was extracted from the freshly isolated or *ex vivo* cultured embryos.

**Genotyping and RT-PCR analysis of pre-implantation stage embryos**

DNA and mRNA were simultaneously extracted from the freshly isolated or *ex vivo* cultured pre-implantation stage embryos using the modified DNA/RNA extraction protocol using the Dynabeads mRNA Direct Kit (Invitrogen). Briefly, pre-implantation stage embryos were collected in a drop of M2 media, rinsed in RNAse Away solution (Molecular Bioproducts) and then placed in 10 ul of the ‘cleared Dynabeads solution’ (DNA lysis buffer) for DNA processing according to the Dynabeads DNA Direct Universal kit protocol (Invitrogen). Following the lysis of the embryo and the DNA/Dynabeads complex formation using 170 ul of the resuspended Dynabeads per embryo, the supernatant was transferred to a clean, pre-chilled tube with 100 ul of the RNA lysis buffer from the Dynabeads mRNA Direct Kit (Invitrogen) and stored on ice for mRNA extraction until the DNA processing was complete. mRNA extraction was carried out using Oligo(dT)25 Dynabeads according to the mRNA Direct Kit protocol (Invitrogen).

For genotyping, the DNA was subjected to multiplex PCR utilizing primer sets recognizing the mutated and wild type *Ctf* alleles. The primer pair for the wild type *Ctf* allele is the following: Forward: 5’ GAG AAA GTA GTT GGT AAT ATG AAC CCT CC 3’ and Reverse: 5’ GGA CAT GTG TAA CTG CAA AGC TCA CAC TG 3’, with a product size of 420 bp. The primer pair identifying the knockout *Ctf* allele includes: Forward: 5’ GGC ATG CTG GGG ATG CCG TGG GCT GTA TGG 3’ and Reverse: 5’ CCA GTG CCC TCT GAT ACA TGA TTT TGA TCG TCC 3’, with a product size of 600 bp. Neo-excised allele genotyping primers are: Forward: 5’ TGA CCT AAG CCT AAC CCT AGC TGA 3’ and Reverse: 5’ TGA AAG TAC CTC TGA GCA AAG GGA 3’, with a product size of 516 bp.

For standard RT-PCR analysis the mRNA extracted from the embryos was reversed transcribed utilizing Superscript III (Invitrogen) under the following conditions. Random hexamers were added to 1–3 µg of RNA and incubated at 70°C for 5 minutes in a thermocycler. The temperature was cycled to 50°C and the RNA/primer mixture equilibrated for 5 minutes. Next, the reaction mixture (nuclease free H₂O, 5× buffer, RNAse inhibitor, dNTPs, DTT, and RT, except for no RT control), pre-equilibrated at 50°C, was added to the RNA/primer mixture and incubated for two hours at 50°C, followed by heat inactivation at 70°C for 5 minutes. *Ctf* expression was analyzed using multiple PCR conditions for both *Ctf* and control Gapdh transcripts. Primers for the *Ctf* transcript (GENBANK U51037.1) included: Sense: 5’ GAG CCT GCT GTA GAA ATT GAA CCT GAG CC 3’, (2188–2216) and Antisense: 5’ CCA ATA ATC GTG GTG GCG CGC GTG ACC AGG GCC CC 3’, (2551–2522) with a product size of 363 bp spanning *Ctf* exons 11 and 12. Primers for the control Gapdh transcript included: Sense: 5’ CGT ATT GGG CGC CGT GTG ACC AGG GC 3’, and Antisense: 5’ GCC ATG AGG TCC ACC ACC CTT GG TCG TGT 3’, with a product size of 950 bp. RNA was tested for DNA contamination using a no-RT control and primers were verified by amplification of genomic DNA. The exact PCR conditions are available upon request.

For the real time RT-PCR analysis, cDNA was generated with SuperScript II reverse transcriptase (Invitrogen) under the following conditions. Random hexamers were added to 1.5 µg of RNA and incubated at 75°C for 5 min then transferred immediately to ice. Next, the reaction mix including 5× SuperScript II buffer, 0.1 M DTT, RNase inhibitor, dNTPs, and SuperScript II RT was added to the RNA/primer mixture and incubated at 45°C for 1 hour, then 50°C for 10 min, followed by heat inactivation at 75°C for 15 min. Real Time PCR analysis was performed on the automated ABI 7900 PCR machine (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) and a *Ctf*-specific Taqman probe and primers spanning the junction between *Ctf* exons 9 and 10 as follows: CTCF_mRNA_F1: 5’-TGG CTT TGT GTG TCT CAA GTG T--3’, probe: 5’-6FAM-ATT CAC CCG CGG GAA CAC AAT GGC A-NFQ-3’, mCTCF_mRNA_R1: 5’-CAG CAC AGT TAT CTG CAT GTC-3’. Amplification of Gapdh was performed in parallel reactions with primers and a Taqman probe as follows: forward primer: 5’-CCCGTAGAACAAAATGGT-GAAGG-3’, probe: 5’-6FAM-CGGTTGAACG-GATTGGCCGTTATT-3BHQ_1-3’, and reverse primer: 5’-AAATGGCAGCCTTGTTGGAATG-3’. PCR was performed according to the manufacturer’s instructions in 20 µL reactions, using 2 µL of 1:1 dilution of cDNA from RT reaction described above. Standard ABI 7900 cycling conditions were followed. Sequence-specific amplification was detected by FAM (reporter dye) fluorescent signal during the amplification cycles. The standard curve assay (as described by Applied Biosystems) with serial dilutions of a standard was used for absolute quantification. Following quantification, *Ctf* mRNA levels were normalized to Gapdh expression. Each sample was assayed in triplicate; data represents mean +/- standard error.

**Immunofluorescence and TUNEL assay**

 Blastocysts were cultured on pre-coated 0.1% gelatinized glass slides or Lab-Tek permannox chamber slides (Nunc), fixed with 3.7% formaldehyde/PBS for 15 minutes and permeablized with 0.5% Triton X-100/PBS at room temperature. The embryos were washed with PBS/PVP (3 mg/ml) and incubated with a blocking solution containing 10% NGS (ImmunoPure normal goat serum, Pierce Biotechnology)/PBS/0.1% Triton X-100 for 1 hour at room temperature followed by overnight incubation with a primary rabbit monoclonal anti-CTCF antibody (Cell Signaling) diluted 1:500 in 2% NGS/PBS/0.1% Triton X-100 at 4°C.
Following a PBS/PVP/0.1% Triton X-100 wash, the embryos were incubated with a secondary goat Texas red-conjugated antirabbit antibody (1:200) (Jackson Laboratory) for 1 hour at room temperature. After washing, slides were counterstained with DAPI. Apoptosis was assessed using the DNA fragmentation end labeling kit (FragEL, Calbiochem) following the manufacturer’s instructions.

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