An upstream secondary DNA motif within the *IL3* insulator CTCF binding site is required for enhancer-blocking insulator activity.

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Short title

CTCF insulator activity requires binding to 2 specific DNA motifs

Abstract

CCCTC-binding Factor (CCTF) is the only known vertebrate protein that functions to organise the genome into distinct functional chromatin domains. A subset of CTCF sites also provide insulator activity and barrier activity to block inappropriate enhancer-promoter communication and spreading of histone modifications and non-coding transcription into loci where it is unwarranted. Paradoxically, other CTCF sites mediate enhancer and promoter communication, partly by supporting DNA loop extrusion within chromatin domains. Despite intensive study and abundant data, it remains poorly understood how CTCF directs these different functions. In this study we provide new data and mine published data that show that CTCF utilises zinc fingers 9 to 11 and an upstream DNA binding consensus sequence resembling CAGCTGTTCC to mediate high affinity binding and enhancer-blocking insulator activity. A single high affinity CTCF binding site from the *IL3* locus is able block *IL3* promoter activation by an upstream enhancer. Mutation of a CTGCAGCTTT sequence upstream of the CTCF core motif abolishes insulator activity. We propose that CTCF is able to confer insulator and barrier activity upon a specific subset of CTCF sites that contain the upstream DNA consensus binding motif, thereby allowing CTCF to function differently in different contexts.

Introduction

CTCF (CCCTC-binding Factor) is a highly conserved, 11-fingered zinc finger (ZF) protein [1-3] able to use different combinations of ZFs to bind to a highly divergent set of DNA sequences [1, 4]. CTCF sites are highly diverse in that their functions range from defining genome domain boundaries, blocking spreading of chromatin modifications, and insulating enhancers from promoters, while conversely some CTCF sites mediate enhancer-promoter communication. CTCF plays a major role in maintaining chromosomal 3D architecture whereby it is found at the borders of independently folded chromatin domains termed Topologically Associating Domains (TADs) that are typically ~1000 kilobase (kb) in size [5-7]. These interaction domains are bordered by a subset CTCF sites that act as roadblocks to directional DNA loop extrusion through the ring of the Cohesin complex at each boundary of the TAD [8-10]. DNA loops extrude through the Cohesin ring on the NH2-terminal or ZF3 side of the bound CTCF complex which faces into the TAD. This provides a mechanism whereby loop extrusion assists enhancer interaction with genes on one side of the CTCF boundary but not the other, and allows some CTCF sites to act as enhancer-blocking insulators in functional enhancer assays [11-13].

It is generally accepted that CTCF is the principal vertebrate insulator-binding protein [14, 15], and it is known to bind to most previously characterized enhancer-blocking insulators, including those in the human *IL3*/*CSF2* locus [11], the chicken beta-globin locus [16], the Igf2/H19 locus [17-20], the HLA-DQA1 locus [21], and the mouse alpha-globin locus [13]. In addition, some CTCF sites, such as ones found in the HOXA cluster and the *FOXJ3* locus, function as barrier elements segregating domains of active chromatin marked by histone H3K4me3 from the spreading of repressive domains of chromatin marked by H3K27me3 [22-24]. The +2.9 and +4.2 kb CTCF sites in the *IL3* insulator also block the spread of read-through non-coding transcription coming from both upstream and downstream [25]. Furthermore, CTCF is the only protein identified thus far that is directly associated with insulator function in vertebrates (other than the CTCF homolog BORIS which

65 is expressed only in germ cells [26]). It is through CTCF that other insulator-associated proteins, such
66 as the Cohesin complex, are recruited to CTCF sites [11, 27-29]. Despite the obvious importance of
67 CTCF, it remains unclear how some CTCF sites can block enhancer function, while others promote
68 enhancer function.

69 CTCF sites account for ~10% of all ubiquitous DNase I hypersensitive sites (DHSs) in the
70 genome [30]. CTCF employs up to 11 ZFs to bind to its target sites, generating considerable
71 complexity in the sequences that it can recognize. Global CTCF chromatin immunoprecipitation
72 (ChIP) studies defined a core sequence resembling CCACCAG(G/A)GGG that is present in all CTCF
73 sites at least 13,000 times in the human genome [4, 31, 32] and this core is now known to interact
74 with CTCF ZFs 4 to 7 [33-35]. However, other studies revealed that in 13 to 15% of cases CTCF sites
75 make additional contacts via ZFs 9 to 11 to a second upstream consensus sequence resembling
76 CAGCTGTTCC, with ZF 8 bridging a flexible length DNA linker region in between [1, 11, 13, 34-44].
77 Individual deletions of ZFs 8 to 11 led to preferential loss of CTCF sites containing the upstream
78 sequence [43]. The functions of the upstream CTCF motif and ZFs 9 to 11 remain poorly defined, but
79 they are likely to support higher affinity binding than CTCF sites containing just the core consensus.
80 Furthermore, an inspection of published studies of CTCF sites with known insulator, repressor or
81 chromatin barrier activity reveals that they typically have this upstream motif (Fig 1) [1, 11, 14, 16,
82 21, 37, 38, 40, 45, 46]. A recent study of CTCF sites in the alpha globin locus found that the CTCF sites
83 that function the most efficiently as enhancer-blocking insulators are also the CTCF sites that have
84 the upstream motif, and this study observed that insulation is greater when the NH2-terminal or ZF3
85 side of the bound CTCF complex faces towards the enhancer [13]. In this orientation of the CTCF site,
86 the enhancer is downstream of the CCACCAG(G/A)GGG sequence, and any extrusion loops are
87 predicted to progress along the genome in the opposite direction and away from the promoter. A
88 similar previous functional screen for the insulator activity of CTCF sites inserted into the *Sox2* locus

89 also showed a required for upstream CTCF motif, but found that 2 to 4 tandem copies of CTCF sites
90 were needed for insulator activity, and that the orientation was less important [12].
91
92 **Figure 1 Legend. Conservation among insulator and boundary CTCF sites.**
93 Comparison of sequence homology between previously characterized CTCF binding sites that have
94 been defined as insulators, repressors or chromatin boundaries [1, 11, 13, 14, 16, 21-24, 28, 37-40,
95 45-47]. The three lines of sequence in the global CTCF consensus above the table represent the
96 predominant alternative bases allowed at each position with the preferred bases shown above in
97 uppercase [41-43]. Regions protected in published DNase I or DMS footprinting assays are
98 highlighted in bold. Underlined bases represent bases where methylation or carboxyethylation
99 blocks binding of CTCF in interference assays. Above the table are the predicted binding sites of Zn
100 finger domains 3-7 and 9-11 (indicated by brackets and numbers) [33]. Zn Finger 8 is not shown
101 because this domain sits away from the DNA above the variable length spacer region. Zn fingers 3-7
102 bind the core CTCF consensus sequence common to all CTCF sites (primary motif) and Zn fingers 9-
103 11 bind the secondary upstream consensus sequence which is present in ~13% of CTCF sites.
104 Underneath the table are shown a web logo [48] derived from the sequences in the table, and the
105 published global consensus sequence for CTCF sites that contain the upstream binding site [42, 43].
106
107 CTCF controls many aspects of chromatin structure, chromosomal architecture and the
108 regulation of transcription. These functions can include regulating the intrusion of histone
109 modifications or non-coding transcription into gene loci, and long-range chromatin looping. The
110 complex nature of CTCF sequences and their functions makes it very difficult to dissect the individual
111 specific functions of CTCF when sites are embedded within chromosomal DNA. Even in circular
112 plasmids it is difficult to evaluate enhancer-blocking insulators unless they are inserted both
113 upstream and downstream of the gene loci under study. To circumvent these difficulties, we

114 previously developed a novel enhancer-blocking insulator assay based of the transfection of
115 linearised luciferase reporter gene plasmids containing insulator elements in between the *IL3*
116 promoter an upstream *CSF2* enhancer [11]. Using this model we previously reported that insertion
117 of 3 tandem copies of the *IL3* +2.9 kb CTCF site completely blocked *CSF2* enhancer activity in linear
118 plasmids, and blocked 80% of activity in circular plasmids [11]. This model system was able to assay
119 for the enhancer-blocking activity of CTCF in isolation from other activities relating to chromatin
120 modifications or chromatin architecture.

121 In this new study we employed one of the enhancer-blocking CTCF sites from the *IL3*
122 insulator [11] as a model to investigate the functions of the secondary upstream CTCF binding motif.
123 We first showed that *in vitro* CTCF binding is greatly reduced in the absence of this upstream DNA
124 motif. We next employed our previously described enhancer-blocking insulator assay based on
125 linearised DNA fragments containing the *CSF2* enhancer and the *IL3* promoter [11]. We
126 demonstrated that a single CTCF site from the *IL3* insulator was sufficient to confer robust enhancer-
127 blocking insulator activity and that the secondary upstream consensus sequence was essential for
128 this activity. This observation now further builds upon our understanding of mechanisms that
129 determine why only a subset of CTCF sites function as insulators.

130 Results

131 The *IL3* insulator encompasses 2 nearly identical CTCF binding sites that include both the core
132 consensus and an upstream CTGCAGTTTC motif (Fig 2A). Each of these motifs is highly conserved
133 across mammalian species for both sites (S3 Table). Our previous study of this insulator employed *in*
134 *vivo* footprinting using dimethyl sulphate to confirm that CTCF bound to both the upstream and core
135 motifs, while the flanking sequences were hypersensitive to DNaseI (summarised in Fig 2A) [11].

137 **Figure 2 Legend. EMSAs of CTCF binding to the *IL3* insulator CTCF site.**

138 (A) Locations of the core and upstream CTCF motifs within CTCF sites found within the human IL-3
139 gene insulator [11]. The boxes represent regions protected from DNase I in footprinting assays. G
140 bases protected from modification by DMS in *in vivo* footprinting assays are indicated by open
141 circles. Regions hypersensitive to DNase I (arrows) or modification by DMS (filled circles) are also
142 indicated.

143 (B) Sequences of CTCF EMSA probes used in this study. Mutated residues are shown in bold
144 lowercase and the two consensus sequences are underlined.

145 (C-E) EMSAs using intact or mutated *IL3* +2.9 kb CTCF sites. EMSAs included 4 µg of Jurkat nuclear
146 extract with either the +2.9, Δ1°, Δ2°(1), Δ2°(2), Δ1°/Δ2°(2), HS4, or ΔHS4 probes (C). Specificity of
147 CTCF binding was confirmed by incubating 4 µg of nuclear extract with 2 µl of anti-CTCF or control
148 IgG antibodies, and by comparison to the chicken beta globin HS4 CTCF site (C). The vertical black
149 lines indicated parts of the gel where 2 irrelevant lanes were deleted. Binding data was quantified by
150 densitometry (D).

151 (E - G) Competition assays of CTCF binding. EMSAs included 4 µg of Jurkat nuclear extract in the
152 presence of increasing amounts unlabelled competitor as indicated above. (H) Densitometric
153 quantification of the EMSAs in panels E and F depicting the efficiency of the mutated +2.9 CTCF sites
154 to compete with wild-type +2.9 site. Values are expressed as the fold decrease in the presence of
155 competitor. The unmanipulated EMSA images are shown in S2 Figure.

156

157 **The upstream CTCF motif promotes high affinity binding to DNA**

158 We employed Electrophoretic Mobility Shift Assays (EMSAs) using Jurkat human T cell nuclear
159 extracts to investigate the relative contributions of the primary core (1°) and secondary upstream
160 (2°) motifs to *in vitro* CTCF binding. Because CTCF is a ZnF protein, EMSAs were performed in the
161 presence of Zn^{2+}, which we previously found to be essential for efficient *in vitro* binding of CTCF

162 using nuclear extracts [11]. We synthesised various mutated derivatives of the +2.9 kb *IL3* CTCF site
163 for use in EMSAs and included intact and mutated versions of the chicken beta-globin HS4 CTCF site
164 as controls (Fig 2B). The identity of the CTCF complex was confirmed using CTCF antibodies and
165 control IgG (Fig 2C). EMSAs demonstrated that *in vitro* CTCF binding was highly dependent on the
166 presence of the conserved core motif, and binding was abolished if the core consensus was mutated
167 in either the *IL3* or the HS4 CTCF site ($\Delta 1^{o}$ and ΔHS4) (Fig 2C). High affinity CTCF binding was
168 dependent upon maintaining the upstream motif intact, whereby binding was reduced 3-fold when
169 it was mutated ($\Delta 2^{o}$) (Fig. 2C). These binding data were quantified by densitometry (Fig 2D). The
170 relative strength of binding of the intact and mutated CTCF sites in EMSAs was also evaluated by
171 including 3, 10 or 30 ng of unlabelled oligonucleotides as competitors (Figs 2E - G). The mutation of
172 the core sequence eliminated all competing binding activity while mutation of the upstream
173 sequence resulted in a modest decrease in competition.
174
175 **The secondary upstream CTCF motif is essential for enhancer-blocking *IL3* insulator function**
176 We next dissected the relative contributions that the upstream and core consensus sequences made
177 towards the enhancer-blocking activity of the *IL3* +2.9 kb insulator CTCF site. We performed
178 transfection assays in Jurkat T cells with plasmids containing a 717 bp fragment of the inducible
179 human *CSF2* enhancer (GME) upstream of the -559 to +50 region of the human *IL3* promoter in front
180 of the luciferase gene in pXPG (Fig 3). The activity of the inducible *IL3* promoter was measured in
181 linearised plasmids 40 hours after transfection into Jurkat T cells, following stimulation for 8 h with
182 20 ng/ml PMA and 1 mM calcium ionophore A23187 to induce T cell receptor signalling. Enhancer-
183 blocking insulation activity was measured after placing a single CTCF site in the reverse orientation in
184 between the enhancer and promoter. In this configuration the CTCF ZF 3 to 7 binding sequence
185 CCACTAGGGGGAGACA is facing towards the enhancer, which is predicted to maximise the insulation
186 activity of CTCF. Insertion of just a single copy of the *IL3* CTCF site was sufficient to almost entirely

187 block *CSF2* enhancer activity (Fig 3). However, mutation of either the upstream motif or the core
188 CTCF consensus sequence abolished enhancer-blocking insulator activity (Fig 3).
189
190 **Figure 3 Legend. Enhancer blocking assay of a CTCF site.**
191 Linearised luciferase reporter gene plasmids containing the human IL-3 gene promoter (pIL3) were
192 assayed in Jurkat T cells. As depicted in the cartoon at the right, derivatives of pIL3H contained either
193 just the *CSF2* enhancer (pIL3-GME) or the enhancer plus a CTCF site inserted between the promoter
194 and the enhancer (pIL3-C-GME). CTCF site plasmids contained either the intact *IL3* +2.9 kb CTCF site,
195 or this CTCF with either the primary or the secondary consensus motif mutated, as indicated below.
196 The CTCF sites are inserted into the plasmids in the opposite orientation to that shown which means
197 that the NH2 terminus of bound CTCF faces towards the enhancer. Luciferase activities were
198 measured 40 h after transfection plus an additional 8 h after stimulating transfected Jurkat cells with
199 PMA and calcium ionophore. Luciferase activities were expressed relative to pIL3-GME having a
200 value of 1, and represent the mean of 6 transfections, using two independently derived DNA clones.
201 Error bars indicate Standard Deviation. Data was normalised by comparison with the cotransfected
202 Renilla luciferase control plasmid pRL-TK.
203
204 **The upstream CTCF motif is a common feature of enhancer insulators and chromatin barriers**
205 The role of the upstream consensus sequence at insulators remains poorly understood. To find
206 further evidence for its significance we performed a brief survey of other well-defined CTCF-
207 dependent insulators and found that they typically included a near ideal match to the 10 bp
208 upstream binding site (highlighted in yellow in Fig 1). Figure 1 also summarises previously published
209 *in vitro* DNA binding data and *in vivo* footprinting data confirming contacts between CTCF and the
210 upstream motif where ZFs 9 to 11 are predicted to bind. Regions protected by CTCF from either
211 DNase I digestion and/or methylation by DMS *in vivo* are highlighted in bold, whereas regions where

212 CTCF binding is blocked by *in vitro* modification of DNA by either methylation or carboxyethylation
213 are underlined. Studies of nuclease accessibility at these sites invariably find that the regions
214 occupied by ZF 4 to 7 and Z F9 to 11 are separated by an accessible variable length linker
215 approximately 8 bp in length where ZF8 is thought to sit away from an accessible region of linker
216 DNA [11, 13, 33, 44].

217 Figure 1 also lists CTCF sites within previously defined chromatin boundary elements from
218 the *HOXA9* and *FOXJ3* loci that similarly include the upstream CTCF motif [22-24]. To find further
219 evidence for the presence of the upstream CTCF motif at histone H3 K27me3 chromatin boundaries
220 we next performed a limited scan of the publicly available genome data from a global study of
221 histone methylation and CTCF binding, provided by the authors online via a link to the UCSC genome
222 browser (https://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcell.aspx) [24, 47]. This allowed us to
223 identify several additional CTCF sites with conserved upstream CTCF motifs at H3K27me3 chromatin
224 boundaries (Fig 1 and S1 Figure). We examined the conservation of a sample of the above sites and
225 found that like the *IL3* insulator (Fig 1 and S3 Table), the upstream CTCF site is highly conserved in at
226 least the UPK2, HOXA9, FOXJ3 and ABCG8 CTCF sites (S4 to S6 Tables).

227 Taken together, the above data collectively provide strong evidence that the upstream CTCF
228 consensus present in ~13-15% of all CTCF sites plays a major role in both enhancer insulator and
229 chromatin barrier function. A sequence logo derived from the insulator sites in Fig 1 is essentially
230 identical to that derived for the upstream consensus, suggesting a strong functional association.

231 Discussion

232 The aim of this study was to further raise the consciousness of the scientific community about the
233 potential role of the upstream CTCF consensus in insulator and barrier function. Despite numerous
234 studies over the last 25 years revealing binding of CTCF to an upstream CTCF motif at known
235 insulators, the role and mechanism of action of this secondary binding site is still not fully
236 understood or appreciated. Our findings are in agreement with another recent study of CTCF sites in

237 the mouse alpha globin locus which similarly found a correlation between the presence of the
238 upstream CTCF motif and the ability of CTCF sites to function as enhancer-blocking insulators when
239 inserted into the genome [13]. They also agree with the findings of a genomic screen for insulators
240 inserted into the mouse *Sox2* locus whereby CTCF sites that functioned as insulators, or were found
241 at TAD boundaries, were also those CTCF sites that included the upstream CAGCTGTTCC-like motif
242 [12]. However, one major difference to our study was that the *Sox2* study found that single CTCF
243 sites had no enhancer-blocking insulator activity and four tandem CTCF sites were required to block
244 38% of gene expression [12]. In our study, a single CTCF site functioned as an insulator to block 74%
245 of enhancer activity in the context of non-integrated linear DNA, although we also previously
246 observed that 100% insulation was only achieved when 3 tandem copies were used [11]. The above
247 study of CTCF sites in the alpha globin locus also found that insulation strength increased with
248 insertion of multiple CTCF sites [13].

249 **The mechanism of insulation by CTCF remains an enigma**

250 The mechanism of action of insulation mediated via the upstream CTCF motif is far from
251 clear. In our study we found that the upstream motif was needed for high affinity binding, but the
252 work of others indicated that high affinity binding alone is not sufficient for insulator activity [12,
253 13]. This raises the possibility that CTCF has 2 independent functional DNA binding domains that
254 contribute different activities. This is supported by the fact that CTCF uses ZFs 4 - 7 and ZFs 9 – 11 as
255 two independent clusters of DNA binding domains whereby the ZFs bind side by side to adjacent 3
256 bp ZF recognition sequences to the two separate motifs which are interrupted by a variable length
257 (~7 – 10 bp) DNA spacer bridged by the more flexible non-bound ZF8. The data suggest that
258 interactions with the core CTCF motif via ZFs 4 to 7 are essential for all CTCF binding whereas binding
259 via ZFs 9 -11 contributes additional functionality at a subset of sites. It is evident that the 2 DNA
260 binding domains bind somewhat independently because the DNA linker is of flexible length and is
261 highly accessible to nucleases when CTCF is bound.

262 CTCF sites that function as insulators or TAD boundaries normally function in a specific
263 orientation whereby the core motif and ZF3 face into the loop [8]. This may be related to the fact
264 that CTCF recruits cohesin, and DNA loop extrusion through the cohesin ring proceeds by drawing in
265 DNA from the ZF3 end of the CTCF/cohesin complex [8, 13], which is downstream and not upstream
266 of the CTCF core motif [10]. As TAD DNA loop extrusion can extend for hundreds of kilobases, most
267 CTCF sites probably have the ability to pass through the cohesin ring. Taken together, these
268 observations support a model whereby high affinity binding of ZFs 9 – 11 to the upstream motif
269 provides a double lock to the CTCF complex, stabilising its binding, and rendering domain boundaries
270 and loop ends more stable. Enhancer-blocking insulation also works most effectively when ZF3 is
271 facing towards the enhancer [13]. However, it seems unlikely that enhancer insulation can be
272 regulated solely on the basis of effects on loop extrusion and enhancer looping to promoters
273 because many insulator assays have the enhancer less than 1 kb from the promoter. Nevertheless,
274 cohesin almost certainly contributes to insulator activity, and co-association with the cohesin protein
275 Rad21 provided a better predictor of insulator activity that just strength of CTCF binding [13].
276 There is also evidence that CTCF binds less rigidly to CTCF sites lacking the upstream motif
277 [49]. Some CTCF sites bind in a highly tissue-specific manner that relies on cooperation with other
278 transcription factors binding to adjacent sequences. A study of these dynamic CTCF sites in blood
279 cells found that only 6% of these sites included the upstream motif whereas most insulator and
280 barrier CTCF sites have it, whereas 13 – 15% of CTCF sites globally include the upstream motif [49].
281 While we still have a poor understanding of the molecular mechanisms of enhancer
282 blocking, there may also be a role for CTCF ZF8 which interacts with the phosphate backbone in the
283 minor groove of the linker DNA separating the core and upstream motifs [33]. The other ZFs interact
284 with the DNA bases in the major groove. It is likely that any structure formed by ZF8 on DNA will
285 itself be much more rigid, and fixed like a bridge above the linker, when both motifs are tightly

286 bound. Such a rigid structure would be expected to behave differently than one where binding via
287 ZFs 8 – 11 is dynamic.

288 **The upstream CTCF binding motif is not conserved in insects**

289 The role of the upstream CTCF binding motif at insulators described here may be restricted to
290 vertebrates. A study of CTCF ChIP peaks in Drosphila S2 cells found no enrichment for the closely
291 linked CTGCAG-like motifs identified in CTCF sites in human cells, but just the same core consensus
292 motif as is found in vertebrates [50]. This is most likely due to the fact that ZFs 9 – 11 are not as
293 highly conserved between human and Drosophila CTCF as are ZFs 4 – 7 [51]. This includes 2 amino
294 acids in ZF9 and 1 amino acid in ZF11 that are predicted to contact DNA in human CTCF [50].
295 Although Drosophila do express both CTCF and cohesin, they may not interact or function in the
296 same way as they do in vertebrates. This is partly because Drosophila utilise multiple insulator
297 proteins and not just CTCF [50, 52]. Drosophila insulator CTCF sites also showed a partial
298 dependency upon additional unidentified flanking sequences located within 90 bp of the core motif
299 [50].

300 **CTCF is a multifunctional protein**

301 Taken together, our data and recent published studies all predict that CTCF can use two distinct
302 modes of binding to contribute very different and seemingly contradictory functions. Binding via just
303 ZFs 4 – 7 may support more dynamic binding, and generate a structure able to pass through cohesin
304 rings. This mode of binding is more likely to fit with the vast majority of CTCF sites that probably
305 assist enhancer-promoter communication. Conversely, binding that also includes tight binding to ZFs
306 9-11 may generate a more rigid structure that is either unable to pass though cohesin rings or is
307 tightly bound to cohesin itself. This mode of binding appears to support insulator or barrier function.
308 Future studies will need to determine the structures that CTCF adopts when bound to DNA together
309 with its partners to gain a better understanding of the mechanisms of insulation.

310 ## Materials and Methods

311 **Electrophoretic Mobility Shift Assays (EMSAs)**

312 Double stranded oligonucleotides containing the wild type sequence, mutations or truncations of
313 the +2.9 kb CTCF binding site from the IL-3/GM-CSF insulator [11] or chicken β-globin gene [14] were
314 labeled by end-filling with α-[32P]-dCTP plus unlabelled dATP, dGTP and dTTP. The oligonucleotide
315 sequences were as follows:

316 +2.9:

317 TCGATATCCTGTGGCATGTCTCCCCCTAGTGGTCCTTCCAGAAACTGCAGCCGCCGCCCCTGCTCCTCCCTCGA

318 Δ2°(1):

319 TCGATATCCTGTGGCATGTCTCCCCCTAGTGGTCCTTCCAGAAAATTGTTATGCCGCCCCTGCTCCTCCCTCGA

320 Δ2°(2):

321 TCGATATCCTGTGGCATGTCTCCCCCTAGTGGTCCTTCCCTTTGATTGTTATGCCGCCCCTGCTCCTCCCTCGA

322 Δ1°:

323 TCGATATCCTGTGGCATGTCATTCCAGAGGAATCCTTCCAGAAACTGCAGCCGCCGCCCCTGCTCCTCCCTCGA

324 HS4:

325 TCGATCCCCCAAAGCCCCCAGGGATGTATTTACGTCCCTCCCCCGCTAGGGGGCAGCAGCGAGCCGCCCCTCG

326 A

327 ΔHS4:

328 TCGATCCCCCAAAGCCCCCAGGGATGTATTTACGTCCCTCCCTTCCTCTGGAATAGCAGCGAGCCGCCCCTCGA

329 Δ1°/Δ2°:

330 TCGATATCCTGTGGCATGTCATTCCAGAGGAATCCTTCCCTTTGATTGTTATGCCGCCCCTGCTCCTCCCTCGA

331 Nuclear extract from unstimulated Jurkat cells were prepared as previously described [53]. 4 µg of
332 nuclear extract was incubated with 4 µg of poly(dI-dC) and 100 ng of an unrelated 22 bp
333 oligonucleotide duplex competitor (containing the GM450 Runx-1 element from the GM-CSF
334 enhancer [54] for 10 minutes before addition of 0.2 ng of labeled oligonucleotide in a 17 µl reaction

335 mixture containing 10% glycerol, 20 mM HEPES, 30 mM KCl, 30 mM NaCl, 0.1 mM ZnSO4, 0.1 mM
336 MgCl2, 1% dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml
337 leupeptin for 15 minutes at room temperature (~ 22 °C). 4% polyacrylamide gels containing 25 mM
338 Tris borate, 0.5 mM EDTA, were pre-electrophoresed at 10 V/cm for 1 h and run at 10 V/cm for 1 h
339 20 min at room temperature. Gels were fixed in 0.1% cetyl trimethylammonium bromide, 50 mM
340 sodium acetate before drying and visualization on a phosphorimager screen. Supershift assays were
341 carried out by addition of 2 µl of CTCF antibody (07-729 Millipore) or IgG antibody (12-370 Millipore)
342 to 4 µg of Jurkat nuclear extract and incubation for 10 min at room temperature before subsequent
343 addition of poly(dI-dC) and oligonucleotide. Competition assays were carried out by addition of the
344 specified amount of unlabelled oligonucleotide competitor in 1 µl to 4 µg of nuclear extract and
345 incubated for 15 mins at room temperature before addition of 0.2 ng of +2.9 labeled
346 oligonucleotide.

347 Relative binding and competition efficiency were determined by densitometric analysis using
348 Quantity One (Bio-Rad) software.

349 **Enhancer-blocking assays**

350 The insulator function of individual CTCF sites was tested using linearised firefly luciferase reporter
351 gene plasmids as previously described [11]. Briefly, gene fragments cloned into pXPG [55] were
352 cotransfected with the Renilla luciferase control plasmid pRL-TK into Jurkat cells which were then
353 cultured for 40 h before stimulation for 8 h with 20 ng/ml Phorbol 12-myristate 13-acetate (PMA)
354 and 1 mM calcium ionophore A23187. Cell extracts were then harvested for dual luciferase activity.
355 The plasmid pIL3 (also known as pIL3H [56]) contains the -559 to +50 region of the human IL-3 gene
356 promoter in the Hind III site of pXPG [55], and pIL3-GME contains the 717 bp Bgl II fragment of the
357 human GM-CSF gene enhancer cloned upstream in the Bgl II site of pIL3. The pIL3-C-GME series of
358 insulator plasmids were made by inserting a single copy of the indicated DNA segments containing
359 the intact or disrupted IL-3 +2.9 kb CTCF site into the Xho I site of pIL3-GME using Xho I adapters at

360 each end. The CTCF sites are inserted into the plasmids in the opposite orientation to that shown in
361 Fig 1A and Fig 2 which means that the NH2 terminus of bound CTCF and ZF3 face towards the
362 enhancer while ZF11 faces towards the promoter. Firefly luciferase plasmids were linearised with
363 Aat II prior to transfection. Relative luciferase activity was calculated as the ratio of firefly and Renilla
364 luciferase activity.

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577 **Supporting information**

578 S1 Figure Locations of CTCF sites at histone H3K27me27 chromatin boundaries.

579 S2 Figure Unmanipulated original images used in figure 2

580 S3 Table Conservation of the IL3 insulator CTCF sites in mammalian species

581 S4 Table Conservation of the UPK2 intronic CTCF site in mammalian species

582 S5 Table Conservation of the HOXA9 chromatin boundary CTCF site in mammalian
583 species

584 S6 Table Conservation of chromatin boundary CTCF sites in mammalian species

Figure 1

														References

Figure 2

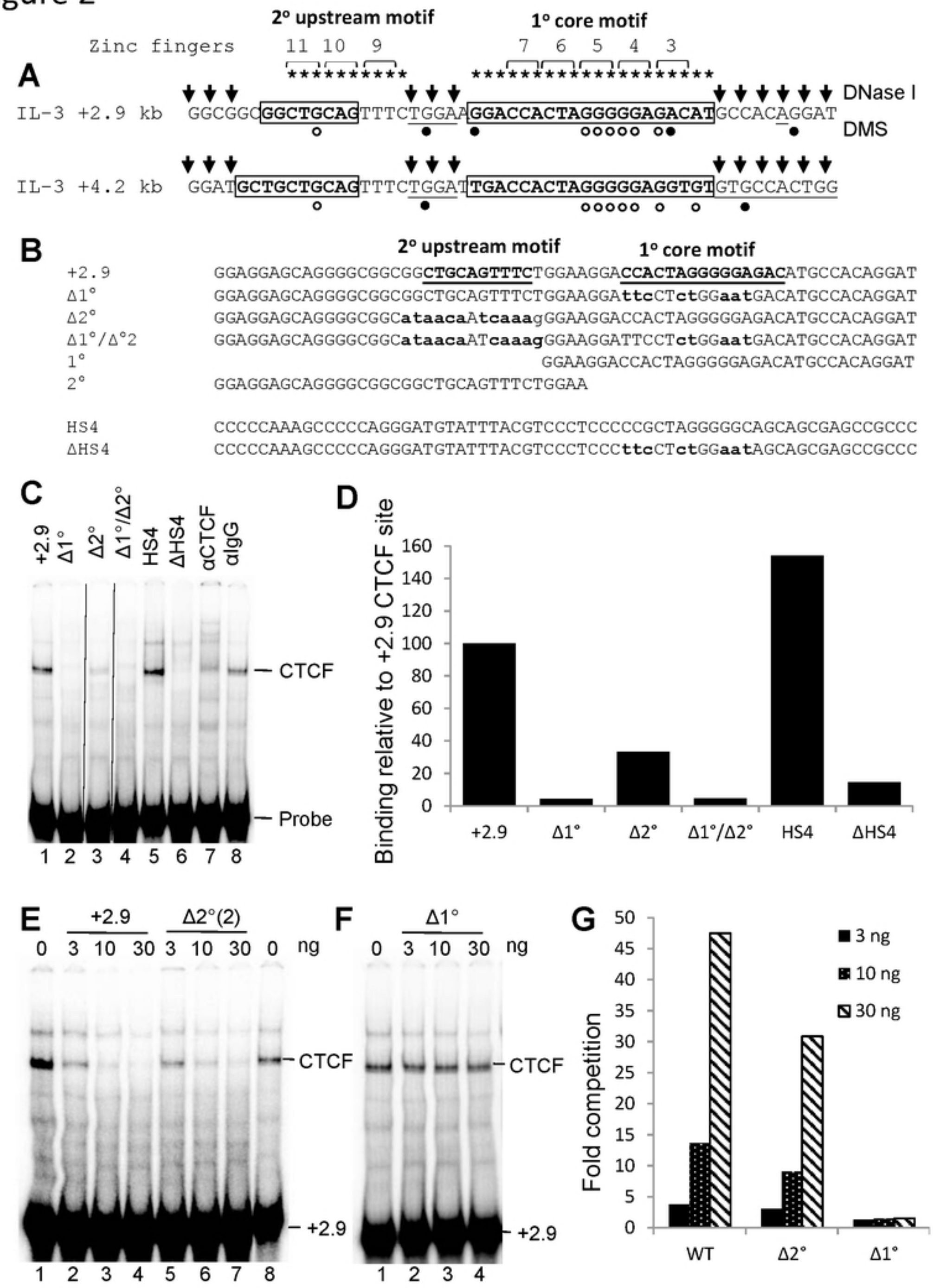
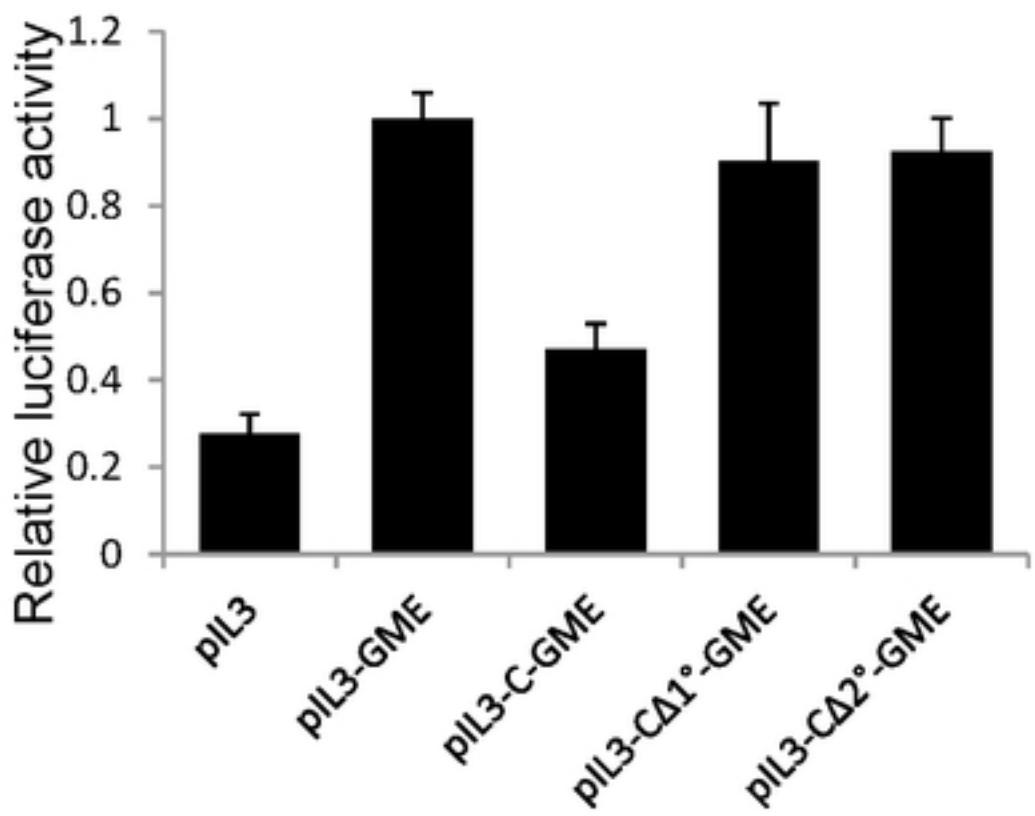
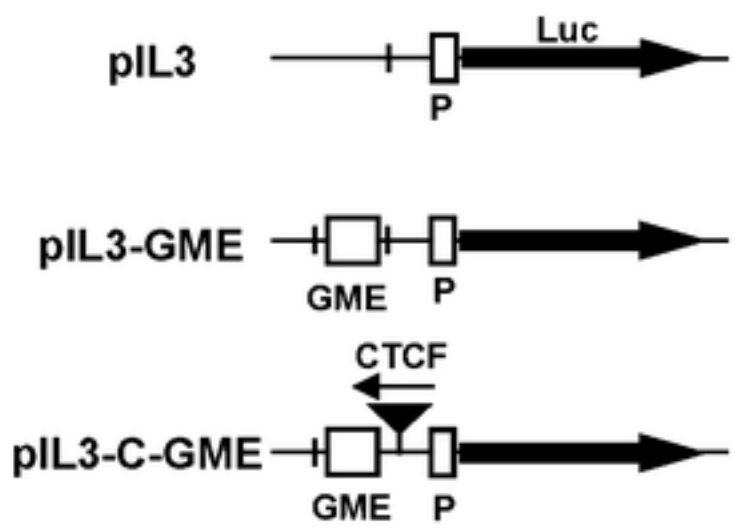


Figure 2

Figure 3





	2° motif	1° motif
C	GGGGCGGCGG**CTGCAGTTTC**TGGAAGGA**CCACTAGGGGGAGA**CATGCCACAGGA	
CΔ1°	GGGGCGGCGGCTGCAGTTTCTGGAAGGA**ttc**CT**ct**GG**aat**GACATGCCACAGGA	
CΔ2°	GGGGCGGC**ataaca**A**tcaaa**gGGAAGGACCACTAGGGGGAGACATGCCACAGGA	

Figure 3