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Maternal depletion of CTCF reveals multiple functions during oocyte and preimplantation embryo development

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CTCF is a multifunctional nuclear factor involved in epigenetic regulation. Despite recent advances that include the systematic discovery of CTCF-binding sites throughout the mammalian genome, the in vivo roles of CTCF in adult tissues and during embryonic development are largely unknown. Using transgenic RNAi, we depleted maternal stores of CTCF from growing mouse oocytes, and identified hundreds of misregulated genes. Moreover, our analysis suggests that CTCF predominantly activates or derepresses transcription in oocytes. CTCF depletion causes meiotic defects in the egg, and mitotic defects in the embryo that are accompanied by defects in zygotic gene expression, and culminate in apoptosis. Maternal pronuclear transfer and CTCF mRNA microinjection experiments indicate that CTCF is a mammalian maternal effect gene, and that persistent transcriptional defects rather than persistent chromosomal defects perturb early embryonic development. This is the first study detailing a global and essential role for CTCF in mouse oocytes and preimplantation embryos.

KEY WORDS: CTCF, Mouse, Oocyte, Preimplantation embryo, Meiosis

INTRODUCTION

CTCF is an 11 zinc-finger DNA-binding protein that is highly conserved among species, and is expressed in almost all cell types (Filippova, 2008; Ohlsson et al., 2001). It was initially isolated as a factor that binds the *Myc* promoter and represses transcription (Lobanenkov et al., 1990). CTCF has since been shown to function as both a classical repressor and activator of transcription. Moreover, it carries out a multitude of other nuclear functions that depend on genomic and epigenetic context. For example, CTCF was identified as an enhancer blocker at the β-globin locus (Bell et al., 1999), and is the only known protein required for insulator activity in vertebrates. It can interact with both DNA and itself, providing a means by which CTCF molecules bound at remote sites in the genome can be brought together physically. According to a model for vertebrate insulator activity, this would prevent enhancer-promoter interactions between elements on different chromatin loops, while allowing or even facilitating those interactions within the same loop (Engel and Bartolomei, 2003; Wallace and Felsenfeld, 2007). Additionally, CTCF can tether its binding sites to the nucleolus, consistent with a model in which CTCF anchors chromatin loops to nuclear substructures (Yusufzai et al., 2004).

Subsequent to its discovery as an enhancer blocker at the β-globin locus, CTCF was shown to bind four elements within the imprinting control region (ICR) of the *H19/Igf2* locus (Bell and Felsenfeld, 2000; Hark et al., 2000; Kanduri et al., 2000; Szabo et al., 2000). CTCF binds to the maternal ICR, where it prevents the activation of *Igf2* by downstream enhancers that are shared by *H19* and *Igf2*. This allows *H19* exclusive access to enhancers on the maternal allele. On the paternal ICR, DNA methylation prevents CTCF from binding, allowing *Igf2* access to the shared enhancers. Thus, CTCF serves as a methylation-sensitive DNA-binding factor that mediates enhancer blocking at the imprinted *H19/Igf2* locus. Furthermore, CTCF binding is essential for preventing ectopic methylation on the maternal ICR (Engel et al., 2006; Pant et al., 2003; Szabo et al., 2004).

Additional CTCF-binding sites are described at other imprinted loci and on the X-chromosome, where they are proposed to play a regulatory role (Chao et al., 2002; Fitzpatrick et al., 2007; Hikichi et al., 2003; Yoon et al., 2005). Other CTCF-binding sites are located at boundaries between active and repressive chromatin, pointing to a role for CTCF at barrier elements (Barski et al., 2007; Cho et al., 2005; Filippova, 2008). Recently, thousands of CTCF-binding sites have been identified throughout the genome, consistent with a global role for CTCF in chromatin organization (Barski et al., 2007; Kim et al., 2007; Xie et al., 2007). Moreover, CTCF-binding sites overlap with cohesin-binding sites, and CTCF is essential for localizing cohesins to defined sites in the genome (Parelho et al., 2008; Stedman et al., 2008; Wendt et al., 2008). However, despite significant advances made in the past year, there are still no published data describing *Ctcf* deletions in mouse. Moreover, although CTCF has been depleted in mammalian cell culture using RNAi, the in vivo consequences of CTCF depletion are largely unknown. Thus, important physiological aspects of CTCF function remain undescribed, particularly the relevance of CTCF-binding sites throughout the genome, in different tissues of an adult organism and during development.

To understand the role of CTCF at the *H19/Igf2* locus, we previously generated transgenic mice expressing Ctcf dsRNA that depletes CTCF from growing oocytes (Fedoriw et al., 2004). The resulting oocytes are hypermethylated at the *H19* ICR. Moreover, the incidence of development to the blastocyst stage is markedly reduced. To gain insight into the molecular basis of these observations, we now identify hundreds of genes that are misregulated in CTCF-depleted oocytes using microarrays. More genes are downregulated than upregulated; moreover,

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downregulated genes are preferentially closer to CTCF-binding sites, especially in their upstream regions. These results are consistent with a major role for CTCF in transcriptional activation and derepression. In the oocyte, CTCF depletion delays the onset of meiosis and reduces meiotic competence, while preventing chiasmata resolution in a small proportion of eggs that have undergone polar body extrusion. After fertilization, CTCF-depletion delays the second mitotic division, perturbs zygotic genome activation, causes abnormal nuclear morphology and finally leads to apoptotic death prior to the blastocyst stage. Maternal pronuclear transfer and *Ctcf* mRNA microinjection experiments indicate that the two-cell delay is a maternal effect that is not caused by persistent chromatin changes arising in the egg, but is more likely to be caused by persistent transcriptional defects. Thus, *Ctcf* is a mammalian maternal effect gene that affects transcription during oocyte growth, and plays important independent roles in meiotic maturation and early embryonic development.

MATERIALS AND METHODS

Collection and culture of eggs and embryos

Transgenic (Tg) and non-transgenic (Ntg) female mice were genotyped as previously described (Fedoriw et al., 2004). Growing oocytes were collected from 10-day-old Tg and Ntg littermates. Fully-grown germinal vesicle (GV) oocytes were collected from 8- to 10-week-old littermates, and cultured in CZB medium at 37°C in an atmosphere of 5% CO_2 and air. Fertilized one-cell embryos were collected from superovulated and naturally plugged 8- to 10-week-old mice, and cultured in KSOM+AA medium (Ho et al., 1995) at 37°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. All experiments involving mice were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Immunofluorescence and TUNEL staining

Cells were fixed with 2% paraformaldehyde for 25 minutes at room temperature. Embryos were TUNEL stained using In Situ Death Detection Kit (Roche). Immunofluorescence staining was done as previously described (Fedoriw et al., 2004). The antibody working dilutions were 1:3 (anti-CTCF, BD Biosciences, 612149), 1:100 (anti-H2AZ, Abcam, ab4174), 1:500 (anti-SMC1, Bethyl Labs, A300-055A) and 1:1000 (anti-dimethylH3K4, Upstate, 07030; anti-HP1-β, Chemicon, MAB3448; anti-α-Tubulin, Sigma, T6199). For SMC1 staining, embryos were pooled and extracted with 0.1% Triton X-100 prior to fixation. Images were acquired using confocal microscopy (Leica) and mean nuclear fluorescence was quantified using ImageJ v1.36b software.

Karyotyping eggs

Chromosome spreads were prepared as previously described (Tarkowski, 1966). Diakinesis spreads were prepared from eggs having undergone germinal vesicle breakdown (GVBD) by 2 hours post-culture. Metaphase II spreads were prepared from eggs having undergone PBE by 16 hours post-culture. Chromosome spreads were prepared from embryos arrested in prometaphase with 0.04 μg/μl colcemid for 1-2 or 16 hours.

RNA isolation and microarray analysis

GV oocytes were collected from 8- to 10-week-old superovulated mice. Five pairs of Ntg and Tg littermates from five different litters were used. Total RNA was extracted from pools of 25 oocytes per mouse using PicoPure RNA Isolation Kit (Acturus). cDNA was synthesized, amplified and biotin-labeled as previously described (Pan et al., 2005). One Affymetrix MOE430 2.0 GeneChip per mouse was probed with 15 μg cDNA and processed according to Affymetrix instructions. GeneChip tabular data are available at the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo; accession #GSE11664). Raw microarray data were analyzed as previously described using MAS5, GeneSpring v7, SAM and EASE software (Pan et al., 2005). We used a 1.4-fold cutoff for EASE analysis because four biological replicates provided sufficient statistical power and confidence to detect a 1.4-fold change in transcript abundance (Zeng et al., 2004).

Real-time PCR

Total RNA was extracted from GV oocytes using Absolutely RNA Microprep Kit (Stratagene), and reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and random hexamer primers. cDNA was quantified by real-time PCR using an ABI Prism 7000 thermocycler and Taqman probes (Applied Biosystems; Ctcf, Mm00484027_m1; Pim1, Mm00435712_m1; Cbfa2t1h, Mm00486771_m1; Gtl2, Mm00522599_m1; Grb10, Mm01180444_m1; Myc, Mm00487803_m1; Boris/Ctcfl, Mm01242223_m1; Slc22a18, Mm00485426_m1; Phlda2, Mm00493899_g1; Fcgr1, Mm00438874_m1; Tlr1, Mm00446095_m1; Ubft, Mm00456972_m1). Crossing points were normalized to UBTF and converted to relative expression values representing the average of three Tg samples over three Ntg samples. Each sample was analyzed in duplicate wells.

TRC upregulation

Fertilized one-cell embryos were collected from 8- to 10-week-old littermates 24 hours post-hCG, and cultured in CZB medium at 37°C in an atmosphere of 5% CO_2 and air. Embryos were examined for cleavage at 30-minute time intervals, and those that cleaved within a 2-hour time period were cultured for an additional 6, 12 and 21 hours. Embryos were then radiolabeled with 1 μCi/μl [^{35}S]-methionine/CZB, as previously described (Conover et al., 1991). Samples were separated using 10% SDS-PAGE and exposed to a phosphoimager. TRC levels were quantified using ImageQuant TL v2005 software.

Maternal pronuclear transfer experiments

One-cell embryos were collected from 8- to 10-week-old littermates 19 hours post-hCG. Maternal pronuclear transfer was carried out as described (Han et al., 2005). Embryos were cultured until 72 hours post-hCG in KSOM+AA medium at 37°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

Production of Ctcf mRNA and microinjection

An optimized Kozak sequence was added upstream of the start codon at position 307 of a mouse CTCF cDNA sequence p5.1 (GB accession #U51037) subcloned into a Bluescript plasmid (Filippova et al., 1996). The CTCF-coding sequence, 66 bp of the modified 5′ UTR, and the first 267 bp of the 3′ UTR were then subcloned into an In-Vitro Transcription plasmid (pIVT) containing a synthetic poly(A) tail. Capped and polyadenylated *Ctcf* mRNA was transcribed using MEGAscript Kit (Ambion). RNA was purified using MEGAclear Kit (Ambion), precipitated and resuspended at 2 μg/μl in water. One-cell embryos were collected from 8- to 10-week-old littermates 19 hours post-hCG, and microinjected with 5-10 pl of Ctcf mRNA at a concentration of 1 or 2 μg/μl. Control embryos were injected with an equivalent amount of *Gfp* mRNA. Embryos were cultured until 72 hours post-hCG in KSOM+AA medium at 37°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

Statistical analysis

Graphpad Prism 4 software was used to calculate statistical significance. Two-tailed *t*-tests, one-way ANOVA or Fisher's exact tests were used as appropriate.

RESULTS

CTCF depletion in oocytes and embryos

We previously generated Tg mice expressing Ctcf dsRNA under the control of the zona pellucida 3 (*Zp3*) promoter (Fedoriw et al., 2004). The transgene depletes CTCF specifically in growing oocytes because the *Zp3* promoter is active only in these cells. Five independent Tg lines have been previously described (Fedoriw et al., 2004). Depending on the line, Ctcf transcripts were reduced in fully-grown germinal vesicle (GV) oocytes by 50% to 99% relative to controls. We selected the three most severely affected lines for further study, all of which produced GV oocytes that were similarly CTCF-depleted (> 99% reduction; Table 1). Nevertheless, the three Tg lines exhibited phenotypic differences, described below, that

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Table 1. Validation of gene misregulation by real-time PCR

Gene	Microarray Line 1	Real-time PCR Line 1	Line 12	Line 21
Ctcf	0.03	0.0094±0.0004	0.0094±0.003	0.0092±0.002
Pim1	0.48	0.62±0.06	0.54±0.26	0.38±0.06
Cbfa2t1h	0.55	0.61±0.06	0.62±0.15	0.69±0.08
Gtl2	0.55	0.53±0.08	0.51±0.02	0.55±0.05
Grb10	0.84	0.78±0.04	0.61±0.15	0.68±0.24
Myc	ND	1.05±0.12	0.91±0.35	0.86±0.18
Boris	ND	A:A	A:A	A:A
Slc22a18	1.77	2.80±0.18	1.88±0.50	1.94±0.21
Phlda2	1.86	2.18±0.23	1.55±0.15	2.16±1.15
Fcgr1	35.9	75.0±6.1	76.4±23.9	73.0±10.8
Tlr1	58.0	1:A	1:A	1:A

Values are transgenic expression relative to non-transgenic expression±s.e.m.
1:A, detected in transgenic oocytes but absent from non-transgenic oocytes.
A:A, absent from both transgenic and non-transgenic oocytes.
ND, not determined.

could have resulted from differences in the time course of CTCF depletion during oocyte growth. We therefore examined CTCF protein levels in growing oocytes, when the *Zp3* promoter controlling transgene expression is initially activated and maternal transcripts are beginning to accumulate. Growing oocytes from Line 1 were the most severely CTCF-depleted (86% reduction), followed by those from Line 21 (59% reduction) and Line 12 (33% reduction; Fig. 1A). CTCF mRNA levels correlated with protein levels (data not shown). Therefore, differences in CTCF depletion were apparent among the three most severely affected lines.

Although the *Zp3* promoter-driven transgene is only active during oocyte growth, RNAi probably persists in the GV oocyte, and for several cell divisions after fertilization (Wianny and Zernicka-Goetz, 2000). Persistent RNAi should affect all embryos derived from Tg females, regardless of whether the embryos themselves inherit the transgene. We therefore determined the protein expression patterns of CTCF in embryos derived from wild-type and Tg female littermates. In embryos derived from Ntg females, CTCF was excluded from nucleoli, but was otherwise diffusely localized in the nucleus throughout preimplantation development (Fig. 1B). CTCF was upregulated gradually from the morula to the late blastocyst stages of development (72 to 120 hours post-hCG). However, in embryos derived from Tg Line 1, CTCF was depleted through the morula and into the early blastocyst stages of development (72 and 96 hours post-hCG, respectively; Fig. 1B). Because zygotic CTCF is transcribed at the two-cell stage (48 hours post-hCG; data not shown), depletion of CTCF from later stages of preimplantation embryo development was most probably due to persistent RNAi (Fig. 1C).

Transcriptional misregulation in CTCF-depleted oocytes

Given the early and severe depletion of CTCF in Tg oocytes, and the multitude of CTCF-binding sites throughout the genome, we expected that many genes would be misregulated. We therefore compared the expression profiles of Tg and Ntg GV oocytes using Affymetrix MOE430 2.0 GeneChips, which cover over 39,000 transcripts and variants. Oocytes from five pairs of Ntg and Tg

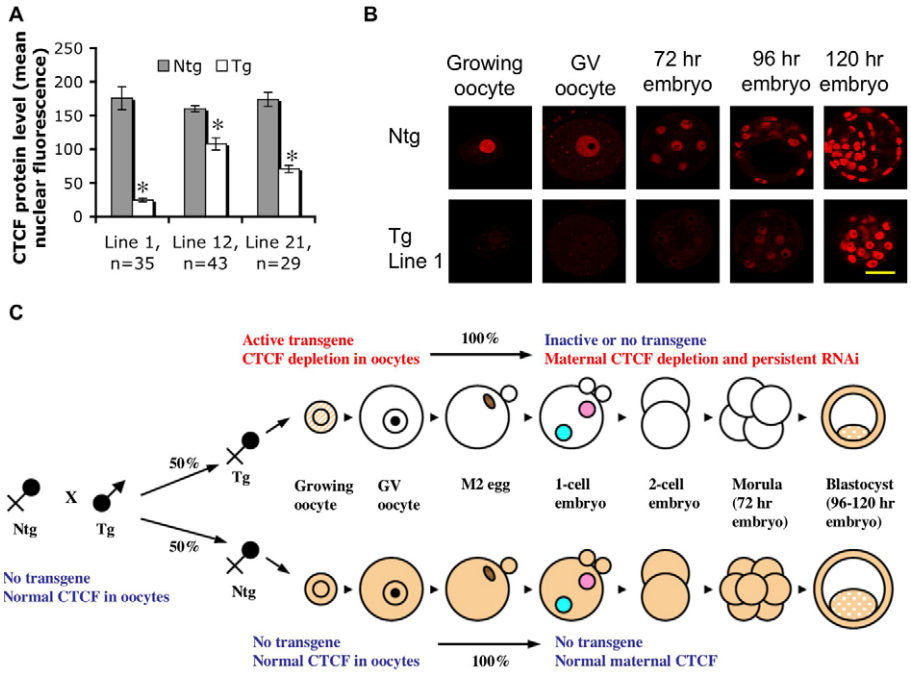

Fig. 1. CTCF depletion in oocytes and embryos. (**A**) Quantification of nuclear CTCF immunofluorescence staining in growing oocytes. Ntg and Tg indicate oocytes derived from 10-day-old Ntg and Tg littermates, respectively. Lines 1, 12 and 21 are shown. **P*<0.0001; *n*=number of oocytes. (**B**) Immunofluorescence images of CTCF-stained oocytes and embryos. One confocal section of an egg or embryo is shown per panel, with red indicating CTCF-stained nuclei. Line 1 is shown. Scale bar: 40 μm. (**C**) Schematic of CTCF depletion in oocytes and embryos. Ntg females were mated to Tg males and ~50% of female progeny inherited the transgene. All oocytes from Tg females were CTCF depleted. CTCF-replete oocytes and embryos are depicted in brown, whereas CTCF-depleted oocytes and embryos are depicted in white. Pink and blue circles represent maternal and paternal pronuclei, respectively. GV, germinal vesicle; hr, hours post-hCG.

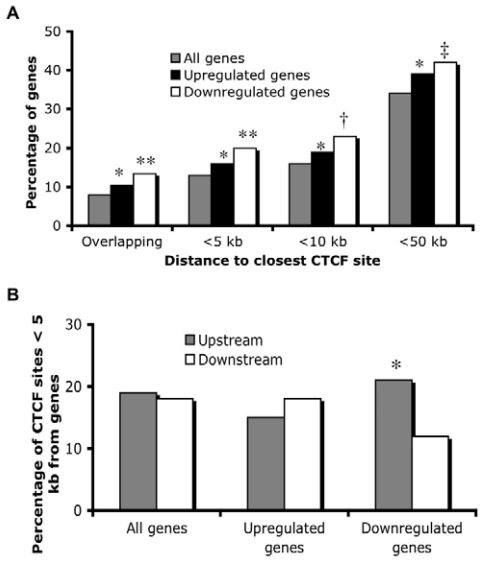

Fig. 2. Transcriptional misregulation in CTCF-depleted oocytes. (**A**) Percentages of genes within 0 (overlapping), 5, 10 and 50 kb of a CTCF-binding site. The proportions of nearby upregulated and especially downregulated genes were significantly higher than background (all genes) within every distance threshold. *$P<0.05$; **$P<10^{-8}$, $^{\dagger}P<10^{-7}$, $^{\ddagger}P<10^{-5}$. (**B**) Percentages of CTCF binding sites within 5 kb of all, upregulated and downregulated genes. Among sites within 5 kb of downregulated genes, the percentage of upstream sites was significantly higher than the percentage of downstream sites, compared with background. *$P<0.05$.

littermates were analyzed independently. To minimize false positives, we restricted analysis to 20,396 transcripts that were 'Present' according to Microarray Analysis Suite 5 (MAS5) software, in four out of five Ntg or Tg replicates. An unsupervised hierarchical cluster analysis revealed that all replicates clustered according to their genotype (see Fig. S1 in the supplementary material). Statistical Analysis of Microarrays (SAM) revealed 1590 significantly upregulated and 2282 significantly downregulated transcripts (FDR<5%). Of these transcripts, 460 were upregulated and 934 were downregulated by more than 1.4-fold, while 115 were upregulated and 278 were downregulated by more than twofold (see Table S1 in the supplementary material). Experimental Analysis Systematic Explorer (EASE) grouped 1.4-fold misregulated transcripts into several over-represented functional categories. (see Tables S2-S10 in the supplementary material). Interestingly, the most significantly over-represented categories were related to embryogenesis, suggesting that transcriptional defects in CTCF-depleted oocytes might affect subsequent embryonic development.

We then determined if CTCF-binding sites were enriched in areas of the genome near misregulated genes. We obtained the genomic locations of 5584 predicted and experimentally identified mouse CTCF sites from CTCFBSDB [(Bao et al., 2008); www.insulatordb.utmem.edu]. We obtained the genomic locations of all mouse genes from Ensemble Biomart (www.ensembl.org). For each mouse gene, we determined the distance to the closest CTCF-binding site, using 0 as the distance between a gene and an overlapping site. We found that upregulated and downregulated genes were both significantly closer to CTCF-binding sites than background, and this trend was strong up to a distance of 10 kb. In addition, the trend was much stronger for downregulated genes. For example, 13% of all genes, 16% of upregulated genes ($P<0.05$) and 20% of downregulated genes ($P<10^{-8}$) were located within 5 kb of a CTCF site (Fig. 2A). Next, we determined if CTCF binding sites were preferentially upstream or downstream of misregulated genes. Among CTCF sites within 5 kb of all genes, 19% were upstream and 18% were downstream of a gene. The remaining sites overlapped with genes. Among CTCF sites within 5 kb of upregulated genes, 15% were upstream and 18% were downstream. Most notably, among CTCF sites within 5 kb of downregulated genes, 21% were upstream and only 12% were downstream ($P<0.05$; Fig. 2B). Thus, among downregulated genes the closest CTCF-binding sites were significantly biased toward upstream locations. These results suggest that downregulated genes were CTCF targets rather than indirect targets or off-targets of RNAi. Moreover, considering that the majority of misregulated genes in CTCF-depleted oocytes was downregulated, these results suggest that CTCF predominantly activates or derepresses transcription in oocytes.

As expected, CTCF was the most highly downregulated gene (Table 1). We validated eight additional misregulated genes using real-time PCR (Table 1). Because several putative CTCF targets were not identified in our analysis, we examined two putative targets (*Myc* and *Ctcfl*/*Boris*) using real-time PCR. We found that although CTCF is a repressor of these genes in cell culture (Filippova et al., 1996; Qi et al., 2003; Vatolin et al., 2005), their expression was not upregulated in CTCF-depleted oocytes (Table 1). This is interesting in light of several studies showing that CTCF binding is largely invariant across cell lines (Gombert et al., 2003; Kim et al., 2007). It is therefore likely that the transcriptional output of CTCF binding varies in a cell-type specific manner, possibly as a consequence of different binding co-factors and post-translational modifications.

Meiotic defects in CTCF-depleted oocytes

Given the high level of transcriptional misregulation in CTCF-depleted oocytes, we examined oocyte nuclei using several anti-chromatin antibodies. Nuclear protein levels of dimethylH3K4, H2Az and HP1-β were unchanged in Tg oocytes derived from Line 1 (see Fig. S2A-F in the supplementary material). We also scored the numbers of surrounded nucleolus (SN)-type and non-surrounded nucleolus (NSN)-type GV oocytes, which can be distinguished based on nuclear morphology. NSN-type nuclei contain several discrete foci of heterochromatin on a background of decondensed euchromatin. Immediately prior to ovulation, NSN-type nuclei may transition to SN-type nuclei that are entirely heterochromatic and transcriptionally silent (Zuccotti et al., 1995). Although both subtypes can undergo meiotic maturation in vitro, SN-type oocytes are more meiotically competent (Liu and Aoki, 2002). Interestingly, we found that the frequency of the SN subtype was slightly but significantly decreased among Tg oocytes derived from all three lines (Table 2).

We therefore determined the rate of oocyte maturation by isolating and culturing GV oocytes, whereupon they resumed meiosis. At the end of prophase (diakinesis), the nuclear membrane dissolves and the apparent disappearance of the nucleus is known as germinal vesicle breakdown (GVBD). Slightly fewer Tg GV oocytes were able to undergo GVBD (data not shown). However, among oocytes that underwent GVBD, 26% of Ntg oocytes and only 2% of Tg oocytes had undergone GVBD by 1 hour post-culture. By 2 hours post-culture, roughly equal percentages of Ntg and Tg oocytes had undergone GVBD (Table 2). Thus, GVBD was delayed in CTCF-depleted oocytes. By 16 hours post-culture, 56% of Ntg oocytes and only 32% of Tg (Line 1) oocytes had extruded a polar body, signifying completion of meiosis I. A similar difference was

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Table 2. Meiotic defects in CTCF-depleted oocytes

Line	Genotype	0 hours* % SN-type (*n*)	1 hour* % GVBD (*n*)	2 hours* % GVBD (*n*)	16 hours* % Ana/telo 1 (*n*)	16 hours* % PBE (n)
1	Ntg	68.7 (131)	26.0 (50)	76.0 (50)	9.5 (63)	55.6 (63)
	Tg	48.0 (98)	2.0 (50)	74.0 (50)	16.1 (62)	32.3 (62)
		P<0.01	*P*<0.001	*P*=1	*P*=0.3	*P*<0.05
12	Ntg	64.5 (110)	ND	ND	3.2 (62)	38.7 (62)
	Tg	57.6 (92)	ND	ND	6.2 (81)	24.7 (81)
		P=0.384			*P*=0.7	*P*=0.099
21	Ntg	81.5 (119)	ND	ND	4.8 (62)	58.1 (62)
	Tg	64.9 (111)	ND	ND	12.1 (66)	28.8 (66)
		P<0.01			*P*=0.21	*P*<0.01

*Germinal vesicle (GV) oocytes were cultured for the indicated number of hours.
SN-type, surrounded nucleolus-type GV oocytes.
GVBD, germinal vesicle breakdown.
Ana/telo 1, anaphase or telophase of meiosis 1.
PBE, polar body extrusion.
Ntg, non-transgenic; Tg, transgenic.
n, number of oocytes.
ND, not determined.

observed in all three Tg lines (Table 2). Longer periods of culture did not increase the incidence of polar body extrusion (PBE; data not shown). Thus, meiotic competence was decreased among CTCF-depleted oocytes.

To determine whether defects in chromosome segregation accompanied delayed meiotic maturation and decreased meiotic competence, we prepared diakinesis and metaphase-II chromosome spreads at 2 and 16 hours post-culture, respectively. By 2 hours post-culture, all Ntg and Tg eggs that underwent GVBD contained roughly 20 bivalents, and no obvious defects in chiasmata number or sister chromatid cohesion were apparent. By 16 hours post-culture, all Ntg eggs having undergone PBE contained roughly 20 univalents. However, although the majority of Tg eggs contained 20 apparently normal univalents, 7% of Tg eggs having undergone PBE contained roughly 20 bivalents (Fig. 3). Thus, PBE occurred in some Tg eggs without chiasmata resolution and chromosome segregation.

We observed no defects in spindle morphology using an antibody against α-tubulin (data not shown). However, we did find an increased proportion of Tg eggs in anaphase or telophase of metaphase 1, which presumably resulted from an overall delay in meiosis (Table 2). Eggs arrested at metaphase I can form 2n polar bodies upon fertilization, leading to digynic triploid embryos that die at various stages post-implantation (Kaufman and Speirs, 1987). Consistent with these observations, 8% of CTCF-depleted eggs formed triploid embryos when fertilized in vivo, whereas no Ntg eggs formed triploid embryos (see Fig. S3 in the supplementary material).

Early mitotic defects leading to apoptosis

We previously showed that although Tg eggs are fertilized in vitro at a normal incidence, fewer develop to the blastocyst stage by 120 hours post-hCG (Fedoriw et al., 2004). To determine when the decrease in developmental competence was first apparent, we cultured in vivo fertilized one-cell embryos and examined development at various time points after hCG injection. CTCF-depleted one-cell embryos cleaved at a normal rate, and by 48 hours post-hCG, almost all control and CTCF-depleted one-cell embryos reached the two-cell stage (data not shown). However, by 72 hours post-hCG, when control embryos were at the four- to eight-cell stage, CTCF-depleted embryos were at the two- to four-cell stage (Fig. 4A). Therefore, the decrease in developmental competence was first apparent at the two- to four-cell transition.

Because zygotic genome activation (ZGA) is essential for development beyond the two-cell stage, we determined whether ZGA was defective in CTCF-depleted embryos. An accepted marker for ZGA is the transcription requiring complex (TRC),

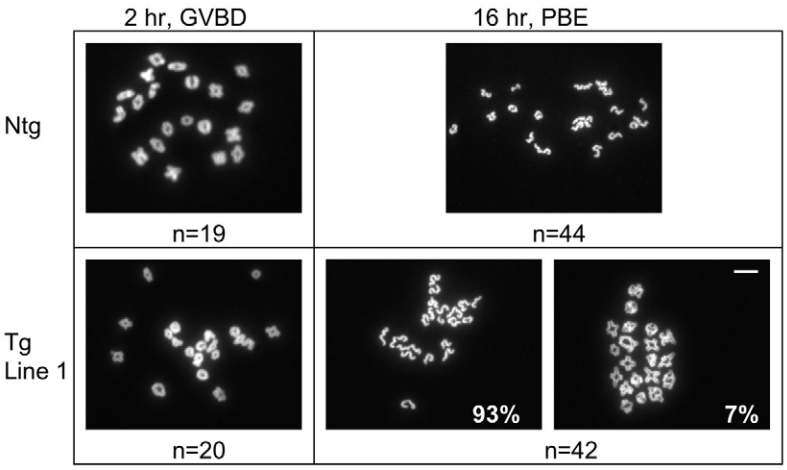

Fig. 3. Meiotic defects in CTCF-depleted oocytes. Eggs having undergone GVBD by 2 hours of culture were processed for diakinesis spreads. Eggs that have undergone PBE by 16 hours of culture were processed for M2 spreads. Line 1 is shown. Seven percent (3/42) of Tg eggs that have undergone PBE contained roughly 20 bivalents. *n*=number of oocytes. Scale bar: 0.1 μm.

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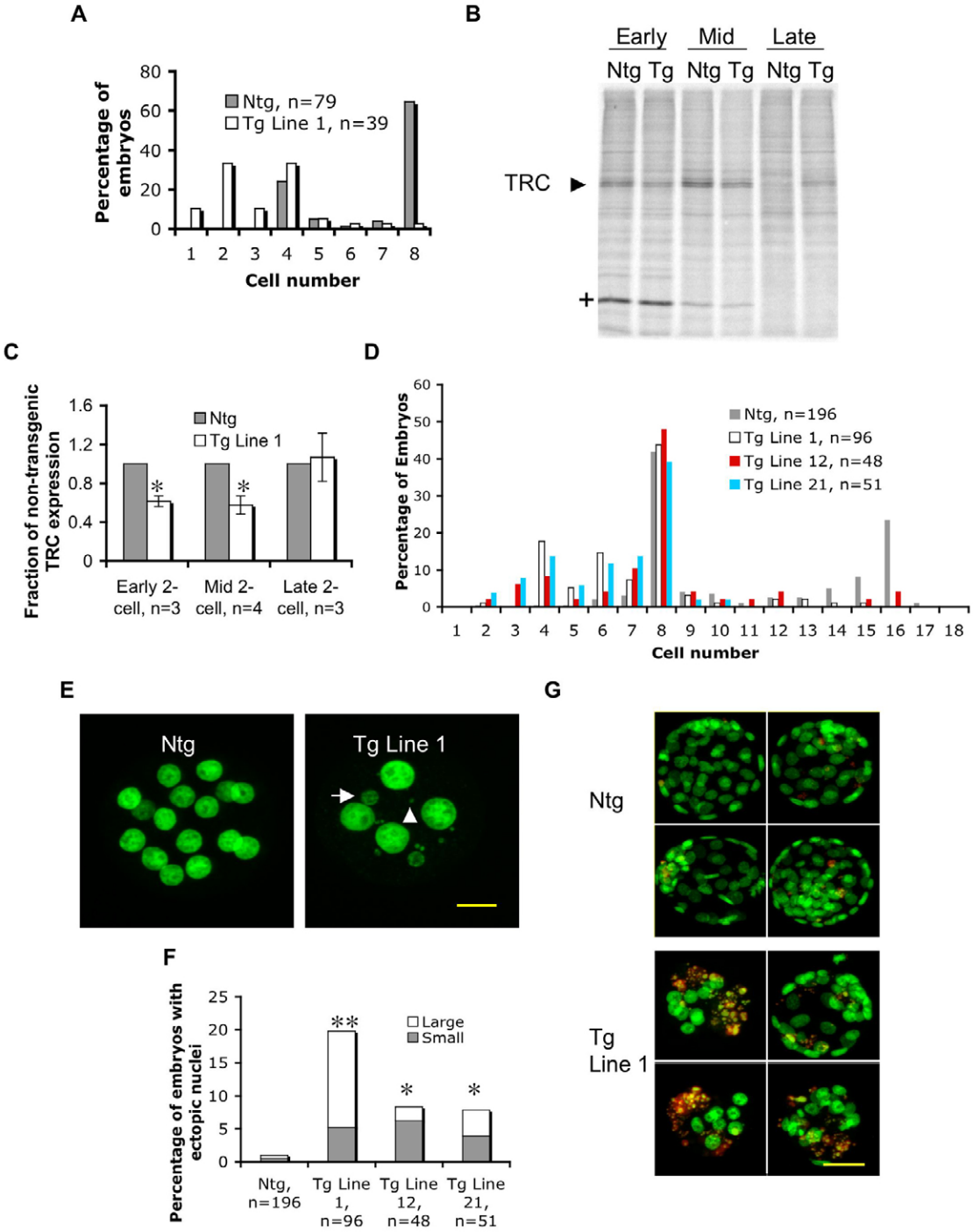

Fig. 4. Early mitotic defects leading to apoptosis. (**A**) Embryo development in culture. One-cell embryos were cultured to 72 hours post-hCG and categorized according to cell number. Line 1 is shown. *n*=number of embryos. (**B**) Autoradiogram and (**C**) quantification of TRC upregulation. Two-cell embryos from Line 1 were radiolabeled at various timepoints post-cleavage. At each timepoint, TRC expression in 3-5 embryos derived from Tg mice were normalized to TRC expression in an equivalent number of embryos derived from Ntg mice (relative expression=1). Early, Mid and Late indicate embryos radiolabeled at 6, 12 and 21 hours post-cleavage; *$P<0.05$; *n*=number of experiments. + indicates Spindlin, an abundant maternal protein. (**D**) Embryo development in vivo. Cleavage-stage embryos were flushed at 72 hours post-hCG and categorized according to cell number. Lines 1, 12 and 21 are shown. *n*=number of embryos. (**E**) Immunofluorescence images of DAPI-stained embryos in D. One confocal section of an embryo is shown per panel, with green indicating DAPI-stained nuclei. Line 1 is shown. Tg embryos have ectopic nuclei. Arrow, large ectopic nucleus; arrowhead, small ectopic nucleus. Scale bar: 20 μm. (**F**) Percentages of embryos in D with ectopic nuclei. Embryos were categorized according to whether large (white bars) or small (gray bars) ectopic nuclei were observed. Lines 1, 12 and 21 are shown. *$P<0.05$; **$P<0.0001$; *n*=number of embryos. (**G**) Apoptosis at 120 hours post-hCG. One-cell embryos were cultured to 120 hours post-hCG and TUNEL stained. One embryo is shown per panel, with green indicating DAPI-stained nuclei and red indicating TUNEL staining. Line 1 is shown. Only 7% (2/28) of embryos derived from Tg mice versus 94% (29/31) of embryos derived from Ntg littermates formed a blastoceol cavity by 120 hours post-hCG. Scale bar: 40 μm.

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which is composed of three α-amanitin-sensitive proteins ~70 kDa in weight (Conover et al., 1991). In control two-cell embryos, TRC expression initiated by 6 hours after the first cleavage, peaked by 12 hours post-cleavage, and was downregulated by 22 hours post-cleavage. In CTCF-depleted two-cell embryos, TRC expression was reduced by 40% relative to control two-cell embryos at both 6 and 12 hours post-cleavage (Fig. 4B,C). It is therefore possible that a disruption in ZGA caused a developmental delay at the two-cell stage.

We also flushed cleavage-stage embryos from oviducts of Ntg and Tg females. By 72 hours post-hCG, embryos flushed from oviducts of Tg females were at the four- to eight-cell stage, whereas embryos flushed from oviducts of Ntg females were at the eight- to 16-cell stage (Fig. 4D). Thus, after spending more time at the two-cell stage, CTCF-depleted embryos were delayed by about one cell division, and this delay persisted at later timepoints (data not shown). Among cleavage-stage embryos flushed at 72 hours post-hCG, some exhibited abnormal ectopic nuclei (Fig. 4E). The frequency of embryos with ectopic nuclei was less than 1% among embryos derived from Ntg females, whereas the frequency was up to 20% among embryos derived from Tg females (Fig. 4F). Abnormal embryos were negative for cleaved caspase 3 and phosphorylated H2Ax (data not shown), indicating an absence of apoptosis or widespread DNA damage, but consistent with a mitotic defect leading to improper chromosome segregation.

In mammalian cell culture, CTCF is essential for localizing cohesins to specific sites throughout the genome; however, this localization is important for gene regulation at interphase rather than for sister chromatid cohesion during mitosis (Parelho et al., 2008; Stedman et al., 2008; Wendt et al., 2008). Consistent with these findings, mitotic chromosomes from CTCF-depleted two- to four-cell embryos exhibited no cohesion defects. Moreover, no significant differences in the rates of sister chromatid resolution or chromatid arm separation during mitotic arrest were observed, indicating that cohesins dissociated from chromatin normally during prophase and prometaphase (see Fig. S4A,B in the supplementary material). Finally, antibody staining of CTCF-depleted and control two- to four-cell embryos showed normal levels of chromatin-bound SMC1 during interphase (see Fig. S4C,D in the supplementary material).

By 120 hours post-hCG, 94% of cultured control embryos were at the blastocyst stage, whereas only 7% of cultured CTCF-depleted embryos were at the blastocyst stage. The remaining embryos were at the morula stage or at various stages prior to morula compaction. A limited amount of apoptosis is normal in blastocysts, and may be required to eliminate suboptimal blastomeres from the embryo prior to implantation. In agreement with previous reports (Fabian et al., 2005), we observed TUNEL-positive cells in the inner cell mass and trophectoderm of control blastocysts, but prior to 120 hours post-hCG only the polar body was TUNEL positive. Similarly, we did not observe apoptosis in CTCF-depleted embryos at earlier timepoints; however, by 120 hours post-hCG many CTCF-depleted embryos arrested at the morula stage exhibited abnormally high levels of apoptosis (Fig. 4G; data not shown). CTCF-depleted embryos having formed a blastoceol cavity appeared small but otherwise normal (Fig. 4G, upper left 'Tg' embryo). Thus, CTCF-depleted embryos failing to reach the blastocyst stage at the time of implantation were probably eliminated by apoptosis. Because CTCF levels were normal at this timepoint, apoptosis was presumably a downstream effect of earlier defects.

Maternal pronuclear transfer and Ctcf mRNA-injection experiments

Given the importance of CTCF in chromatin organization, we initially hypothesized that CTCF depletion caused a persistent nuclear change in the egg that resulted in the early mitotic defect. Such persistent changes could have included defects in meiosis or genome-wide epigenetic changes passed down from the egg and inherited by the embryo. To test this hypothesis, we performed maternal pronuclear transfer experiments in which maternal pronuclei of CTCF-depleted one-cell embryos were exchanged with maternal pronuclei of control one-cell embryos. By 48 hours after transfer, unmanipulated control embryos were at the four- to eight-cell stage, whereas unmanipulated CTCF-depleted embryos were at the two-cell stage. In control experiments, as expected, exchanging maternal pronuclei among control embryos or among CTCF-depleted embryos did not affect development. Surprisingly, introducing CTCF-depleted maternal pronuclei into control embryos resulted in development to the four- to eight-cell stage. These reconstructed embryos had normal nuclear CTCF protein levels by 48 hours after transfer, presumably because control embryos were replete with cytoplasmic stores of CTCF mRNA (Fig. 5A; data not shown). This result suggests that persistent nuclear changes arising in the egg did not cause the early mitotic defect, or that the changes could be reversed at the one-cell stage.

By contrast, introducing control maternal pronuclei into CTCF-depleted embryos resulted in development to the two-cell stage, suggesting that the early mitotic defect was caused by the depletion of maternal CTCF transcripts and possibly other maternal transcripts stored in the cytoplasm (Fig. 5A). However, persistent RNAi depleted CTCF until at least 48 hours after transfer, well after zygotic transcription of CTCF had begun (data not shown). Therefore, in order to separate maternal effects from the effects of persistent RNAi, we injected mouse *Ctcf* mRNA into one-cell embryos. By 48 hours after injection, control Gfp-injected embryos reached the four- to eight-cell stage, whereas CTCF-depleted Gfp-injected embryos reached the two- to four-cell stage. Injecting *Ctcf* mRNA into CTCF-depleted one-cell embryos restored nuclear protein levels by 6 and 48 hours after injection, but the resulting embryos were delayed at the two-cell stage (see Fig. S5 in the supplementary material; Fig. 5B,C). Therefore, maternal Ctcf transcripts were important for embryonic development, suggesting that CTCF is a maternal-effect gene. In addition, the data suggest that the two-cell delay was a consequence of transcriptional misregulation in the oocyte rather than a direct effect of CTCF depletion in the embryo.

DISCUSSION

Maternal-effect genes are transcribed in the oocyte, and are essential for normal embryonic development. To date, relatively few maternal effect genes have been identified in mammals. These include *Mater*, *Hsf1*, *Dnmt1o*, *Pms2*, *Stella*, *Npm2*, *Zar1*, *Formin2*, *mHR6a* and *E-cadherin*, all of which have been studied using traditional knockout technology (Burns et al., 2003; Christians et al., 2000; De Vries et al., 2004; Gurtu et al., 2002; Howell et al., 2001; Leader et al., 2002; Payer et al., 2003; Roest et al., 2004; Tong et al., 2000; Wu et al., 2003). However, useful this approach has been, it only uncovers maternal effect genes with relatively minor functions in other essential processes. Because ZGA in mammals occurs long before lineage specification and embryo patterning occur, many mammalian maternal effect genes could have important general functions that preclude the analysis of homozygous null females. More recent studies have used promoter-driven transgenes to deplete

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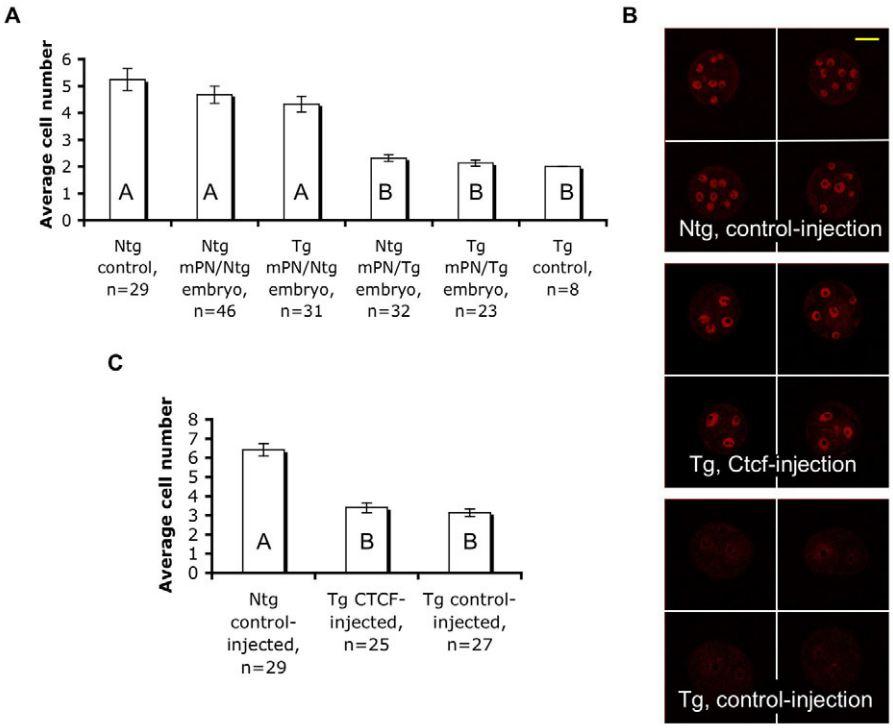

Fig. 5. Maternal pronuclear transfer and Ctcf mRNA-injection experiments. (**A**) Maternal pronuclear transfer experiments. Maternal pronuclei (mPNs) were reconstructed with one-cell embryos and cultured until 72 hours post-hCG. The average cell number per transfer-type at 72 hours post-hCG is indicated. Ntg and Tg (Line 1) indicate mPNs or embryos derived from Ntg and Tg littermates, respectively. Bars labeled A are significantly different from bars labeled B (overall $P<0.0001$). n=number of embryos. (**B**) Immunofluorescence images of CTCF-stained embryos after cytoplasmic mRNA microinjection. One-cell embryos from Line 1 were microinjected with CTCF or control GFP mRNA, and cultured until 72 hours post-hCG. One confocal section of an embryo is shown per panel, with red indicating CTCF-stained nuclei. Scale bar: 40 μm. (**C**) Embryo development after cytoplasmic mRNA microinjection. The average cell number per microinjection-type at 72 hours post-hCG is indicated. Bars labeled A are significantly different from bars labeled B (overall $P<0.0001$). n=number of embryos.

maternal transcripts without affecting maternal viability. For example, *Ezh2* and *Brg1* have been knocked out in growing oocytes using tissue-specific Cre/LoxP (Bultman et al., 2006; Erhardt et al., 2003), whereas basonuclin transcripts have been depleted from growing oocytes using transgenic RNAi (Ma et al., 2006).

Presently, there is no clear consensus on how directly a maternal gene influences embryonic processes in order for it to be considered a 'maternal-effect' gene. In many cases, maternal effects are believed to be independent of potential defects in oocyte growth and meiotic maturation, although this has only been proved in the case of *Brg1* (Bultman et al., 2006). However, in the case of *Formin2*, the maternal effect is clearly downstream of meiotic defects that lead to polyploidy and post-implantation lethality in the embryo (Leader et al., 2002). In many other cases, the molecular functions of maternal-effect genes and the full extent of their effects on oocyte development are simply unknown. Using transgenic RNAi, we have severely depleted CTCF from growing oocytes and identify hundreds of misregulated genes. However, despite such a high degree of gene misregulation in growing oocytes, the importance of CTCF does not become grossly apparent until meiotic maturation and the two-cell embryonic stage. In addition, we show that the two-cell mitotic defect is not the result of earlier meiotic defects or other persistent nuclear defects arising in the egg. Thus, *Ctcf* can be added to a short list of mammalian maternal effect genes, a list that will probably grow with the use of oocyte-specific gene-targeting methods.

Three recent studies have identified thousands of CTCF-binding sites throughout the human and mouse genomes (Barski et al., 2007; Kim et al., 2007; Xie et al., 2007). In the first study, a ChIP-Chip approach was used to identify 13,804 CTCF-binding sites, over 75% of which share a consensus motif (Kim et al., 2007). In the second study, a computational approach was used to identify 233 conserved noncoding elements (CNEs) (Xie et al., 2007). Three CNEs are bound by CTCF, and are found at 14,987 sites in the genome, which constitutes ~25% of the total number of sites identified. In the third study, a ChIP-Seq approach was used to identify 20,262 CTCF-binding sites (Barski et al., 2007). We have identified hundreds of misregulated genes in CTCF-depleted oocytes. The number of downregulated genes is greater than the number of upregulated genes, especially among highly misregulated genes. Moreover, downregulated genes are enriched for nearby CTCF-binding sites, especially in their upstream regions. Although it is likely that some transcriptional changes are indirect effects of CTCF depletion, the results suggest that many downregulated genes are direct CTCF targets. Moreover, the results are consistent with recent findings that CTCF-binding sites are enriched for active histone marks, and may activate genes by recruiting RNA polymerase II (Chernukhin et al., 2007) or by preventing the spread of heterochromatin. By contrast, upregulated genes are not as strongly associated with CTCF-binding sites, and nearby sites are not biased toward their upstream regions. These data could be consistent with CTCF acting as an enhancer-blocker to repress gene transcription from a distance, possibly by competing with promoters for nearby enhancers (Yoon et al., 2007) or by stalling the linear transfer of activating factors (Zhao and Dean, 2004). However, this mechanism of repression is less likely in oocytes and embryos prior to two-cell ZGA because, lacking a required co-factor, they apparently do not use enhancers (Majumder et al., 1997).

Given the multitude of CTCF-binding sites throughout the genome, and its diverse roles in nuclear organization, it is notable that in the absence of appreciable levels of CTCF, oocyte growth is apparently not perturbed, and defects in meiotic maturation are relatively minor. For example, only 7% of Tg oocytes fail to segregate chromosomes at anaphase I, and all oocytes can be fertilized regardless of meiotic defects. By contrast, after fertilization most CTCF-depleted embryos are delayed at the two-cell stage, and only 7% of CTCF-depleted embryos are able to form a blastoceol cavity. Moreover, maternal pronuclear transfer and RNA microinjection experiments suggest that persistent transcriptional defects rather than persistent chromatin defects result in the early

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mitotic delay. Overall, these results point to an important role for CTCF in transcriptional regulation rather than in chromatin structure per se.

With respect to specific misregulated genes, several are imprinted. The maternally expressed transcript *Gtl2* is downregulated, consistent with a role for CTCF in transcriptional activation at the *Gtl2/Dlk1* locus (Paulsen et al., 2001). In addition, the maternally expressed gene *Grb10* is downregulated, and although CTCF is hypothesized to function as an insulator on the paternal allele (Hikichi et al., 2003), our results suggest that CTCF may activate *Grb10* on the maternal allele. However, two adjacent maternally expressed genes, *Slc22a18* and *Phlda2*, are upregulated. This is consistent with a role for CTCF in transcriptional repression at the *Kcnq1* locus (Fitzpatrick et al., 2007). Furthermore, the non-imprinted gene *Cbfa2t1h* is downregulated by almost twofold. *Cbfa2t1h* is a putative maternal effect gene that was identified as such because its transcripts are enriched in oocytes and one-cell embryos (Mager et al., 2006). However, *Cbfa2t1h* cannot easily account for the phenotypes because its protein levels appear only slightly decreased (data not shown). Nevertheless, it is possible that a combination of many transcriptional defects contribute to the phenotypes in CTCF-depleted embryos, or that a few highly misregulated genes have undiscovered maternal effects.

The two-cell delay and disruption of ZGA are consistent with a maternal effect because maternally derived products are presumed to regulate these early processes. We have yet to determine whether TRC expression in CTCF-depleted embryos is reduced throughout the two-cell stage, or if TRC expression peaks at a timepoint between 12 and 22 hours post-cleavage. In addition, we have not yet determined if other markers of ZGA are disrupted. However, with respect to TRC expression, there is a high degree of heterogeneity among small groups of CTCF-depleted two-cell embryos, and this heterogeneity increases as the two-cell stage progresses. This suggests that transcriptional defects in the oocyte may be compounded to varying degrees in individual embryos. Near the end of preimplantation development, a small number of embryos survive and implant. This may be a consequence of transcriptional heterogeneity during ZGA, or it may reflect differences in the time course of CTCF depletion among individual growing oocytes. This also implies that CTCF can restore chromatin organization and transcription de novo, but we do not know whether more subtle consequences of early CTCF-depletion persist. It will be interesting to determine what long-term epigenetic effects persist in CTCF-depleted embryos subsequent to implantation.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/16/2729/DC1

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