

Mediation of CTCF transcriptional insulation by DEAD-box RNA-binding protein p68 and steroid receptor RNA activator SRA

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CCCTC-binding factor (CTCF) is a DNA-binding protein that plays important roles in chromatin organization, although the mechanism by which CTCF carries out these functions is not fully understood. Recent studies show that CTCF recruits the cohesin complex to insulator sites and that cohesin is required for insulator activity. Here we showed that the DEAD-box RNA helicase p68 (DDX5) and its associated noncoding RNA, steroid receptor RNA activator (SRA), form a complex with CTCF that is essential for insulator function. p68 was detected at CTCF sites in the *IGF2/H19* imprinted control region (ICR) as well as other genomic CTCF sites. In vivo depletion of SRA or p68 reduced CTCF-mediated insulator activity at the *IGF2/H19* ICR, increased levels of *IGF2* expression, and increased interactions between the endodermal enhancer and *IGF2* promoter. p68/SRA also interacts with members of the cohesin complex. Depletion of either p68 or SRA does not affect CTCF binding to its genomic sites, but does reduce cohesin binding. The results suggest that p68/SRA stabilizes the interaction of cohesin with CTCF by binding to both, and is required for proper insulator function.

[Keywords: RNA-binding protein; noncoding RNA; CTCF; insulator function]

Supplemental material is available at <http://www.genesdev.org>.

Received July 8, 2010; revised version accepted September 20, 2010.

Chromatin insulators are DNA elements that can protect a gene from outside influences, which might lead to either inappropriate activation or silencing of the gene (Kellum and Schedl 1991; Chung et al. 1993; Geyer and Corces 1992; Bushey et al. 2008). One kind of insulator action can block interaction between an enhancer and a promoter when it lies between them; a number of such DNA elements and their associated protein factors have been identified in *Drosophila* (Moon et al. 2005; Wallace and Felsenfeld 2007), most notably the *Gypsy* element and its associated DNA-binding protein, Suppressor of Hairy-wing, which recruit multiple cofactors essential to the insulator activity (Geyer and Corces 1992; Georgiev and Kozycina 1996; Pai et al. 2004). In vertebrates, the CCCTC-binding factor CTCF is the principal protein with well-established insulator function (Bell et al. 1999;

Bell and Felsenfeld 2000; Hark et al. 2000; Kanduri et al. 2000). Work in many laboratories has shown that CTCF-binding sites are widely distributed in vertebrate genomes (Barski et al. 2007; Kim et al. 2007; Xie et al. 2007; Cuddapah et al. 2009). Recent studies suggest that their primary function is to establish contacts between these sites, stabilizing long-range interactions (Gaszner and Felsenfeld 2006; Phillips and Corces 2009; Sandhu et al. 2009), and either separating or bringing together distant regulatory elements. In this view, insulation is a consequence of a particular configuration in which the insulator lies between the enhancer and the promoter and prevents their interaction.

CTCF is a highly conserved 11-zinc-finger, DNA-binding protein (Ohlsson et al. 2001) implicated in diverse regulatory functions, including transcriptional activation/repression and X chromosome inactivation (Filippova et al. 1996; Vostrov and Quitschke 1997; Chao et al. 2002; Phillips and Corces 2009). The role of CTCF in mediating enhancer-blocking insulation was initially identified at the 5' DNase-hypersensitive site 4 (5'HS4) insulator of the chicken β -globin locus (Bell et al. 1999). CTCF was

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Article published online ahead of print. Article and publication date are online at <http://www.genesdev.org/cgi/doi/10.1101/gad.1967810>.

subsequently found to control, through its insulator activity, allele-specific expressions of *IGF2* and *H19* in the mouse and human *IGF2/H19* loci (Bell et al. 1999; Bell and Felsenfeld 2000; Hark et al. 2000; Kanduri et al. 2000). It has been shown that CTCF binds to multiple sites on the maternal allele within the imprinted control region (ICR) that lies between *IGF2* and the endodermal enhancers controlling its expression, effectively blocking those enhancers and silencing *IGF2* expression. In contrast, DNA methylation of the ICR on the paternal allele prevents CTCF binding and allows *IGF2* expression (Bell and Felsenfeld 2000; Hark et al. 2000; Kanduri et al. 2000; Holmgren et al. 2001). Depletion of CTCF or mutation of its binding sites results in loss of imprinting of *IGF2* and *H19* (Engel et al. 2008; Wendt et al. 2008) and alters the pattern of long-range intranuclear contacts (Engel et al. 2008; Yoon et al. 2007).

CTCF insulator activity requires a number of protein cofactors that interact with CTCF. Earlier studies have identified the SNF2-like chromodomain helicase protein CHD8 and the Polycomb group subunit Suz12 as mediating CTCF insulator function, although the mechanisms of their action have not been reported (Ishihara et al. 2006; Li et al. 2008). Recent attention has focused on the cohesin complex, which interacts with CTCF and is found at a large fraction of CTCF sites in vivo (Parelho et al. 2008; Rubio et al. 2008; Wendt et al. 2008). Depletion of cohesin subunit concentration in cells strongly inhibits the insulator action of CTCF, affecting both gene expression and long-range physical contacts in the surrounding region (Hadjur et al. 2009; Nativio et al. 2009; Hou et al. 2010). Given the known properties of cohesin in bringing sister chromatids together during S phase and through G2 phase into mitosis, one attractive hypothesis is that cohesin serves an analogous function in bringing together distant CTCF-occupied sites during interphase. However, it is not known what other factors may be involved in establishing or maintaining such structures.

In this study, we report that the DEAD-box RNA helicase p68 (DDX5) and its associated noncoding RNA, steroid receptor RNA activator (SRA), are both essential in vivo for insulator function at the *IGF2/H19* ICR. p68/SRA is present at the ICR in mouse and human cells. Our evidence suggests that it is important because it binds to both CTCF and cohesin and helps stabilize the cohesin-CTCF interaction.

Results

RNA helicase p68 interacts with insulator-binding protein CTCF

To identify previously unknown CTCF-associated cofactors, Flag-HA-tagged CTCF was purified by tandem affinity purification of nuclear extracts (NEs) from a stably transduced HeLa cell line, and copurifying factors were identified by mass spectrometry (MS). Results from MS analysis identified the DEAD-box RNA helicase protein p68, also known as DDX5, as a factor that copurified with Flag-HA-CTCF (data not shown). The MS analysis also

identified p72, a protein partially homologous to p68, as well as components of the cohesin complex, which has been shown to interact with CTCF (Rubio et al. 2008) and which colocalizes with it at CTCF-binding sites in vivo (Parelho et al. 2008; Wendt et al. 2008). To confirm the MS results, we carried out Flag coimmunoprecipitation (co-IP) using NEs from the same cell line. p68 was retained in Flag-HA-CTCF-expressing cells following IP with an anti-Flag antibody, but not from cells expressing Flag-HA alone (Fig. 1A). To further verify the endogenous interaction of p68 with CTCF, co-IP analysis was carried out using NEs from wild-type HeLa cells. Both p68 and CTCF were pulled down using a rabbit anti-p68 antibody, but not with rabbit IgG (Fig. 1B). This interaction was not confined to HeLa cells; similar co-IP experiments in murine NIH3T3 NEs demonstrated that murine p68 could be copurified with CTCF using a rabbit anti-CTCF antibody (Fig. 1C). In addition, we confirmed colocalization of endogenous CTCF and p68 proteins by double immunostaining; localization was primarily nuclear (Supplemental Fig. 1).

Domain mapping

To investigate whether p68 also interacts with CTCF in vitro, we performed glutathione-S-transferase (GST) pull-down assays using GST-CTCF and GST-p68 fusion proteins expressed and purified from *Escherichia coli* BL21 cells. The results showed that GST-CTCF but not GST could pull down p68 (Fig. 1D). Similarly, GST-p68 but not GST could pull down CTCF (Fig. 1E). We undertook domain mapping of both CTCF and p68 using GST fusion proteins and HeLa NEs to identify the regions of the proteins responsible for their interaction. Full-length GST-CTCF and a GST construct containing only the zinc fingers (amino acids 291–576) pulled down endogenous p68 in HeLa NEs, whereas GST constructs of the N-terminal (amino acids 1–291) or C-terminal (amino acids 576–727) regions of CTCF did not pull down p68 (Fig. 1F,G). To determine the domains of p68 responsible for binding to CTCF, a series of GST-p68 deletion constructs was used to pull down CTCF from HeLa NEs (Fig. 1H). Both the N-terminal and C-terminal domains of p68 (amino acids 1–96 and amino acids 475–614, respectively) bound to CTCF (Fig. 1I). In contrast, the central RNA helicase core domain (amino acids 94–477) did not (Fig. 1J).

The binding of p68 to CTCF is RNA-dependent

p68 is an RNA-binding protein and is required for processing of rRNA and a subset of microRNAs (Fukuda et al. 2007). To examine whether the binding of p68 to CTCF was DNA- or RNA-dependent, we first performed GST or GST-CTCF pull-down assays with HeLa NEs in the presence or absence of DNase I or RNase A. Interactions between p68 and CTCF were not changed following DNase I digestion (Fig. 2A), indicating that this interaction was DNA-independent. In contrast, we found that the binding of p68 to CTCF decreased compared with control when RNase A was included in the reaction, indicating that RNA may play a role in this interaction (Fig.

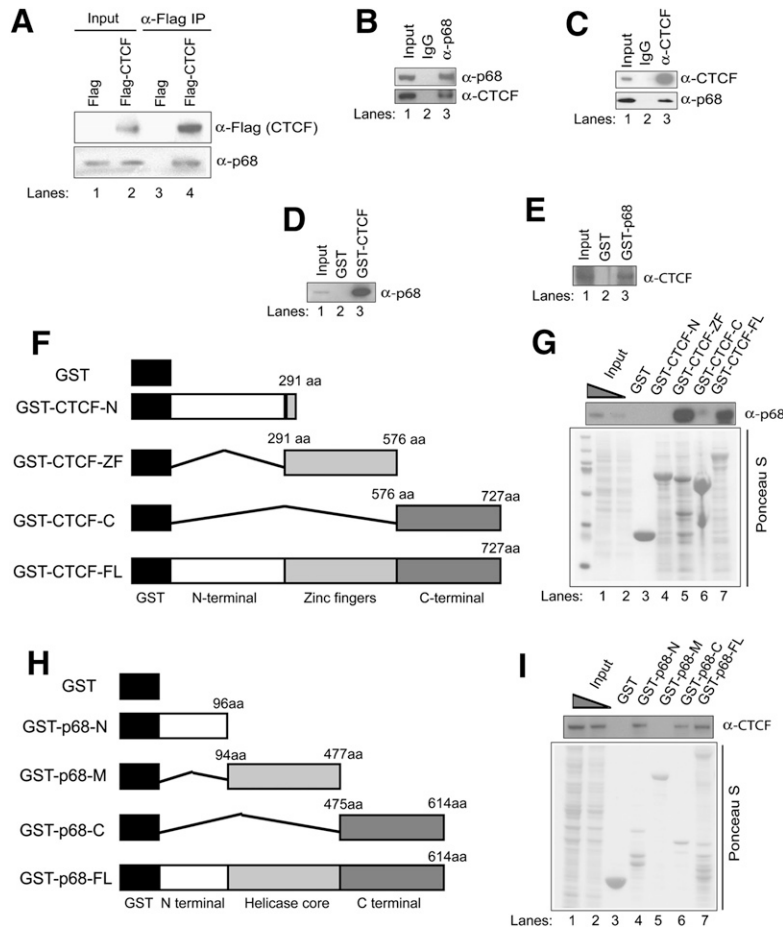


Figure 1. RNA-binding protein p68 interacts with insulator-binding protein CTCF. (A) NEs from HeLa cells stably expressing Flag or Flag-tagged human CTCF were used in Flag co-IP experiments and analyzed by immunoblotting with anti-Flag and anti-p68 antibodies. (B) HeLa NEs were precipitated with rabbit IgG or rabbit anti-p68 antibody, shown on the *top* of the panel, and precipitates were immunoblotted with antibodies, shown on the *right* of the panel. (C) NIH3T3 NEs were precipitated with mouse IgG or mouse anti-CTCF antibody, shown on the *top* of panel, and precipitates were immunoblotted with antibodies, shown at the *right* of panel. (D) Western blot of input, GST-associated, and GST-CTCF-associated proteins isolated from HeLa NEs probed with anti-p68 antibody. (E) Western blot of input, GST-associated, and GST-p68-associated proteins isolated from HeLa NEs probed with anti-CTCF antibody. (F) Schematic representation of GST-CTCF and its deletions used in GST pull-down assays. (G, *top* panel) GST pull-down assay using immobilized GST only or recombinant GST-CTCF N terminus (GST-CTCF-N), zinc fingers (GST-CTCF-ZF), C terminus (GST-CTCF-C), and full-length (GST-CTCF-FL) proteins probed with anti-p68 antibody by Western blot. (*Bottom* panel) Ponceau S stain showing total protein present on the membrane before Western blot. Lanes 1 and 2 had 5% and 2% of the input protein samples, respectively. (H) Schematic representation of GST-p68 and its deletions used in GST pull-down assays. (I, *top* panel) GST pull-down assay using immobilized GST only or recombinant GST-p68 N terminus (GST-p68-N), RNA helicase core region (GST-p68-M), C terminus (GST-p68-C), and full-length (GST-p68-FL) proteins probed with anti-CTCF antibody by Western blot. (*Bottom* panel) Ponceau S stain showing total protein present on the membrane before Western blot.

(GST-p68-FL) proteins probed with anti-CTCF antibody by Western blot. (*Bottom* panel) Ponceau S stain showing total protein present on the membrane before Western blot.

2B). To further confirm this result, we treated magnetic bead immunoprecipitated CTCF complexes with RNase A and then isolated the tightly bound proteins of the bead fraction and the released proteins of the supernatant fraction. Although incubation at 37°C in the absence of RNase A partially released CTCF-bound p68, in the presence of RNase A, 1.51-fold to 1.88-fold more p68 was released from the CTCF-associated bead fraction with the increasing amount (50–200 $\mu\text{g}/\text{mL}$) of RNase A in the reaction (Fig. 2C). A corresponding decrease of p68 in the CTCF-associated bead fraction was seen, consistent with the results in Figure 2B and suggesting that RNA mediates the binding of p68 to CTCF.

Noncoding SRA is present in a CTCF complex

Previous studies have shown that p68/p72 interacts with the noncoding RNA SRA (Watanabe et al. 2001). SRA can be present as both an RNA transcript and a stably expressed protein (SRAP), and appears to be the first example of a molecule active at both the RNA and protein level (Lanz et al. 1999; Chooniedass-Kothari et al. 2004; Colley et al. 2008; Colley and Leedman 2009). Most

recently, it has been shown that SRAP is a transcriptional modulator of estrogen receptor α (ER α) and is recruited to specific promoter regions where it acts as a transcriptional repressor (Chooniedass-Kothari et al. 2010a,b); however, the function of SRAP still remains largely unknown. In order to determine if either SRA or SRAP was present in the CTCF–p68 complex, cell extracts from HeLa cells expressing Flag only or Flag-CTCF were immunoprecipitated with anti-Flag antibody and separated into two fractions for RNA and Western blot analysis. We could not detect a band using an antibody for SRAP in the Flag-CTCF co-IP, although it was present in the input (Fig. 3A), indicating that the protein encoded by SRA was not associated with CTCF. Noncoding SRA transcripts generated through alternative splicing of intron 1 have been characterized previously (Hube et al. 2006; Leygue 2007). Extracted RNAs from the co-IP material were treated with DNase I and subjected to reverse transcription, and the cDNA was amplified with primers specific for either SRA or GAPDH (as a negative control). Our data showed that the noncoding RNA for SRA coimmunoprecipitated with Flag-CTCF, but not with Flag alone (Fig. 3B). In addition, we found that SRA coimmunoprecipitated with

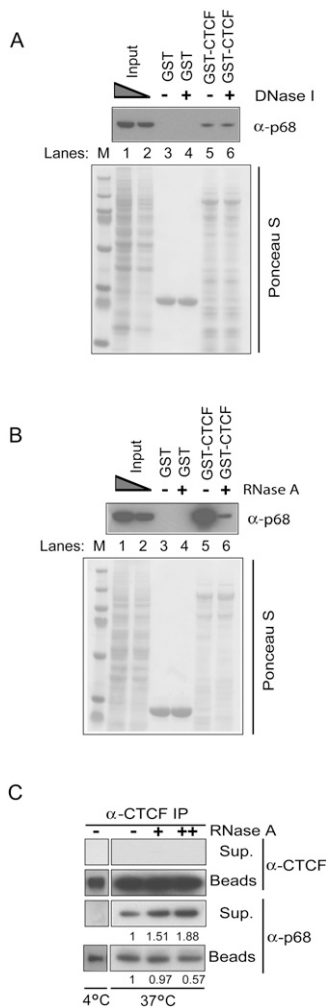


Figure 2. p8 binding to CTCF is RNA-dependent. HeLa NEs were incubated with purified GST or full-length GST-CTCF fusion protein immobilized on glutathione beads overnight at 4°C in the presence (+) or absence (–) of DNase I (A) or RNase A (B). Blots were then probed with anti-p68 antibody. Ponceau S stains in A and B show total protein present on the membranes before Western blotting. (C) CTCF co-IPs from HeLa NEs were digested with increasing amounts of RNase A, separated into supernatant (Sup.) and bead (Beads) fractions, and then subjected to immunoblot with antibody to either CTCF or p68.

the endogenous human CTCF following CTCF co-IP in HeLa cells, but SRAP did not (Fig. 3C,D, respectively). Since CTCF is a DNA-binding protein and SRA binds directly to p68 (Watanabe et al. 2001), the recruitment of SRA to the CTCF complex may be mediated by p68.

Next, we investigated if SRA mediates the interaction between p68 and CTCF. We performed co-IP using an anti-CTCF antibody after SRA knockdown in HeLa cells and found that the binding of p68 to CTCF was decreased by ~30% following SRA knockdown (Fig. 3E), suggesting that either small amounts of SRA present after knockdown are sufficient to give partial CTCF/p68 interaction, or one or more other RNA species can partially substitute for SRA.

p68 and SRA are required for CTCF insulator function

Previous studies have shown that CTCF cofactors such as CHD8 and the cohesin complex are required for CTCF-mediated transcriptional insulation (Ishihara et al. 2006; Parelho et al. 2008; Wendt et al. 2008). The observation that p68, SRA transcript, and CTCF have the potential to form an RNA–protein complex prompted us to test whether either p68 or SRA was involved in CTCF-mediated insulator function. To test insulator activity, we used the previously published pHLIE reporter construct, which carries a luciferase gene driven by the H19 promoter and is flanked by wild-type mouse ICR insulator sequences (Fig. 4A, top panel), and the pHLME control reporter, in which insulating activity is destroyed by mutation of the CTCF-binding sites of one of the ICRs (Fig. 4A, bottom panel) (Ishihara et al. 2006). When CTCF is bound to the ICR sequences of pHLIE, enhancer activity from a nearby SV40 enhancer is blocked. The HeLa cells were first transduced with either shRNA lentiviral particles targeted to p68 or CTCF, or siRNA targeted to SRA, then the pHLIE or pHLME plasmid was cotransfected into HeLa cells with a renilla luciferase control plasmid to measure relative luciferase activity. The knockdown efficiencies of CTCF, p68, and SRA RNA in pHLIE or pHLME transfected HeLa cells were determined by quantitative RT–PCR (qRT–PCR) (Supplemental Fig. 3). Our data indicated that the knockdown of p68, SRA, or CTCF enhanced luciferase activity by twofold in cells transfected with pHLIE compared with that in control shRNA or siRNA (Fig. 4B,C). When the pHLME control reporter was used (Fig. 4A, bottom panel)—in which insulating activity was destroyed by mutation of the CTCF-binding sites—neither p68, SRA, nor CTCF knockdown had any effect on luciferase activity (Fig. 4B,C).

Our laboratory previously identified and described the CTCF-dependent enhancer-blocking insulator properties at the 5'HS4 of the chicken β -globin locus (Bell et al. 1999; Recillas-Targa et al. 1999). Studies have also shown that expression of a *neomycin* (*neo*) resistance gene contained within a chicken β -globin core insulator plasmid is significantly reduced when flanked by insulators, whereas loss of the insulator activity through CTCF knockdown leads to full *neo* gene expression (Recillas-Targa et al. 1999; Parelho et al. 2008). To examine whether the insulator function of 5'HS4 is affected by either p68 or SRA, we transiently transfected pNI control or pNI-core insulator plasmids (Fig. 4D) into CTCF-, p68-, or SRA-depleted K562 cells (Supplemental Fig. 4A–C). As expected, in cells not depleted of these factors, *neo* gene expression was reduced in the pNI-core insulator plasmid when compared with the uninsulated pNI control plasmid, and depletion of CTCF returned *neo* gene expression to that of the uninsulated control (Fig. 4E,F). Interestingly, like CTCF depletion, depletion of either p68 or SRA also returned *neo* expression to levels comparable with that seen in the uninsulated control (Fig. 4E,F), suggesting that both p68 and SRA are required for 5'HS4 insulator function. In addition, we also found that knockdown of p72 increases *neo* gene expression in pNI-core transfected K562 cells, suggesting that

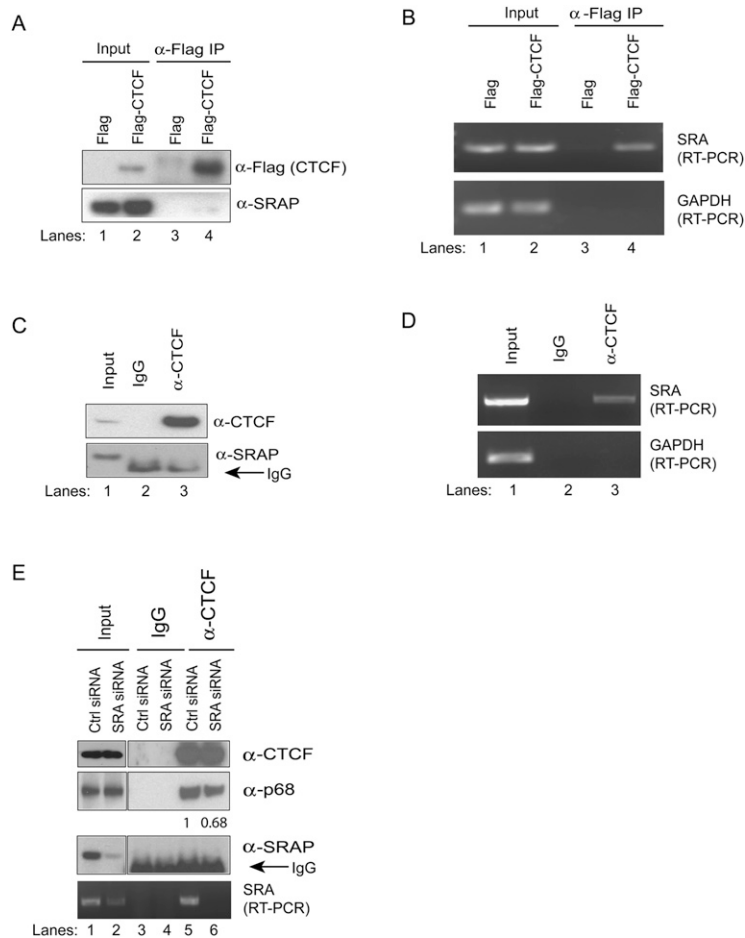


Figure 3. The noncoding RNA SRA binds to CTCF. NEs from HeLa cells stably expressing Flag or Flag-tagged human CTCF were used in Flag co-IP experiments and either analyzed by immunoblotting with anti-SRAP and anti-Flag antibodies (A), or total RNA was extracted and subjected to RT-PCR with the primers specific to SRA and GAPDH (B). (C,D). HeLa NEs were precipitated with mouse IgG or mouse anti-CTCF antibody. Samples were then either immunoblotted with anti-CTCF or anti-SRAP antibodies (C), or total RNA was extracted and subjected to RT-PCR with the primers specific to SRA and GAPDH (D). (E) Control (Ctrl) siRNA- and SRA siRNA-depleted HeLa NEs were precipitated with mouse IgG or mouse anti-CTCF antibody and immunoblotted with anti-p68 antibody (top) or anti-SRAP antibody (middle), or RNA was extracted and samples were analyzed by RT-PCR with the primers specific to SRA (bottom).

p72, which typically forms a heterodimer with p68 (Caretta et al. 2006), also affects insulator activity (Supplemental Fig. 5). Taken together, our data show that knockdown of either p68/p72 or SRA results in the loss of the insulator function activities at both the *H19* ICR and 5'HS4 insulators.

p68 and SRA regulate imprinted expression at the IGF2/H19 locus

p68 and SRA are required for CTCF-mediated enhancer-blocking insulator function in the plasmid assays described above. Next, we asked whether p68 and SRA affect "parent of origin"-specific transcription of the imprinted genes at the *IGF2/H19* locus, which is controlled to a large extent by the *IGF2/H19* ICR and mediated by CTCF binding. The ICR ensures that *IGF2* is expressed from the paternal allele (DeChiara et al. 1991); *H19* is transcribed from the maternal allele (Bartolomei et al. 1991). Imprinting at this locus depends mostly on the insulating activity of CTCF, which binds to ICR sites on the maternal allele, but not on the paternal allele, which is methylated (Bell and Felsenfeld 2000; Hark et al. 2000; Kanduri et al. 2000).

HeLa cells have been used previously to examine imprinting of the *IGF2/H19* locus following cohesin de-

pletion (Wendt et al. 2008). To determine whether p68 regulates imprinting at the *IGF2/H19* locus, we measured the transcript levels of *H19* (Fig. 5A) and *IGF2* (Fig. 5B) in CTCF- or p68-depleted HeLa cells (Supplemental Fig. 2A,C). Although *IGF2* expression is relatively low compared with *H19* expression in control cells, *H19* and *IGF2* transcripts could be detected by RT-qPCR. *H19* transcript levels were reduced by factors of ~0.7 and 0.5 in HeLa cells after CTCF and p68 depletions, respectively (Fig. 5A), while *IGF2* transcript levels were increased ~1.5-fold to twofold in CTCF- or p68-depleted HeLa cells (Fig. 5B).

Primary mouse embryonic fibroblasts (MEFs) in which the maternal and paternal *IGF2/H19* alleles can be distinguished have also been used to study imprinting at the *IGF2/H19* locus (Engel et al. 2008). To investigate whether p68 and CTCF also regulate the imprinting of *IGF2* and *H19* in these MEF cells, we depleted the expression of p68 as well as CTCF (as a positive control) by using either murine CTCF-shRNA or p68-shRNA (Supplemental Fig. 2B,D), and examined the transcript levels of *IGF2* and *H19* in these cells. shRNA-mediated knockdowns of CTCF or p68 decreased *H19* transcript levels ~50% and 40%, respectively (Fig. 5C), while *IGF2* expression was increased ~1.76-fold and 1.35-fold, respectively, in MEF cells (Fig. 5D).

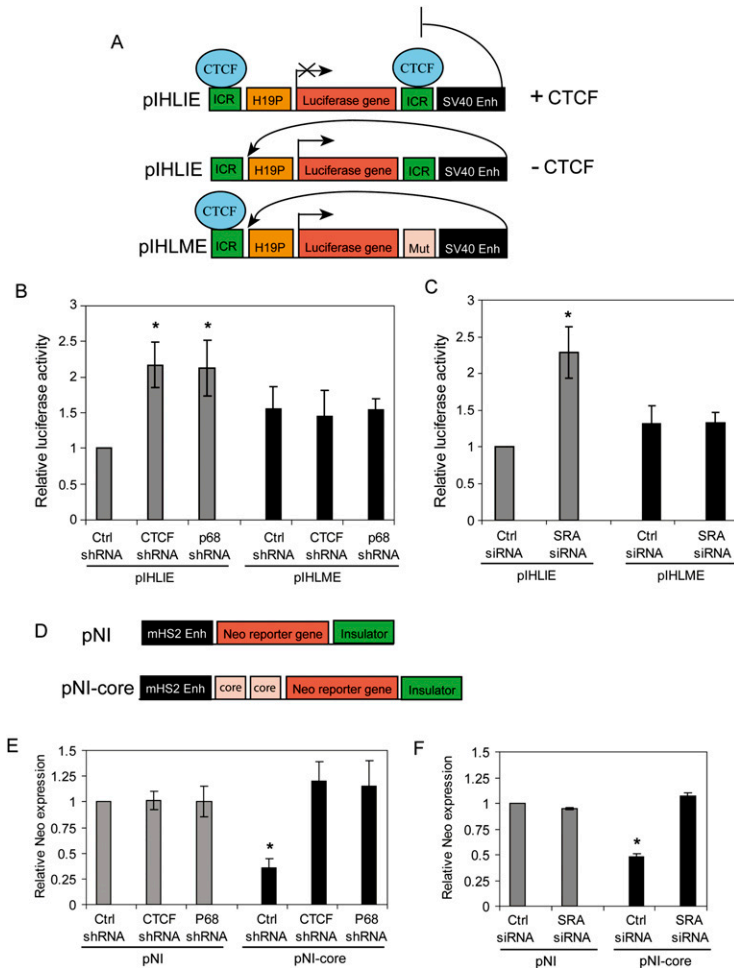


Figure 4. Both p68 and SRA are required for CTCF-mediated insulator function. (A) Schematic diagrams of the reporter constructs pIHLIE and pIHLME that were used in enhancer-blocking assays. In pIHLIE, CTCF binds to the H19 ICR and represses luciferase gene expression by blocking enhancer access to the promoter. In pIHLME, CTCF-binding sites have been mutated. (Enh) Enhancer; (H19P) mouse H19 promoter; (Mut) mutated ICR. (B) CTCF or p68 shRNA-depleted HeLa cells were transfected with either pIHLIE or pIHLME and a renilla luciferase control plasmid. Cells were harvested and luciferase activities were measured 48 h after transfection of the luciferase plasmids. Relative luciferase activity was determined for each cell lysate and normalized against control shRNA. (C) HeLa cells were depleted by SRA siRNA and then, 24 h later, transfected with either pIHLIE or pIHLME and a renilla luciferase control plasmid. Cells were harvested and luciferase activities were measured 24 h after transfection of luciferase plasmids. Relative luciferase activity was determined for each cell lysate and normalized against control siRNA. (D) Schematic representation of the pNI control and pNI-5'HS4 core insulator plasmids that were used in insulator assays. The plasmid backbone of pNI or pNI-core is pGEM4Z. pNI-core contains two copies of the chicken β -globin HS4 core insulator fragments, which are located between the mouse HS2 enhancer and *Y-neomycin* (*Neo*) reporter gene and which block the contact between the HS2 enhancer and *Neo* reporter gene. (E) K562 cells were depleted by CTCF or p68 shRNA and transfected with either pNI or pNI-core plasmid together with a GFP plasmid. Cells were harvested and RNAs were isolated for reverse transcription to examine the expression of the Neo reporter gene after 24 h of transfection. The expression of Neo was normalized against the expression of GFP. (F) SRA siRNA-depleted K562 cells were transfected with either pNI or pNI-core. Cells were harvested and

RNAs were isolated for reverse transcription to examine the expression of the Neo reporter gene after 24 h of transfection. Relative neomycin expression was determined for each cell lysate and normalized against control shRNA (E) or control siRNA (F) samples. Data were analyzed by Student's *t*-test. For all panels, bars represent mean \pm SD of *n* = 3. (*) *P* < 0.01.

To investigate whether SRA has the same effect as p68 on *H19* and *IGF2* transcript levels, we depleted SRA expression in HeLa cells (Supplemental Fig. 2E). Transcript levels of *H19* were decreased ~25% (Fig. 5E) and that of *IGF2* was increased about twofold (Fig. 5F). Similar effects of SRA depletion were observed in MEF cells (data not shown). Taken together, these data suggest that both p68 and SRA may have an important role in regulating imprinting at the *IGF2/H19* locus, possibly through their association with CTCF.

p68 binds to the IGF2/H19 ICR at the maternal allele

Since we found that p68 interacted with CTCF and regulated the imprinting of *IGF2* and *H19*, we asked whether p68 could be found at CTCF-binding sites at the *IGF2/H19* ICR in HeLa cells. Chromatin immunoprecipitation (ChIP) was carried out using an anti-CTCF antibody and each of three different anti-p68 antibodies, followed by qPCR. Both CTCF and p68 were bound in vivo at the *IGF2/H19* ICR, but not at a CTCF-negative

control site (Fig. 6A,B; Wendt et al. 2008). In order to determine if p68 binding to the *IGF2/H19* ICR was allele-specific, we performed similar ChIP assays in MEF cells and found that 92.8% of the CTCF-bound fraction and 79% of p68-bound fraction were at the maternal allele (Fig. 6C). These data support the role of p68 in CTCF-mediated insulation at the *IGF2/H19* locus.

Coincidence of p68, CTCF, or cohesin binding on a genome-wide scale

We wanted to determine whether other CTCF-binding sites in the genome are also associated with p68. We identified 10,837 p68-binding sites in HeLa cells using a preliminary ChIP-seq analysis not intended to reveal all such sites. Twenty-two percent of these p68-binding sites overlap a non-tissue-specific CTCF-binding site (Fig. 6D; Schmidt et al. 2010), far more than the expected 2% (*P* < 10^{-250} , one-sided binomial test) (Supplemental Material). This overrepresentation was also observed when we performed the same analysis using CTCF sites derived

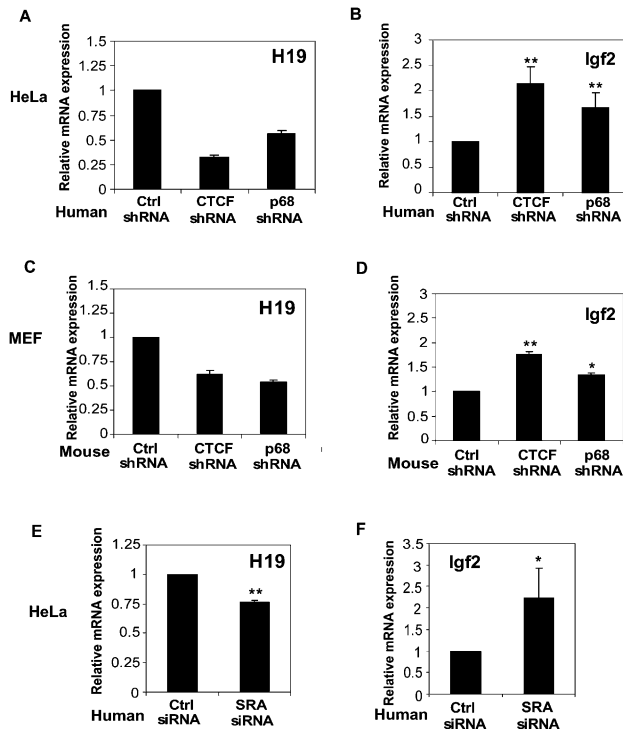


Figure 5. p68 and SRA regulate imprinting at the *IGF2/H19* locus. RT-qPCR analysis of human *H19* (A) and *IGF2* (B) transcripts in p68-shRNA- and CTCF-shRNA-depleted HeLa cells, mouse *H19* (C) and *IGF2* (D) transcripts in p68-shRNA- and CTCF-shRNA-depleted mouse primary MEF cells, and human *H19* (E) and *IGF2* (F) transcripts in control and SRA siRNA-depleted HeLa cells. Transcript levels were normalized to human or mouse HPRT1 levels and then the control shRNA or siRNA sample. Data were analyzed by Student's *t*-test. Error bars represent mean \pm SD. (*) $P < 0.05$; (**) $P < 0.01$.

specifically from HeLa cells (data not shown). ChIP-qPCR validations were carried out on nine selected p68 peaks that overlapped with CTCF peaks. All of these CTCF peaks were enriched for p68, eight quite strongly (Supplemental Fig. 6A,B).

Recently, it was shown that cohesin colocalizes with ER α at DNA-binding sites independently of CTCF (Schmidt et al. 2010). Since it has been reported that p68 also interacts with ER α (Endoh et al. 1999), we asked whether p68 could also bind to sites occupied by ER α and cohesin, but not CTCF. We found that, indeed, p68 binding is significantly overrepresented at these sites ($n_{\text{overlap}} = 257$; $P = 10^{-198}$, one-sided binomial test).

The recruitment of p68 to CTCF-binding sites is both CTCF- and SRA-dependent

To further investigate the relationship between p68 and CTCF, ChIP analysis of the *IGF2/H19* ICR was performed using anti-p68 and anti-CTCF antibodies following knockdown of p68 (Supplemental Fig. 7A) or CTCF (Fig. 6E). Knockdown of p68 or CTCF does not affect SRA expression (Supplemental Fig. 8). As expected, the recruitment of p68 to the CTCF ICR-binding sites was

reduced by p68 knockdown in HeLa cells (Supplemental Fig. 7B). However, p68 depletion did not affect the binding of CTCF to the *IGF2/H19* ICR sites (Supplemental Fig. 7C). ChIP assays were also performed following depletion of either CTCF (Fig. 6E) or SRA (Fig. 6H). As expected, CTCF depletion decreased the fraction of recovered CTCF at the ICR-binding sites (Fig. 6F). The recruitment of p68 to the same ICR sites was decreased not only by CTCF depletion, but also by SRA depletion (Fig. 6G,J). Conversely, the recruitment of CTCF to *IGF2/H19* ICR was not affected by SRA depletion (Fig. 6I). These results indicate that p68 localization to the CTCF-binding sites in the *IGF2/H19* ICR may require CTCF or SRA, but that CTCF binding does not depend on either p68 or SRA.

p68 and cohesin binding at the H19 ICR are interdependent

Since CTCF has also been shown to interact with the cohesin complex (Rubio et al. 2008), we next tried to determine if p68 also interacts with cohesin. To this end, we performed GST pull-down assays between p68 and components of the cohesin complex. We found that SMC1, one subunit of the cohesin complex, interacted strongly with p68 both in vivo and in vitro (Fig. 7A). Interaction with other cohesin subunits could not be detected by this method (data not shown).

We asked whether the interaction between p68 and cohesin was important for binding of either protein to CTCF-binding sites in vivo. We measured the occupancy of cohesin components at the *IGF2/H19* ICR by ChIP analysis after depleting either p68 or SRA. We found that p68 depletion in HeLa cells had only a small effect on the expression levels of the cohesin subunits Rad21, SA2, SMC1, and SMC3 (~20% decrease for SMC3, less for the others), and SRA depletion had no effect on the expression levels of cohesin subunits (Supplemental Fig. 9A,B). In addition, pull-down of cohesin by CTCF does not depend on the presence of p68 (data not shown); however, the recruitment of the cohesin subunits SMC1 or SMC3 to the *IGF2/H19* ICR was reduced by p68 knockdown (Fig. 7B). SMC3 recruitment is also impaired when SRA RNA is depleted (Fig. 7C). These data suggest that p68/SRA may play an important role in facilitating cohesin binding at the *IGF2/H19* ICR. Next, we depleted SA2 by using SA2 shRNA constructs shRNA-1 through shRNA-5. We found that cells highly depleted in SA2, using SA2 shRNA-4, failed to divide (data not shown), but cells with only moderate SA2 depletion (shRNA-3 and shRNA-5) were still able to divide and grow (Supplemental Fig. 10A). In addition, cohesin depletion in HeLa cells had no effect on the expression levels of p68 (Supplemental Fig. 10B). We performed ChIP-qPCR assays on the control shRNA and SA2 shRNA-5-depleted cells using p68 antibody, and found that the occupancy of p68 at the *IGF2/H19* ICR was reduced (Fig. 7D). Thus, the disruption of the cohesin complex through SA2 depletion also interferes with p68 binding to this CTCF site (Fig. 7D). These results show that, as well as interacting individually with CTCF, p68

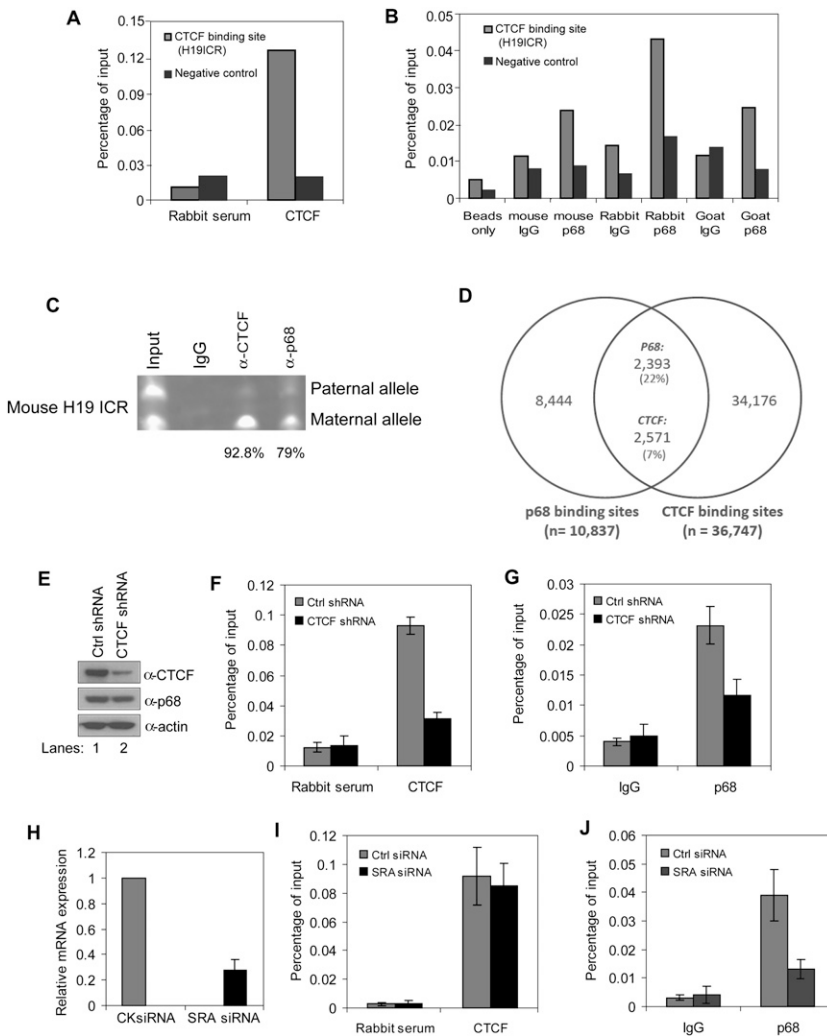


Figure 6. Binding of p68 to the maternal allele of *IGF2/H19* ICR is both CTCF- and SRA-dependent. (A) Cross-linked DNA–protein complexes from HeLa cells were immunoprecipitated with anti-CTCF or normal rabbit serum, followed by qPCR amplification with primers specific to the *IGF2/H19* ICR CTCF-binding region and a negative control region (chr11:1983833–1983994) (Wendt et al. 2008). Values are presented as percent of input. (B) ChIP analysis of p68 occupancy at the *IGF2/H19* ICR CTCF-binding site was performed using chromatin immunoprecipitated from HeLa cells with mouse anti-p68, rabbit anti-p68, and goat anti-p68 antibodies or IgG alone. Precipitated DNA was analyzed by qPCR. (C) Allele-specific ChIP assays were carried out with antibodies against CTCF, p68, or IgG in MEF cells. Input and precipitated samples were subjected to semi-qPCR for the paternal and maternal *H19* ICR alleles and then digested with a restriction enzyme, Tsp45I, a polymorphic restriction site. (D) Venn diagram showing overlap of p68- and CTCF-binding sites. (E) Western blot analysis of CTCF and p68 levels in NEs from CTCF shRNA or empty vector stably expressed HeLa cells. β -Actin was used as a loading control. (F,G) ChIP-qPCR analysis of isolated DNA associated with CTCF (F) and p68 (G) at *IGF2/H19* ICR in CTCF-depleted HeLa cells (mean \pm SD of $n = 3$). (H) RT-qPCR analysis to SRA knockdown efficiency in HeLa cells (mean \pm SD of $n = 3$). (I,J) ChIP-qPCR analysis of isolated DNA associated with CTCF (I) and p68 (J) on the CTCF-binding site at *IGF2/H19* ICR in SRA-depleted HeLa cells (mean \pm SD of $n = 3$).

and cohesin interact with each other in a way that appears to stabilize the binding of both cohesin and p68 to CTCF.

Depletion of p68 or CTCF increases the contact between endodermal enhancer and *IGF2* promoters

Chromatin conformation capture (3C) studies have shown that long-range interactions between the downstream *H19* enhancer and the *IGF2* promoter region are inhibited on the maternal allele, and that the inhibition depends on the enhancer-blocking properties of CTCF bound to the ICR of that allele (Yoon et al. 2007; Engel et al. 2008). To test if p68 was involved in the CTCF-dependent organization of the *IGF2/H19* locus, we depleted either mouse p68 or CTCF by shRNA in MEF cells and measured the change in contacts among sites in the locus, using the endodermal enhancer as an anchor (Supplemental Fig. 11A). Knockdown of CTCF increased the association between the *H19* endodermal enhancer and *IGF2* promoters compared with the control (Supplemental Fig. 11B). These results were expected based on similar measurements with mutated CTCF sites (Engel et al. 2008). While our previous experi-

ments determined that depletion of p68 had no effect on CTCF recruitment to the *IGF2/H19* site (Supplemental Fig. 7C), knockdown of p68 did result in increased contact between the *H19* enhancer and the region containing the *IGF2* promoters, similar to what is seen with CTCF depletion. These results are consistent with those described above showing that depletion of p68 results in overexpression of *IGF2*, paralleling the effects of CTCF depletion.

Discussion

CTCF is implicated in multiple roles in gene regulation, including context-dependent promoter activation/repression, enhancer-blocking and/or barrier insulation, genomic imprinting, hormone-responsive silencing, and, perhaps more fundamentally, long-range intra- and interchromosomal interactions (for review, see Gaszner and Felsenfeld 2006; Phillips and Corces 2009). A number of investigations have examined the role of factors that interact with CTCF and affect its function as an insulator protein. The chromodomain helicase protein CHD8 has been shown to be important for insulation, although its mode of action is

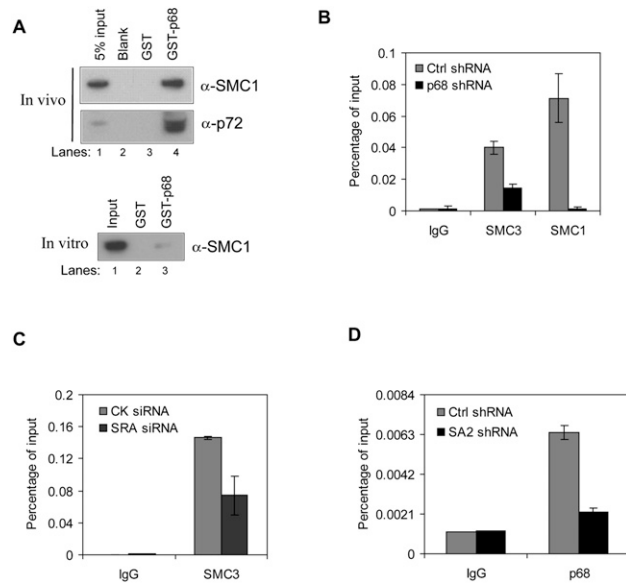


Figure 7. Functional relationships between p68 and cohesin. (A) GST pull-down assay from HeLa NEs (*top* panel) or in vitro translated SMC1 (*bottom* panel) using immobilized GST only or recombinant GST-p68. Bound proteins were analyzed by Western blot for the cohesin subunit SMC1; p72 served as a positive control for in vivo analysis. (B) ChIP-qPCR analysis of isolated DNA associated with the cohesin subunits SMC1 and SMC3 at the CTCF-binding site of *IGF2/H19* ICR in p68-depleted HeLa cells. (C) ChIP-qPCR analysis of isolated DNA associated with SMC3 at the CTCF-binding site of *H19* ICR in SRA-depleted HeLa cells. (CK) Control. (D) ChIP-qPCR analysis of isolated DNA associated with p68 at the CTCF-binding site of *IGF2/H19* ICR in SA2-depleted HeLa cells.

not known (Ishihara et al. 2006). Most recently, it has been shown that CTCF recruits the cohesin complex to its binding sites, and that the presence of cohesin is essential to insulator activity, probably because it stabilizes long-range intranuclear interactions between CTCF sites (Li et al. 2008; Parelho et al. 2008; Wendt et al. 2008). In this study, we show that the DEAD-box RNA-binding protein p68 (DDX5) interacts with CTCF both in vivo and in vitro, and that the noncoding RNA SRA, a functionally important RNA known to associate with p68, immunoprecipitates with CTCF. We also showed that both p68 protein and SRA are necessary for the activity of CTCF as an insulator element in vivo.

The DEAD-box RNA helicase p68 and the partially homologous protein p72 are RNA-binding proteins that are involved in a wide variety of regulatory and biosynthetic functions. p68 is required for ribosome biogenesis, and its ATPase/helicase activities are important for pre-mRNA splicing and microRNA processing (Lin et al. 2005; Jalal et al. 2007; Salzman et al. 2007). Notably, p68 is an essential component of the Drosha complex (Fukuda et al. 2007). p68 also functions as a cofactor for a variety of transcriptional regulatory proteins, including ER α , the p53 tumor suppressor, and MyoD (Endoh et al. 1999; M \acute{e} tivier et al. 2003; Bates et al. 2005; Caretti et al. 2006). At least some of these do not require the helicase

activity of p68, and probably involve a distinct independent mechanism. In many of these cases, the active form of p68 may involve a complex with p72 (Caretti et al. 2007; Fuller-Pace and Ali 2008). Although we focused on the role of p68, data in Supplemental Figure 5 suggest that p72 also plays a role in CTCF function. The p68/p72 protein specifically bind SRA (Watanabe et al. 2001), a functionally important RNA, and it has been shown that the coactivation of MyoD by p68 depends on the presence of SRA (Caretti et al. 2006). SRA has also been shown to bind other proteins and modulate their activity (Colley and Leedman 2009). Since some splice variants of SRA code for protein, it is necessary to distinguish the activity of the RNA from that of the protein.

We report here that the CTCF-p68 interaction is critical to CTCF function as an enhancer-blocking insulator, as demonstrated by transient expression experiments with a reporter carrying CTCF-binding sites in which p68 is depleted by shRNA. Additionally, ChIP experiments show that p68 is present at the ICR of the human *IGF2/H19* locus on chromosome 11 in HeLa cells, as well as the equivalent site on mouse chromosome 7 in MEF cells. Depletion of p68 results in an increase in *IGF2* expression and a decrease in *H19* expression, similar to that observed in HeLa cells upon depletion of cohesin components (Wendt et al. 2008). Loss of p68 also results in an increase in genomic contacts, as measured by 3C, between the endodermal enhancer and sites upstream of *IGF2*, consistent with loss of insulator function. The loss of p68 is not accompanied by a decrease in CTCF binding. We also found that the binding of p68 to CTCF is RNA-dependent: Depletion of ssRNA by RNase A or down-regulation of SRA inhibited the CTCF-p68 interaction (Figs. 2, 3E). This is similar to the behavior of the interaction between p68 and p53 (Suzuki et al. 2009). It is therefore not surprising that the ability of CTCF to act as an insulator also depends on SRA. The protein SRAP did not coprecipitate with CTCF either in vitro or in vivo, suggesting that it is not involved in the CTCF-p68 interaction.

It has been reported that the *Drosophila* DEAD-box putative RNA helicase protein Rm62, which is homologous to p68, interacts physically with the DNA-binding insulator protein CP190 in an ssRNA-dependent manner and negatively regulates *gypsy* insulator function (Lei and Corces 2006). It is striking that, in the case of *Drosophila*, the interacting factors are different from (CP190 rather than CTCF) and the effects are the opposite of (inhibitory rather than activating) those in vertebrates. These results hint at a common mechanism of action that has diverged.

What is the role of p68 in CTCF-dependent insulator function? In Figure 7A, we showed that, in addition to its interaction with CTCF, p68 also bound to a component of the cohesin complex in vitro. As discussed above, cohesin interacts with CTCF and is essential to insulator function. Previous studies have shown that loss of cohesin does not affect CTCF binding at most sites (Parelho et al. 2008). Similarly, depletion of p68 or its associated RNA, SRA, did not affect CTCF binding to the *IGF2/H19* ICR in vivo (Fig. 6; Supplemental Fig. 7); however, depletion of

either did result in loss of cohesin from those sites (Fig. 7B,C), showing that the interactions observed *in vitro* are important *in vivo*. These data support a model in which cohesin binding to CTCF at the *IGF2/H19* locus is further stabilized by cohesin interaction with p68/SRA. We suggest that the effects on insulator function that we observe when p68/SRA is depleted reflect, at least in part, the loss of cohesin from the site. As noted above, p68 is also found at sites occupied by ER α and cohesin. It will be interesting to determine whether, at such sites, p68 plays a role in stabilizing cohesin localization. In addition, it will be necessary to examine the findings of Schmidt et al. (2010) in light of the more recent report of cohesin recruitment to Nipbl/mediator-binding sites (Kagey et al. 2010).

Our results show that CTCF sites, the majority of which recruit cohesin, may require additional components to establish long-range interactions and maintain an active insulator complex. It remains to be determined whether p68/SRA, known to have multiple regulatory activities, contributes in other ways to CTCF function.

Materials and methods

Cell culture

HeLa-S3 and NIH3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Invitrogen). A Flag-tagged CTCF HeLa S3 stable cell line was established following methods in Yusufzai et al (2004). K562 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin–streptomycin. Primary MEF cells were C57BL/6J by C7 (CAST Chromosome 7 in a B6 background), which were kindly provided by Dr. Marisa Bartolomei (University of Pennsylvania School of Medicine) and maintained in DMEM medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin–streptomycin. All cell cultures were maintained in a 5% CO₂ humidified incubator at 37°C.

Antibodies

The following antibodies were used in this study: rabbit anti-CTCF (Abcam, ab70303) and mouse anti-CTCF (Abcam, ab37477) for Western blot and co-IP; rabbit anti-CTCF (Upstate Biotechnologies, 07-729) for ChIP; Flag M2 affinity gel-purified immunoglobulin (Sigma, A2220) for Flag-IP and Western blot; rabbit anti-SMC1 (Abcam, ab9262) for Western blot and ChIP; rabbit anti-SMC3 (Abcam, ab9263) for ChIP; anti-p68/DDX5 (Abcam, ab21696, ab10261, and ab53216) for Western blot, co-IP, and ChIP; goat anti-SA2 antibody (Abcam, ab4463) and rabbit anti-p72 (Abcam, ab66764) for Western blot.

Plasmid constructs

GST CTCF full-length, GST-CTCF N terminus (amino acids 1–291), GST-CTCF zinc finger (amino acids 291–576), and GST-CTCF C terminus (amino acids 576–727) were cloned into the pGEX5T1 vector (GE Healthcare). pcDNA3.1-p68 and pGEX4T1-p68 full-length constructs were kindly provided by Dr. Frances V. Fuller-Pace at University of Dundee, UK. GST-p68 N terminus (amino acids 1–96), GST-p68 RNA helicase core (amino acids 94–477), and GST-p68 C terminus (amino acids 475–614) were amplified by sticky-end PCR and cloned into the pGEX4T1 vector (GE Healthcare), then confirmed by sequencing.

Immunoprecipitation and immunoblot assay

HeLa NEs were prepared as described (Yusufzai et al. 2004). For IP, cleared NEs were incubated with the indicated antibodies plus Protein A magnetic beads (Active Motif) for 4 h or overnight. For RNase A digestion, the immunoprecipitated protein-bound magnetic beads were washed four times with IP buffer and suspended in the RNA digestion buffer containing RNase A (Ambion). The samples were incubated for 1 h at 37°C and quickly separated by a magnetic bar into two fractions: beads containing the still-bound proteins, and supernatant containing the released proteins. The separated protein fractions were subjected to SDS-PAGE and transferred to PVDF membrane (Invitrogen). Immunoblotting was performed with the indicated antibodies.

GST pull-down assays

GST, GST-CTCF full-length and its deletions (N terminus [amino acids 1–291], zinc finger [amino acids 291–576], and C terminus [amino acids 576–727]); GST-p68 full-length and its deletions (N terminus [amino acids 1–96]; and RNA helicase core domain [amino acids 94–477]; and C terminus [amino acids 475–614]) were expressed in *E. coli* strain BL21 cells (Invitrogen). The expressed fusion proteins from bacteria were purified using glutathione sepharose 4 fast flow beads (GE Healthcare) and then incubated with HeLa NE in the PBS-T buffer supplemented with protease inhibitors (1 mM PMSE, protease inhibitor cocktail) for 4 h or overnight at 4°C. Beads were washed four times with PBS-T buffer, boiled in SDS loading buffer, resolved on SDS-PAGE, and transferred to nitrocellulose membrane (Invitrogen). The transferred proteins were monitored by Ponceau S staining. Then the membrane was washed with TBS-T buffer to remove Ponceau S. Immunoblotting was performed with the indicated antibodies.

RT-qPCR assays

Total RNA was extracted from cells using the RNeasy mini kit (Qiagen). RNA was treated with DNase I (Ambion) for 30 min at 37°C. cDNA was synthesized using AffinityScript qPCR cDNA Synthesis kit (Stratagene). Amplification of cDNA was monitored with the SYBR green PCR master mix (Applied Biosystems) on the ABI PRISM 7900 PCR Detection System (Applied Biosystems) according to the manufacturer's instructions. Transcript levels were normalized to HPRT1 levels. Primers used for qRT-PCR are shown in Supplemental Table 1.

Luciferase and insulator assays

The *H19* ICR reporter plasmids (pIHLIE and pIHLME) were reported previously (Ishihara et al. 2006; Wendt et al. 2008) and were kindly provided by Dr. Mitsuyoshi Nakao at Kumamoto University, Japan. To perform luciferase reporter assays, the firefly luciferase reporter plasmid (pIHLIE or pIHLME) and a renilla luciferase control plasmid were cotransfected into p68-shRNA- or CTCF-shRNA-depleted or SRA siRNA-depleted HeLa cells using Fugene 6 (Roche). After 24 or 48 h, cells were harvested and lysed. The activities of both luciferases were detected using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions, and luciferase activity was determined with a Victor Multilabel Counter (PerkinElmer). Renilla luciferase was used as a transfection control. All assays were done in triplicate and repeated at least two times.

pNI empty and pNI-5'HS4-core insulator plasmids have been described previously for use in insulator analysis (Recillas-Targa et al. 1999; Parelho et al. 2008). p68-shRNA-, CTCF-shRNA-, or SRA siRNA-depleted K562 cells were transfected with pNI

empty or pNI-core insulator plasmid, and the expressions of the neomycin gene were detected by RT-qPCR after 24 h. There were three replicates for each treatment, and the experiments were repeated at least twice.

ChIP and qPCR

ChIP was performed using the indicated antibodies. Briefly, cells (5×10^6) were cross-linked with 1% paraformaldehyde for 10 min at 37°C and lysed in buffer containing 50 mM Tris (pH 8.0), 10 mM EDTA, 1% SDS, and protease inhibitors. Chromatin was sheared by sonication to generate 200- to 1000-base-pair (bp) DNA fragments and used for IP steps. Indicated antibodies were incubated with Dynabeads G (Invitrogen) with rotation for 20 min at room temperature. Chromatin preparations were then incubated with the complex of antibody magnetic beads overnight at 4°C. Immune complexes were washed with low-salt buffer, high-salt buffer, LiCl buffer, and TE buffer two times each. Antibody-bound chromatin was reverse-cross-linked, and the ChIP DNA samples were purified and used for qPCR. The PCR primers used for ChIP are listed in Supplemental Table 2.

Allele-specific ChIP and semi-qPCR

MEF cells were used for allele-specific ChIPs. ChIPs were carried out with antibodies against CTCF, p68, or IgG. Precipitated DNA samples and input DNA were subjected to PCR. PCR products were purified and digested with a restriction enzyme, Tsp45I, that is polymorphic between maternal and paternal alleles. Then, digested PCR products were resolved on 8% TBE gels, and gels were stained using 0.5 μ g/mL ethidium bromide in 1 \times TBE buffer for 30 min. The PCR primers used for ChIP are listed in Supplemental Table 2.

Solexa sequencing and validation of ChIP-seq

Thirty-six-base-pair single-read high-throughput sequencing was performed using an Illumina GAI Genome Analyzer. Tags were aligned to the human genome (hg18) using the GAI analysis pipeline (version 1.5.0), yielding 23.3 million quality-filtered, aligned tags from the p68 ChIP sample and 35.5 million from the input DNA sample. Data have been deposited in the Gene Expression Omnibus with accession number GSE24126.

To validate our ChIP-seq results, we used real-time PCR with SYBR green PCR master mix (Applied Biosystems) to amplify ChIP samples and input DNA for nine specific sites that were strongly positive in the ChIP-seq analysis (Supplemental Fig. 5), and calculated percentage of input for each region. It should be noted that the *IGF2/H19* locus did not give a significant peak in the ChIP-seq analysis, consistent with the observation that, in the direct ChIP experiments, it gave a rather low signal compared with other sites (cf. Fig. 6B and Supplemental Fig. S5B). Primer sequences are listed in Supplemental Table 3.

Identification of ChIP-enriched regions

Input DNA ChIP-seq tags were subsetted randomly to match the number of tags in the p68 ChIP-seq set. MACS version 1.3.7 (Zhang et al. 2008) was used to identify p68-enriched sites by comparing the p68 tags with the tag-matched input tags. As the signal:noise ratio in this sample was low, a conservative false discovery rate of 1% was used to filter called peaks. Adjacent peaks separated by less than half the width of either peak were merged.

Acknowledgments

We thank Drs. Frances Fuller-Pace and Zhi-Ren Liu for p68 constructs, Dr. Mitsuyoshi Nakao for luciferase constructs, Drs. Chunhui Hou and Ann Dean for the mouse CTCF shRNA construct, Dr. Marisa Bartolomei for MEF cells, and Drs. Chunhui Hou and Zhixiong Xu for suggestions in 3C experiments. We also acknowledge Drs. Keith Giles and Zhixiong Xu for their critical reading of the manuscript, and all other members of the Felsenfeld laboratory for helpful discussions. This work was supported by the intramural research program of the NIH, National Institute of Diabetes and Digestive and Kidney Diseases.

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