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CTCF mediates long-range interaction between silencer Sis and enhancer Ei and inhibits VJ rearrangement in pre-B cells

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Abstract

Igκ VJ rearrangement occurs only in one allele. It is not fully understood how this allelic exclusion is regulated. We previously identified a recombination silencer and heterochromatin targeting element in the mouse Igκ gene locus, which is termed Sis. Using chromosome conformation capture (3C) technology, here, we demonstrate that Sis and Igκ gene intronic enhancer (Ei) exhibits physical interaction with the intervening DNA looping out. Knock-down of CCCTC-binding factor, CTCF, Ei-Sis interaction is lost and enhancement of VJ rearrangement is detected. Therefore, we propose that the interaction between Sis and Ei mediated by CTCF inhibits the VJ rearrangement and participates in the monoallelic silencing aspect of allelic exclusion regulation.

Key words: *3C, CTCF, Ei, Sis, VJ rearrangement.*

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Introduction

It is becoming increasingly evident that precise regulation of eukaryotic gene expression requires communication between *cis*-acting elements through alterations in higher order chromatin structure [1-4]. The mouse *Igκ* light chain gene locus represents an ideal system to elucidate a paradigm for nuclear reorganization during cellular differentiation [5, 6]. The *Igκ* locus contains 95 potentially functional V_κ genes, four functional and one nonfunctional J_κ regions, a single C_κ region. The locus also possesses a silencer Sis and three transcriptional enhancers: an intronic enhancer (Ei), a 3' enhancer (E3'), and a further downstream enhancer (Ed) [7]. In pre-B cells, one V_κ gene carrying its own promoter is semi-randomly rearranged to one J_κ region. This VJ rearrangement results in transcriptional activation because it puts the V_κ gene promoter under the control of three powerful enhancers. Sis and Ei are reported to regulate VJ rearrangement. Sis inhibits VJ rearrangement through targeting the *Igκ* locus to pericentromeric heterochromatin whereas Ei has a positive role in VJ rearrangement through activation of germline transcription [8, 9].

CCCTC-binding factor (CTCF) is a highly conserved zinc finger protein implicated in diverse regulatory functions, including transcriptional activation/repression, insulation, imprinting [10-13]. Moreover, CTCF mediates intra- and interchromosomal contacts at several developmentally regulated genomic loci [14-17]. It is reported that CTCF associates with Sis in pre-B cells. However, the role of CTCF is not clear. Here we demonstrate that CTCF associates with Sis and Ei, mediates long-range chromatin interaction between Sis and Ei, inhibits germline transcription and downregulates VJ rearrangements in pre-B cells.

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Material and methods

Cell culture

70Z/3 and P815 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 50 μM 2-ME, 100 U of penicillin/ml, and 100 μg of streptomycin/ml at 37°C with 5% CO_2.

Chromosome conformation capture

3C assays were performed as described previously [18]. Cells (10^6) were cross-linked, lysed, and nuclei were digested with *BglII*. After ligation and subsequent DNA purification, the cross-linking frequencies between the anchor and test fragments, as measured by the amount of the corresponding ligation product, were estimated by PCR reactions relative to standards. DNA including Sis, Ei, E3' and Ed was purified from *E.coli* cells and was ligated at high concentration following *BglII* digestion to generate equamolar mixtures of all possible ligation products, and was used to generate standards. The cross-linking and ligation efficiencies between different samples and different experiments were normalized by setting the highest cross-linking frequency for each experimental series to 1.0. Error bars represent the SDs from three to five independent experiments as indicated in the figure legends. Primers used in this study are listed in Table 1.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described previously [19]. Antibody against CTCF was purchased from Millipore. Results were quantified by real-time PCR with SYBR Green dye using the ABI Prism 7500 system (Applied Biosystems). All PCR signals from immunoprecipitation samples were referenced to their respective inputs to normalize for differences in primer efficiencies. α-actin was treated as a negative control and its enrichment fold was considered as 1.0. The enrichment of every test fragment was referenced to α-actin. Primers used in this study were listed in Table 1.

Table 1. Primer sequences used in this study

Primer for 3C assays
1 5'-ccc tgg tgg caa gtg agc aa-3'
2 5'-tca tca atg ctc ctg aca cat-3'
3 5'-tgg tca cca tcc aag aga ttg-3'
4 5'-cca tcc tat ctt ccc ttc taa gg-3'
5 5'-cat caa agg tga ggc cat-3'
Primer for ChIP
1(f)(Sis(f)) 5'-cct tac aca tac aca cac gc-3'
1(r)(Sis(r)) 5'-gaa gtc tgt tta gag ttc tc-3'
2(f)(Ei(f)) 5'-cag agg gga ctt tcc gag agg cc-3'
2(r)(Ei(r)) 5'-acc ctg gtc taa tgg ttt gta ac-3'

(f) forward; (r) reverse

Short hairpin RNA (shRNA) knockdown of CTCF

shRNA sequence for RNA interference of CTCF were described previously [20]. The effective sequence proved to be 5'-GATCCCCGCAGAGAAAGTAGTTGG TAATTTCAAGAGAATTACCAACTACTTTCTCT-GCTTTTTA-3'. The lentiviral transfer construct pCCL. PPT.hPGK.GFP.Wpre, as well as helper viruses pMD2. VSVG, pMDLg/pRRE, and pRSV-REV were transfected into Fx293T cells. Lentiviruses were harvested and infected into target cells 70Z/3 as previously described [21].

Immunoblots

The following antibodies were used: CTCF (Millipore, Billerica, MA, USA) and Actin (Millipore, Billerica, MA, USA).

Real-time PCR for germline transcription

Total RNA, extracted from 10×10^5 cells using TRIzol reagent (Invitrogen, Carlsbad, CA), was reverse transcribed into cDNA with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Real-time PCR was performed with the following primers: sense 5'-GCACACTTAGCTCT-CATTTCCCACCCCAG-3' and antisense 5'-ATGAACTA GAAATTAATATTGTTTGTCTG-3'.

PCR amplification assays for V_κ-J_κ joining

Genomic DNA was purified from cell populations as described previously [22]. For the assay of $V_\kappa$hf24 rearrangement to $J_\kappa1$ and $J_\kappa2$ only, the reverse primer used was 5'-TCATACAAAGGACACAATGGGAC-3'. The family-specific forward Vκhf24 primers used were 5'-TGGGTCAGGAACTGCTTTCACACT-3'. PCR products were separated in 1% agarose gels.

Results and discussion

Recombination silencer, Sis, exhibits interaction with an intronic enhancer, Ei, in 70Z/3 cells

Sis (silencer in the intervening sequence) was discovered as a cluster of four DNase I hypersensitive sites within *Igκ*-locus-chromatin that reside in the intervening sequence between the closest V_κ gene and the J_κ region [23]. In subsequent studies we showed that Sis acted as a recombination silencer and could target germline *Igκ* transgenes to pericentromeric heterochromatin in pre-B cells [8]. The intronic enhancer Ei resides within the J_κ and C_κ region and it exhibits a significant function in activating VJ rearrangement. Subsequent studies showed that Ei activates germline transcription to open *Igκ* chromatin to facilitate the association of recombination complexes. To explore how Sis and Ei cooperate to regulate VJ recombination, we

studied the proximity of these two *cis*-regulatory elements using chromosome conformation capture (3C) technology in pre-B cells 70Z/3. In brief, cross-linked chromatin was digested at a commonly-occurring site with *BglII*, diluted, re-ligated, and long-range association frequencies were assessed with PCR. When the Ei fragment was used as a PCR anchor, a very strong association was observed between Ei and Sis in 70Z/3 cells. In P815 cells, a non-B cell-negative control with inactive *Igκ* loci, however, the interaction between Ei and Sis was not detected (Fig. 1A, B). Therefore, silencer Sis and intronic enhancer, Ei, physically interact with each other in pre-B cells.

CTCF binds to Sis and Ei elements and mediates Sis-Ei physical interaction

We next asked what protein mediates this Sis-Ei physical interaction. Feeney and co-workers have found that Sis possesses bound CTCF in pre-B cells, a protein known to mediate silencing and DNA looping in other systems [24]. Consistently with this report, our ChIP analysis detected the association of CTCF in Sis in 70Z/3 cells and besides that, CTCF was also found to bind to Ei (Fig. 2A). It should be pointed out that Ei does not contain CTCF binding site, the detection of association of CTCF with Ei might be indirect. For example, CTCF associates with Sis. Sis physically interacts with Ei. The whole looping complex was pulled down by CTCF antibody. This result suggests that CTCF may be present in the Sis-Ei looping complexes.

To explore the role of CTCF in Sis-Ei loop formation, we knocked down CTCF in 70Z/3 cells. As shown by the western blot in Fig. 2B, about 90% of CTCF was knocked down.

We then employed 3C to examine the change of Ei-Sis physical interaction in the presence or absence of CTCF. When the Sis fragment is used as a PCR anchor, a very strong association was detected between Sis and Ei in 70Z/3-iluc cells. But this interaction was lost in 70Z/3-iCTCF cells (Fig. 2C). The same result was observed with Ei as an anchor fragment (Fig. 2D). These results indicate that CTCF plays an essential role in mediating the interaction between Sis and Ei.

CTCF inhibits VJ rearrangement through repressing germline transcription

It is the first report showing that the silencer and enhancer can physically interact with each other. To address the role of Sis-Ei physical interaction in VJ recombination, we disrupted the colocalization of Sis and Ei by knocking down CTCF. We then used PCR assay to analyze the VJ rearrangement. The forward primer resides within V_κ gene hf24 that is the most upstream V_κ gene. The reverse primer locates downstream of $J_\kappa 2$ region. PCR products generated from these two primers represent the rearrangement of $V_\kappa$hf24 to $J_\kappa 1$ or $J_\kappa 2$. To quantitate results, we performed PCR assays with one, two, and three times template DNA concentrations. Fig. 3A showed that a two-fold increase of hf24-J1 and hf24-J2 rearrangement in CTCF knock-down

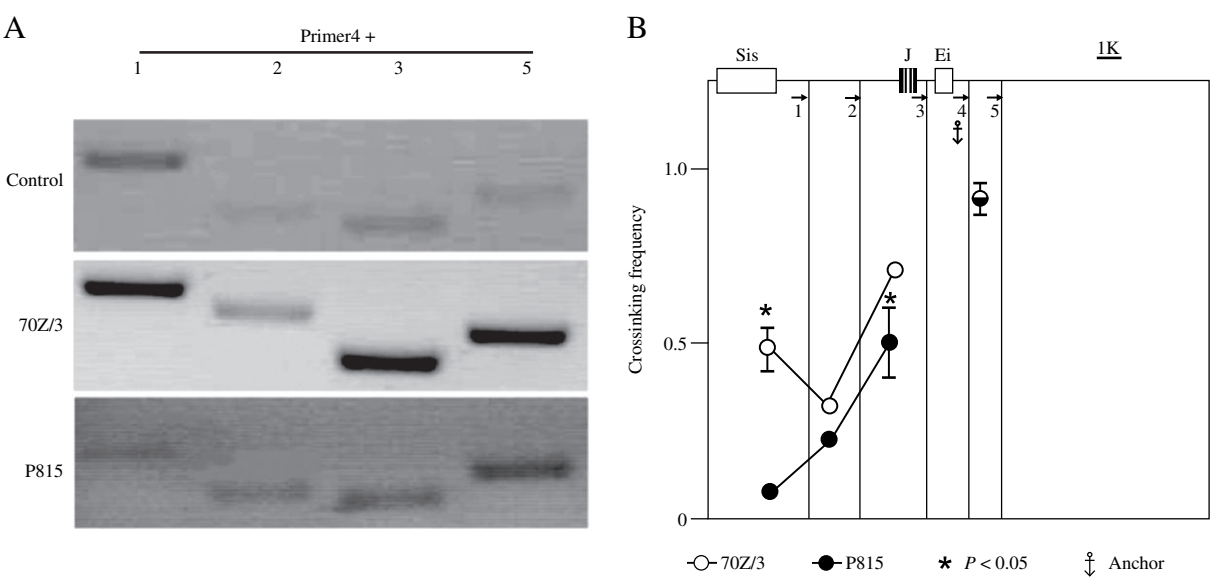

Fig. 1. Sis exhibits interaction with Ei in 70Z/3 cells. (A) PCR-amplified ligation products of 3C. Primer 4 (Ei) was used as an anchor and paired with all the other primers. To normalize the efficiency of PCR amplification process between different primer pairs, the PCR-amplified ligation products from BAC were used as a control. (B) PCR signal quantification. Vertical lines represent *Bgl* α sites, arrows indicate PCR primer sites and direction. Cross linking frequency between different segments and Ei is shown. Standard deviations indicate three independent chromatin preparations

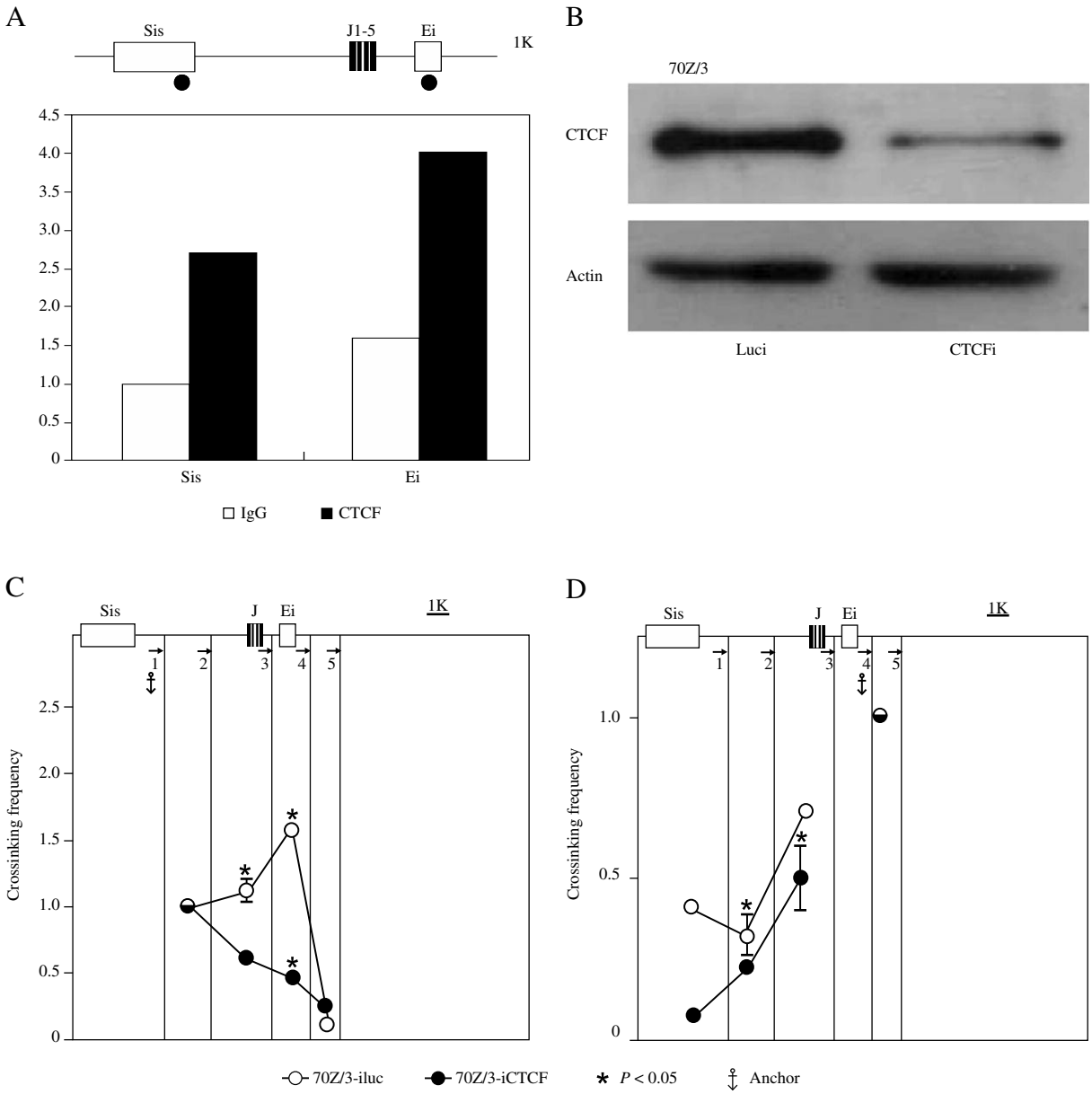

Fig. 2. CTCF mediates physical interaction between Sis and Ei in 70Z/3 cells. (A) Real-time PCR assays of CTCF association with Sis and Ei in 70Z/3 cells. Map of the *Igκ* locus with the sites assayed for PCR amplification in ChIP samples indicated by the underlying solid circles. Fold enrichment refers to the sequence abundance in the immunoprecipitated sample divided by the corresponding sequence abundance in input DNA. (B) Western blot of CTCF in 70Z/3 cells knock-down of CTCF. Actin is an internal control. (C) and (D) 3C assays of proximity between Sis and Ei with Sis as anchor (C) or Ei as anchor (D) in CTCF knock-down 70Z/3 cells. Standard deviations indicate three independent chromatin preparations

70Z/3 cells, indicating that CTCF mediated Sis-Ei looping interaction inhibits VJ rearrangement. This result implies that the interaction between silencer and enhancer may interrupt the function of enhancer.

Since VJ rearrangement requires germline transcription in both V_κ gene and J_κ region and Ei plays an important role in activation of germline transcription. We next examined the change of germline transcription. Two germline transcription starting sites were identified in the upstream of J_κ region. The transcription generated from these two starting sites are termed 5'germline transcription and 3'germline transcription. As shown in Fig. 3B, the level of 5'germline

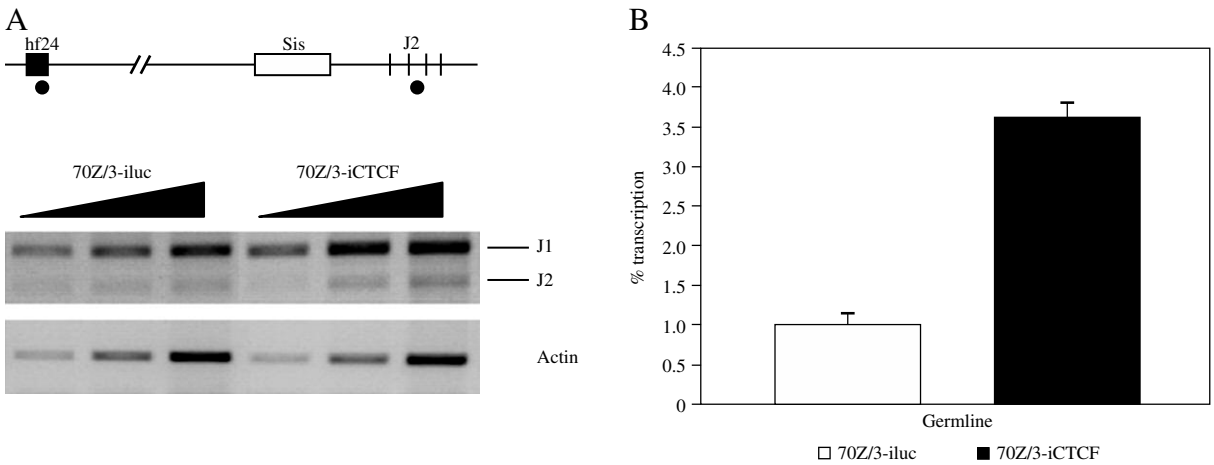

Fig. 3. CTCF inhibits VJ rearrangement and germline transcription in 70Z/3 cells. (A) PCR assays of VJ rearrangement in either 70Z/3 cells or 70Z/3 CTCF knock-down cells. The upper panel shows positions of the primers. Three times serial dilution of the template was used. (B) Real-time PCR assay for germline transcription. Transcript levels were calculated using the DCt method according to the manufacturer's instructions and normalized to the cDNA levels of the mouse GAPDH gene

transcripts increased 3-fold in 70Z/3-iCTCF cells compared with those in 70Z/3-iluc cells, indicating that CTCF mediated Sis-Ei interaction represses germline transcription.

70Z/3 is a late pre-B cell line. It contains a rearranged V_{κ}4-J1 gene. The VJ rearrangement in one allele inhibits activation of the other allele and this is termed allelic exclusion. Allelic exclusion plays an essential role in ensuring that a single B cell expresses only one kind of antigen receptor (BCR). This is important not only for optimizing antibody-antigen specificities by single cells but also for preventing autoimmune reaction because autoimmunity could result if a single cell expressed at least two different BCRs, one directed at an infectious agent and the other against a self-antigen. Previous studies have shown that allelic exclusion of the mouse *Igκ* locus occurs by the monoallelic silencing and a low level of monoallelic activation for rearrangement combined with a negative feedback loop blocking additional functional rearrangements due to down-regulation of RAG expression [25, 26]. Our results lead us to suggest that CTCF-mediated physical interaction between Sis and Ei blocks the function of Ei in stimulating germline transcription and promoting VJ joining. Thus, we revealed a novel mechanism that participates in the monoallelic silencing aspect of allelic exclusion regulation.

The authors declare no conflict of interests.

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References

1. Fraser P, Bickmore W (2007): Nuclear organization of the genome and the potential for gene regulation. Nature 447: 413-417.
2. Li B, Carey M, Workman JL (2007): The role of chromatin during transcription. Cell 128: 707-719.
3. Sadeh R, Allis CD (2011): Genome-wide "re"-modeling of nucleosome positions. Cell 147: 263-266.
4. Sexton T, Schober H, Fraser P, et al. (2007): Gene regulation through nuclear organization. Nat Struct Mol Biol 14: 1049-1055.
5. Schlissel MS (2003): Regulating antigen-receptor gene assembly. Nat Rev Immunol 3: 890-899.
6. Schlissel MS (2004): Regulation of activation and recombination of the murine Igkappa locus. Immunol Rev 200: 215-223.
7. Brekke KM, Garrard WT (2004): Assembly and analysis of the mouse immunoglobulin kappa gene sequence. Immunogenetics 56: 490-505.
8. Liu Z, Widlak P, Zou Y, et al. (2006): A recombination silencer that specifies heterochromatin positioning and ikaros association in the immunoglobulin kappa locus. Immunity 24: 405-415.
9. Xu Y, Davidson L, Alt FW, et al. (1996): Deletion of the Ig kappa light chain intronic enhancer/matrix attachment region impairs but does not abolish V kappa J kappa rearrangement. Immunity 4: 377-385.
10. Chen X, Xu H, Yuan P, et al. (2008): Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell 133: 1106-1117.

11. Ohlsson R, Renkawitz R, Lobanenkov V (2001): CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. Trends Genet 17: 520-527.
12. Phillips JE, Corces VG (2009): CTCF: master weaver of the genome. Cell 137: 1194-1211.
13. Wan LB, Pan H, Hannenhalli S, et al. (2008): Maternal depletion of CTCF reveals multiple functions during oocyte and preimplantation embryo development. Development 135: 2729-2738.
14. Guo C, Yoon HS, Franklin A, et al. (2011): CTCF-binding elements mediate control of V(D)J recombination. Nature 477: 424-430.
15. Kurukuti S, Tiwari VK, Tavoosidana G, et al. (2006): CTCF binding at the H19 imprinting control region mediates maternally inherited higher-order chromatin conformation to restrict enhancer access to Igf2. Proc Natl Acad Sci U S A 103: 10684-10689.
16. Ling JQ, Li T, Hu JF, et al. (2006): CTCF mediates interchromosomal colocalization between Igf2/H19 and Wsb1/Nf1. Science 312: 269-272.
17. Ribeiro de Almeida C, Stadhouders R, de Bruijn MJ, et al. (2011): The DNA-binding protein CTCF limits proximal Vkappa recombination and restricts kappa enhancer interactions to the immunoglobulin kappa light chain locus. Immunity 35: 501-513.
18. Liu Z, Ma Z, Terada LS, et al. (2009): Divergent roles of RelA and c-Rel in establishing chromosomal loops upon activation of the Igkappa gene. J Immunol 183: 3819-3830.
19. Liu Z, Garrard WT (2005): Long-range interactions between three transcriptional enhancers, active Vkappa gene promoters, and a 3' boundary sequence spanning 46 kilobases. Mol Cell Biol 25: 3220-3231.
20. Sekimata M, Perez-Melgosa M, Miller SA, et al. (2009): CCCTC-binding factor and the transcription factor T-bet orchestrate T helper 1 cell-specific structure and function at the interferon-gamma locus. Immunity 31: 551-564.
21. Wang ZV, Schraw TD, Kim JY, et al. (2007): Secretion of the adipocyte-specific secretory protein adiponectin critically depends on thiol-mediated protein retention. Mol Biol Cell 27: 3716-3731.
22. Xiang Y, Garrard WT (2008): The Downstream Transcriptional Enhancer, Ed, positively regulates mouse Ig kappa gene expression and somatic hypermutation. J Immunol 180: 6725-6732.
23. Liu ZM, George-Raizen JB, Li S, et al. (2002): Chromatin structural analyses of the mouse Igkappa gene locus reveal new hypersensitive sites specifying a transcriptional silencer and enhancer. J Biol Chem 277: 32640-32649.
24. Degner SC, Wong TP, Jankevicius G, et al. (2009): Cutting edge: developmental stage-specific recruitment of cohesin to CTCF sites throughout immunoglobulin loci during B lymphocyte development. J Immunol 182: 44-48.
25. Goldmit M, Ji Y, Skok J, et al. (2005): Epigenetic ontogeny of the Igk locus during B cell development. Nat Immunol 6: 198-203.
26. Liang HE, Hsu LY, Cado D, et al. (2004): Variegated transcriptional activation of the immunoglobulin kappa locus in pre-b cells contributes to the allelic exclusion of light-chain expression. Cell 118: 19-29.