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Methylation of a CTCF-dependent boundary controls imprinted expression of the *Igf2* gene

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The expression of the insulin-like growth factor 2 (*Igf2*) and *H19* genes is imprinted. Although these neighbouring genes share an enhancer[1], *H19* is expressed only from the maternal allele, and *Igf2* only from the paternally inherited allele[2,3]. A region of paternal-specific methylation upstream of *H19* appears to be the site of an epigenetic mark that is required for the imprinting of these genes[4,5]. A deletion within this region results in loss of imprinting of both *H19* and *Igf2* (ref. 5). Here we show that this methylated region contains an element that blocks enhancer activity. The activity of this element is dependent upon the vertebrate enhancer-blocking protein CTCF. Methylation of CpGs within the CTCF-binding sites eliminates binding of CTCF *in vitro*, and deletion of these sites results in loss of enhancer-blocking activity *in vivo*, thereby allowing gene expression. This CTCF-dependent enhancer-blocking element acts as an insulator. We suggest that it controls imprinting of *Igf2*. The activity of this insulator is restricted to the maternal allele by specific DNA methylation of the paternal allele. Our results reveal that DNA methylation can control gene expression by modulating enhancer access to the gene promoter through regulation of an enhancer boundary.

Maternal transmission of a 1.6-kilobase (kb) deletion, within a differentially methylated region which is located between the mouse *Igf2* and *H19* genes, results in expression of the normally silent *Igf2* allele[5]. We examined the ability of this deleted fragment (DMD) to act as a positional enhancer-blocking element by inserting it at various locations relative to an enhancer in our model system[6–8], described in Methods. Insertion of the 1.6-kb DMD fragment between the enhancer and the promoter results in an 8–10-fold drop in colony number, similar to the 8-fold drop observed with the previously characterized 1.2-kb chicken *β-globin* insulator[7]. These results are not attributable to the increased distance between the enhancer and the promoter, as insertion of up to 2.3-kb of heterologous DNA between them has little effect on colony number[7]. Furthermore, like the *β-globin* insulator[8], placing the DMD outside the enhancer–promoter path, either upstream of the enhancer or downstream of the promoter, has little effect on expression (Fig. 1b). From these results, we conclude that the DMD has the position-dependent enhancer-blocking properties of an insulator.

The DMD fragment is part of a larger (~2-kb) imprinted control region (ICR) that is methylated exclusively on the paternal allele throughout development[4,9]. Allele-specific alterations in chromatin structure also occur in this region[10–12]. Two nuclease-hypersensitive regions are located on the maternal allele (HS1 and HS2 in Fig. 1a), whereas the chromatin on the paternal allele is methylated and nuclease insensitive[12]. Both HS1 and HS2 remain hypersensitive throughout development and are present independent of tissue type. We tested the enhancer-blocking potential of fragments spanning HS1, HS2 and a larger fragment that spans the entire ICR. All of these fragments confer enhancer-blocking activity (Fig. 1b). HS1 and HS2 individually show considerable enhancer-blocking activity, a fragment that contains both HS1 and HS2 essentially eliminates the enhancer's influence on expression (Fig. 1b, compare NIΔE with ICR).

A binding site (FII) for CTCF within the core of the chicken *β-globin* insulator is an essential component of the enhancer-blocking activity of that element[6]. A comparison between FII and 2.6-kb of mouse sequence spanning the ICR revealed a 13/16

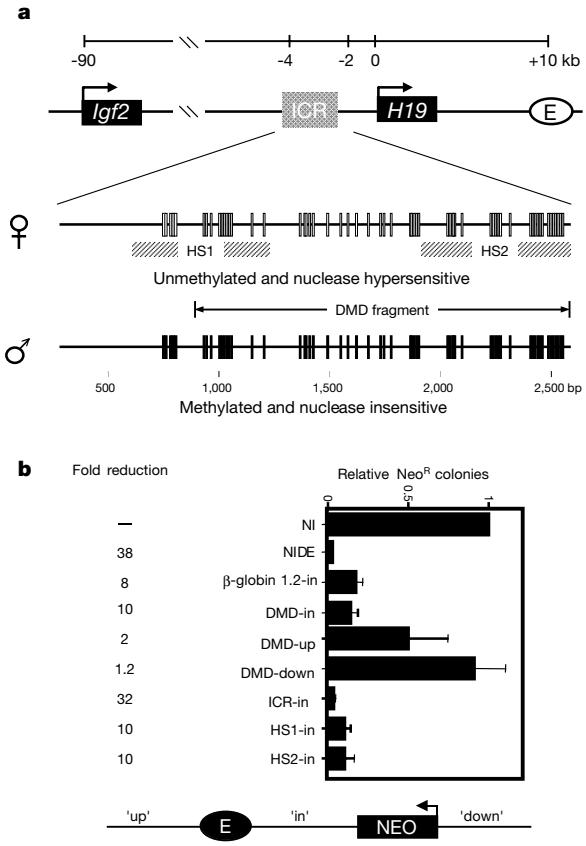

Figure 1 A differentially methylated region upstream of *H19* has the enhancer-blocking properties of an insulator. **a**, Neighbouring mouse *Igf2* and *H19* genes. On the maternally inherited chromosome, the ICR is unmethylated (white rectangles) and contains two nuclease-hypersensitive regions (hatched boxes, HS1 and HS2); on the paternally inherited chromosome, the ICR is methylated (black rectangles) and contains no hypersensitive sites[12]. Deletion of a 1.6-kb fragment of the ICR (the DMD fragment) eliminates HS2 and most of HS1 (ref. 5). **b**, Enhancer-blocking activity was measured by a standard assay[6–8] in which fragments of the ICR were inserted at defined positions relative to the enhancer and promoter in stably integrated constructions (see Methods). For each construction, colony number was normalized to un-insulated control, NI. Data are the average of three independent measurements.

base-pair (bp) match between the 3′ end of FII and a sequence at the 5′ edge of HS2 (m3 in Fig. 2a). By searching the remainder of the ICR with the m3 sequence, we identified four homologous sequences (m1–4 in Fig. 2a). Sequences homologous to these mouse sites are also found upstream of the human and rat *H19* genes, indicating that they are functionally important (aligned in Fig. 2a). The conservation of sequences overlapping those shown here has been noted[13,14]. In humans, a region of paternal-specific DNA methylation upstream of *H19* has also been described[15,16] and the homologous sequences defined here are part of a larger repeating element found in that region. An alignment of all of the sites from rat, human and mouse reveals a 12-bp consensus sequence that is shared among them (Fig. 2a). This consensus sequence bears a 11/12-bp match with the sequence at the 3′ end of *β-globin* FII. Notably, this 3′ region of FII makes an essential contribution to CTCF binding and enhancer blocking[6]. Consistent with these observations, we find that each of these mouse sites and a representative human site bind to both purified chicken CTCF (P in Fig. 2c) and *in vitro* translated human CTCF (I in Fig. 2c). Like FII, a fragment spanning a single mouse ICR site confers position-dependent enhancer-blocking activity *in vivo* (Fig. 2b). Furthermore, in gel shifts with K562 nuclear extracts (E in Fig. 2c) (the cells in which the enhancer-blocking assays were performed), a complex that co-migrates with the FII/CTCF complex was observed with each of these mouse and human ICR sites (Fig. 2c), and an antibody raised against CTCF supershifted this complex (Fig. 2d). Consistent with our attribution of enhancer-blocking activity to CTCF, this complex was competed by FII, but not by a mutant of FII in which both enhancer blocking and CTCF binding have been eliminated (FIIx3′ in Fig. 2d)[6]. Alteration of the base pairs shared between the ICR sites, and FII, in the context of one of these mouse sites, resulted in loss of competition for binding to CTCF (m1x3′ in Fig. 2d). Mutation of the same base pairs in the context of a human ICR site has the same effect on CTCF binding[17].

In the mouse *H19* ICR, HS1 and HS2 each contain two CTCF sites. We sequentially deleted each of these sites (Fig. 3), and measured the enhancer-blocking activities of the resulting fragments. As the enhancer-blocking activities of HS1 and HS2 are dependent upon their orientations (data not shown), deletion analyses were carried out with the orientation that gave the strongest activities. In each case, a deletion that eliminates either of the CTCF sites results in a reduction in enhancer-blocking

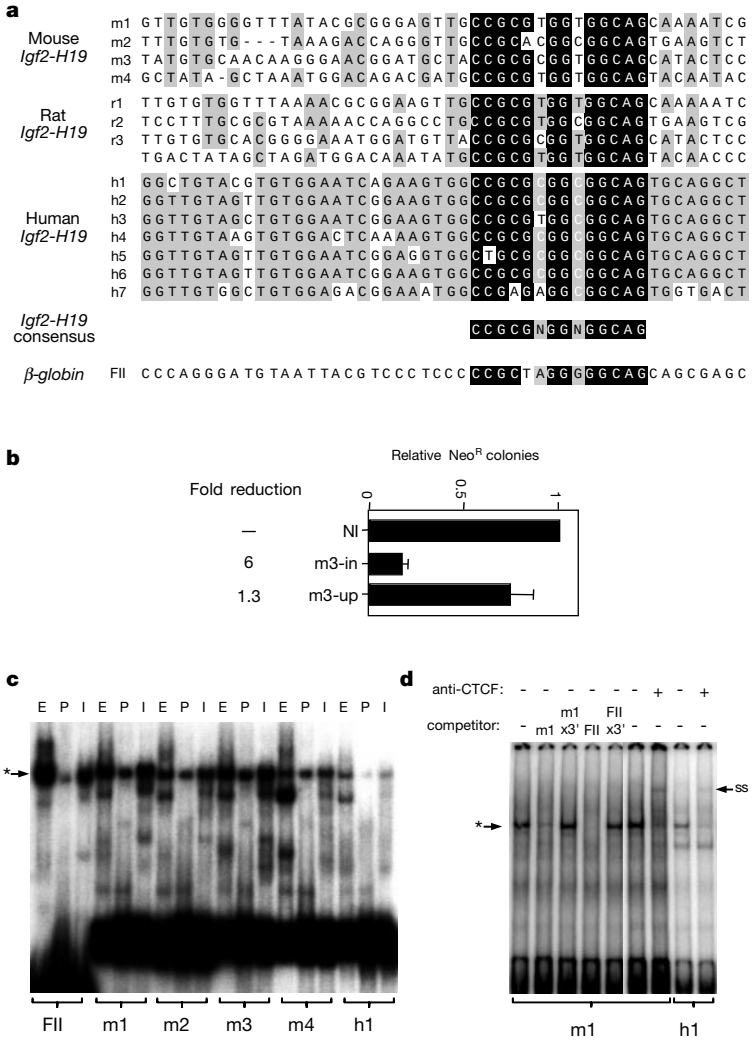

Figure 2 Conserved CTCF sites within the *H19* ICR. **a**, Sequences of the CTCF sites clustered upstream of the mouse, rat and human *H19* genes. Shading indicates identity among the sites: grey, species-specific identities; black, cross-species sequence conservation among these sites. **b**, Enhancer-blocking activity of a fragment spanning only m3 from the mouse ICR. Data are the average of three independent experiments. **c**, Gel mobility-shift analysis of β-globin FII (60-mer) and 83–91-mer duplexes spanning mouse and human ICR sites binding to K562 nuclear extract (E), partially purified (chicken) CTCF (P) and *in vitro* translated chicken CTCF (I). Asterisk, the position of the CTCF–DNA complex. Labelled DNA probes are indicated at the bottom. **d**, Analysis of CTCF binding to representative mouse and human ICR sites. DNAs were incubated with K562 nuclear extract in the presence of a 50-fold excess of unlabelled competitors as indicated or with an anti-CTCF antibody. SS, position of the supershifted CTCF complex.

activity, whereas deletion of both sites from either HS1 or HS2 eliminates their activity (Fig. 3a). The deletions that we made span sequences that are larger than the average 53-bp CTCF footprint. Among these sequences, however, the only significant similarity is within the CTCF sites. These similarities define a consensus sequence for CTCF binding (Fig. 2a, d), and we have shown that this consensus sequence is essential for the enhancer-blocking activity of β-globin FII[6]. We have also shown that single CTCF sites from several other loci (including the mouse ICR, Fig. 2b) alone confer enhancer-blocking activity in our assay[6]. Consistent with these findings, a human ICR site also confers enhancer-blocking activity and point mutations that eliminate the CTCF consensus from this site result in loss of enhancer-blocking activity *in vivo*[17].

We have shown that sequences within the mouse H19 ICR have the enhancer-blocking properties of an insulator. Several observations suggest that this activity is directly involved in the regulation of *Igf2*. If the *H19* enhancer is moved from its genomic location downstream of *H19* to a new location upstream of the ICR, the normally silent maternal allele of *Igf2* is expressed[18]. This indicates that the enhancer's position downstream of the *H19* locus may prevent activation of the maternal *Igf2* allele. Competition between the *H19* and *Igf2* promoters cannot explain this result because deletion of the *H19* promoter has no effect on *Igf2* expression[19]. Instead, the enhancer's position relative to the ICR restricts its action[18]: a deletion within the ICR results in biallelic expression of *Igf2* (ref. 5). This proposal is further supported by the observation that maternal inheritance of the relocated enhancer results in loss of expression of the normally active *H19* allele. In this case, because the ICR is now located between the enhancer and the *H19* promoter, it blocks their interaction. Thus, the dependence of *H19* and *Igf2* expression on the position of the *H19* enhancer is explained by a model that posits the existence of an insulator within the ICR[5,18,20].

The influence of the ICR on expression of *Igf2* depends upon the allele's parent of origin. It has been proposed that this is because the *H19* locus contains an insulator that is active only on the unmethylated (maternal) allele[20]. In this model, inheritance of paternal-specific CpG methylation in the ICR results in inactivation of the insulator and thus the *H19* enhancer is free to activate *Igf2* on this allele. Direct support for a role of DNA methylation in activation of *Igf2* comes from the observation that in DNA methyltransferase-1-deficient mouse embryos, both alleles of *Igf2* are silent[21].

The results of our deletion analysis of HS1 and HS2 indicate that the conserved CTCF sites in these elements may be responsible for their enhancer-blocking activities. One model that could explain why CpG methylation abolishes this activity is that CTCF cannot bind these sites when they are methylated. To test this, we made the corresponding oligomers with 5-methyl cytosine incorporated at each CpG, and assessed the ability of the resulting duplex to compete for the binding of CTCF to the unmethylated form. Methylation of each of the mouse sites, and a representative human site, greatly reduces their ability to compete for binding of CTCF to an unmethylated site, even at a 50-fold molar excess (Fig. 3b). Methylation of *β-globin* FII has a similar effect. Because FII and the ICR sites have only one CpG in common, we examined the influence of methylation at only this site (on both strands) in several ICR sites (Fig. 3b, right). In fact, methylation of this CpG alone significantly reduces CTCF binding to all of these sites (Fig. 3b, M1 lanes). This result indicates that enhancer access could, in principle, be regulated by a single (perhaps targeted) methylation event. Further examination of the influence of methylation on gene expression will require a system that allows the establishment and maintenance of partially methylated transgenes *in vivo*.

We have shown that the *H19* ICR is an enhancer-blocking element. CTCF-binding sites are required for this activity and when these sites are methylated they no longer bind the insulator protein CTCF. Our results, and those of ref. 17, provide direct evidence for a mechanism for Igf2 imprinting, in which differential methylation of an enhancer boundary allows epigenetic control of *Igf2* expression in the embryo (Fig. 4). In humans, a causal link between overexpression of *Igf2* and the pathogenesis of some cases of Beckwith–Wiedemann syndrome has been suggested[22–25]. Beckwith–Wiedemann syndrome, or fetal overgrowth syndrome, is a disorder of prenatal overgrowth and predisposition to embryonic malignancies such as Wilms tumour. There is a correlation between loss of imprinting of *Igf2* in Wilms tumour and Beckwith–Wiedemann

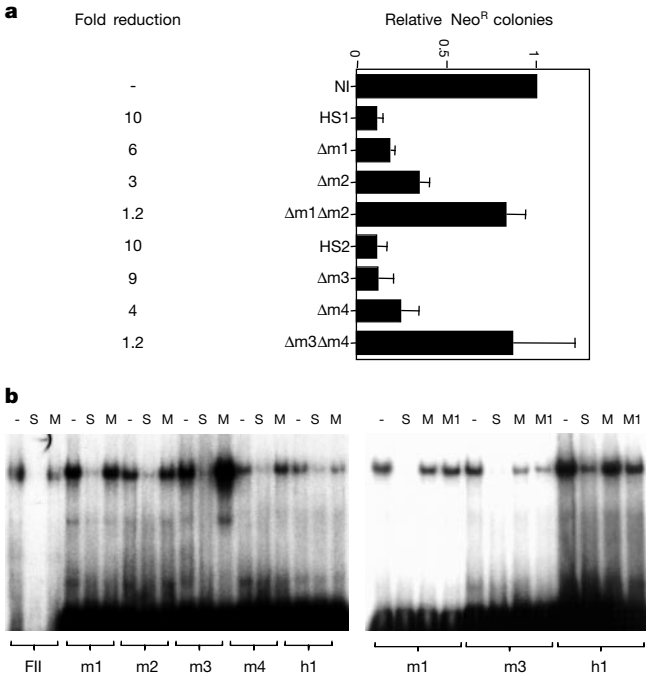

Figure 3 CTCF is responsible for the methylation-sensitive enhancer-blocking activities of the mouse and human ICRs. **a**, Enhancer-blocking activities of fragments of the mouse ICR after sequential deletion of the sequences spanning individual CTCF sites. Results are the average of two to three independent measurements. **b**, Left, effect of CpG methylation on binding of partially purified chicken CTCF to various sites in the absence of competitor DNA (–), or in the presence of a 50-fold excess of unlabelled duplex DNA (S, self) or a 50-fold excess of unlabelled duplex of identical sequence with 5-methyl C incorporated at every CpG (M, uniformly methylated) (identical results were obtained with human CTCF (data not shown)). Labelled DNA probes are indicated at the bottom of the panel. Right, the effect of 5-methyl C substitution at a single site (M1, singly methylated at the first CpG in the black-shaded region of Fig. 2a).

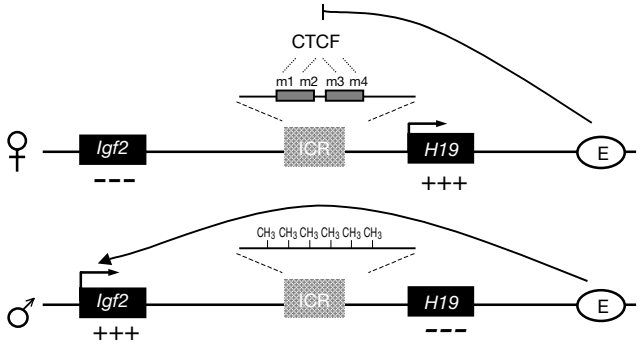

Figure 4 A model for methylation-dependent modulation of insulator action in the epigenetic regulation of *Igf2*. On the maternally inherited chromosome, the ICR is unmethylated. This allows binding of CTCF to its sites (m1–4), two in each nuclease-hypersensitive region (shaded boxes), and the resulting insulator blocks activation of the maternal copy of *Igf2* by the *H19* enhancer. On the paternally inherited chromosome, the ICR is methylated. This prevents CTCF binding, thereby inactivating the insulator and allowing the *H19* enhancer to activate *Igf2*.

syndrome and increased methylation of the maternal *H19* allele[26–29]. In Wilms tumour, this aberrant methylation pattern includes the CTCF sites illustrated in Fig. 2a[16]. These sites are consistently methylated on both alleles in Wilms tumours with loss of *Igf2* imprinting. Our data are consistent with the idea that the loss of *Igf2* imprinting observed in those tumours is caused by inactivation of a CTCF-dependent insulator in that locus.

In *Drosophila*, the activity of an insulator can be modulated by adjacent *cis*-acting sequences[30]. Our results reveal that in vertebrates the activity of enhancer boundaries can be controlled by DNA methylation. Some insulators may act not only as fixed boundaries, but also as switches that provide a new kind of modulated gene regulation. □

Methods

Constructions

The 2.5-kb ICR fragment and the 1.6-kb DMD fragment were generated by PCR on genomic DNA with ICRR, 5′-AGGCGCGCCAAGCTTTGTCACAGCGGACCCCAACCT ATG, and ICRF, 5′-AGGCGCGCCCAGAGCTCTTTCTCCACCACTTGTCTAAGT, and DMDF, 5′-AGGCGCGCCGGTACCTCGTGGACTCGGACTCCCAAATCA, primers, respectively. The approximate 800-bp HS1 fragment was generated with ICRF and HSIR, 5′-GGCGCGCCATAGTAGCTATACTTCAATTTTCA, and the HS2 fragment was generated with HS2F, 5′-AGGCGCGCCTTTATAAGAGGTTGGAACACTTGT, and ICRR. We deleted m1 and m2 from HS1 by PCR using the following additional primers: HS1Δm1F, 5′-CCCTATTCTTGGACGTCTGCTGAATCTATTGGAATTCACAAATGGCAATGC; HS1Δm1R, 5′-GATTCAGCAGACGTCCAAGAATAGGG; HS1Δm2F, 5′-GACTCGGACT CCCAAATCAACAAGGACGGATTGCAACTGATTGAGTTTC; HS1Δm2R, 5′-CCTTGTTGATTTGGGAGTCCGAGTC; HS2Tm4R, 5′-AGGCGCGCCAAGACTGAAGG AGCTACCCAAGAA; HS2F, 5′-AGGCGCGCCTTTATAAGAGGTTGGAACACTTGT; HS2Tm3F, 5′-AGGCGCGCCAGAGAACTTGACTCATTCCCTACAC; HS2Δm3F, 5′-AGAAGCTGTTATGTGCAACAAGGGAGCGATTCATTCCCAGCAATATCC; HS2Δm3R, 5′-CCCTTGTTGCACATAACAGCTTCT. The fragments Δm3 and Δm4 are roughly 200-bp truncations of the 5′ and 3′ ends of HS2 and are generated by PCR with the primer pairs HS2Tm3F/ICRR and HS2F/HS2Tm4R, respectively. In fragment Δm3Δm4, roughly 90 bp spanning the m3 site were internally deleted, the deletion of m4 results from a 3′ truncation. This was accomplished by two-step overlapping PCR using the primer pairs HS2Δm3F/HS2Tm4R and HS2F/HS2Δm3R on a DMD clone template. The products of these reactions were gel-purified and mixed, and the final product was amplified by PCR with primer pairs HS2F/HS2Tm4R. Internal deletions of about 90-bp fragments spanning m1 and m2 from HS1 were generated by first amplifying with primer pairs HS1F/HS1Δm1R, HS1Δm1F/HSIR, HS1Δm1F/HS1Δm2R, HS1F/HS1Δ2R and HS1Δm2F/HSIR. To generate singly or doubly deleted fragments, the products of these reactions were gel-purified, mixed accordingly and amplified with HS1F/HS1R. The resulting fragments were sub-cloned into pNI[6] after addition of the appropriate linkers where necessary. For enhancer-blocking assays with m3, 5′-GCTGTTATGTGCAACAAGGGAACGGATGCTA CCGCGCGGTGGCAGCATACTCCTATATATCGTGGCCCAAATGCTGCCAACTT GGGGGAGCGATTCATTC was directly synthesized with the appropriate restriction sites at its ends and cloned into pNI at either the *Asc*I or *Nde*I sites.

Enhancer-blocking assays

Assays were performed as described[6]. Each reporter carried a gene for neomycin resistance driven by the human ^{A}γ-globin promoter and a strong enhancer derived from a mouse β-globin LCR element. This was stably transfected into the human erythroleukaemia line K562, and the number of colonies resistant to G418 was counted. As in earlier studies[6–8], when the fragment being tested was in the 'up' or 'in' position, a 1.2-kb chicken insulator element was placed 3′ of the promoter to block the effects of an enhancer on an adjacent tandemly integrated promoter. The 1.2-kb insert was not present when the test fragment was in the 'down' position, as the purpose was only to measure silencing activity on the adjacent promoter.

DNA–binding assays

Protein preparations and gel mobility-shift assays were performed as described[6]. We generated *in vitro* translated chicken CTCF by transcription/translation of a clone provided by A. West. We incubated 20 μl reactions at room temperature with 1–2 μl of protein and 20 fmol of labelled duplex DNA. Probes were annealed with duplexes of the following sequences: m1, AGGCGCGCCGTTGTGGGGTTTATACGCGGGAGTTG CCGCGTGGTGGCAGCAAAATCGATTGCGCCAAACCTAAAGAGCCGGCGCGCCT; m2, AGGCGCGCCAATCCTTTGTGTGTAAAGACCAGGGTTGCCGCACGGCGGCAG TGAAGTCTCGTACATCGCAGTCCGGCGCGCCT; m3, AGGCGCGCCCTGTTATGT GCAACAAGGGAACGGATGCTACCGCGCGGTGGCAGCATACTCCTATATATCG TGGCCCAAAGGCGCGCCT; 4m, AGGCGCGCCACGCTGTGCAGATTTGGCTATAG CTAAATGGACAGACGATGCCGCGTGGTGGCAGTACAATACTACATATGGC GCGCCT; h1, GCCCTGATGGCGCAGAATCGGCTGTACGTGTGGAATCAGAA GTGGCCGCGCGGCGGCAGTGCAGGCTCACACATCACAGCCCGAGCACGCC; FII, AGGCGCGCCCCCAGGGATGTAATTACGTCCCTCCCCCGCTAGGGGGCAGCAG GCGCGCCT; FIIx3′, AGGCGCGCCCCCAGGGATGTAATTACGTCCCTCCAAAT AGCTTTTCAGCAGGCGCGCCT; m1x3′, AGGCGCGCCTGCTGAATCAGTTGT GGGGTTTATACGCGGGAGTTGAATATGTTGTTACTCAAATCGATTGCGCCAAA CGGCGCGCCT. Oligomer modified with 5-methyl C were generated by direct synthesis with the appropriate phosphoramidite (Glen Research) on an ABI synthesizer. Competitions were carried out by the simultaneous addition of a 50-fold excess of unlabelled DNA duplex. We carried out supershifts by adding antibody raised against a conserved carboxyl-terminal CTCF peptide (APNGDLTPEMILSMMD) to a standard gel-shift reaction of 2 μl followed by 2 h incubation at 23 °C.

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