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The role of CTCF in regulating V(D)J recombination

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Abstract

V(D)J recombination in B and T cells is required for the generation of receptors with a broad spectrum of specificity to foreign antigen. A total number of three *immunoglobulin (Ig)* and four *T* cell receptor (*Tcr*) loci can be targeted by the recombinase enzyme (RAG1/2) in a defined series of recombination events, which drive the progression of B and T cell development. This process is regulated at multiple levels to ensure lineage specific, ordered rearrangement and allelic exclusion [1]. One key component of this is modulation of chromatin looping and locus contraction, which is important in bringing widely separated gene segments into close contact with each other to enable synapse formation for lineage and stage specific V gene rearrangement [2,3,4•,5,6•]. Recent studies provide new insight into looping and its role in these processes. In this review we focus on the contribution of the 11 zinc finger nuclear protein, CTCF, in mediating loop formation and conformational changes that are important for the regulation of *Ig* and *Tcr* rearrangement.

Introduction

Chromatin organization in eukaryotic cells is known to have a fundamental impact on gene regulation [7]. Classical studies combining genetic analyses and DNA/RNA FISH provided the first insights into the organization of genes relative to each other and to nuclear compartments and chromosome territories. The antigen receptor loci have afforded a rich model system for studying locus dynamics. DNA FISH studies first demonstrated that large-scale locus contraction (which occurs through chromatin looping) is known to be a common feature of V(D)J recombination [2,3]. Several factors, including the B cell specific transcription factor, Pax5, the polycomb group protein Ezh2, Ikaros and Yin Yang 1 (YY1) were identified as being required for *Igh* contraction and distal V_H gene rearrangement [4•]. Further painstaking FISH analyses by Kees Murre's lab elucidated the fine details of the *Igh* looping substructure and they proposed a model in which the contracted *Igh* locus adopts a rosette like structure containing clusters of loops separated by linkers [8].

The advent of chromosome conformation capture and genome wide sequencing has enabled us to look in greater detail at inter- and intra-chromosomal interactions. One particular protein that has emerged as a key player in implementing chromatin conformation is the CCCTC-binding factor, CTCF. It can act both as a transcriptional repressor or activator, depending on the context. To explain these contrasting effects it has been proposed that CTCF is an insulator protein that plays a role in establishing regulatory domains. Indeed, a recent study examining CTCF in a global setting confirmed and extended the observations of many other labs showing that it connects and separates different chromatin domains through loop formation [9]. Handoko *et al.* combined ChIP, chromatin conformation capture (3C) and high throughput genome wide sequencing (ChIA-PET) and found five distinct

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chromatin domains associated with CTCF in mouse embryonic stem cells (Figure 1). These indicate the various functions of CTCF in higher-order nuclear organization.

Identification of CTCF sites in the *Igh* locus

In the V(D)J recombination field, several groups have shown that CTCF sites within antigen receptor loci have a fundamental regulatory role. Using ChIP-chip, the Feeney lab found CTCF sites throughout the V_H regions of the *Igh* locus and identified two additional sites close to the most 5′ D_H gene, DFL16.1 [10] (Figure 2a). However, CTCF binding was similar in pro-B, pre-B and thymocytes, so this could not explain their hypothesis that these elements were involved in regulating changes in locus conformation, which is distinct in the three cell types. To analyze this further they looked at changes in the binding pattern of the CTCF cofactor, cohesin, which is known to play a role in holding sister chromatids together during mitosis and in regulating gene expression through binding to CTCF sites in a lineage and stage specific manner [11•,12•,13]. Degner *et al.* [10] discovered that cohesin binding at CTCF-sites in the *Igh* locus follows a more cell-type specific pattern than CTCF alone (Figure 2a), which lent support to their proposal that CTCF regulates locus contraction.

PAIR elements link CTCF with Pax5, a key mediator of *Igh* locus contraction

To investigate the role of CTCF in locus contraction the Busslinger lab performed ChIP-chip to map active chromatin within intergenic regions of the *Igh* locus [14•]. They identified 14 Pax5-activated intergenic repeat elements (PAIR elements) within the distal V_H gene region, most of which contain functional CTCF, E2A and Pax5 binding sites (Figure 2b). 11 out of the 14 PAIR elements were found immediately upstream of V_H3609 genes interspersed within the distal V_HJ558 gene family. Detailed analysis of three PAIR elements (PAIRs 4, 6 and 7) show binding of Pax5, E2A and CTCF in pro-B cells (Figure 2b, green box). Ebert *et al.* demonstrated that the presence of Pax5 at these elements is essential for an active chromatin signature and antisense transcription within this region. In pre-B cells depletion of Pax5 binding correlates with loss of antisense transcripts, although E2A and CTCF remain bound. These transcription sites are distinct from those previously proposed by the Corcoran lab as being important in opening up the locus [15]. Since locus contraction and decontraction follow the same pattern as Pax5 binding at the pro-B and pre-B cell stage, respectively, these PAIR elements are implicated in Pax5 mediated changes in locus conformation that are known to be essential for most V_H–DJ_H rearrangements [3,4•].

Conserved CTCF sites within the *Igh* locus act as insulators

Additional insight into the role of CTCF in the *Igh* locus came from studies performed by the Corcoran lab. They demonstrated that two conserved CTCF binding sites located upstream of the DFL16.1 gene are embedded in adjacent DNase hypersensitive sites (HS4/5) that have enhancer blocking activity in the *Igh* locus, and their presence marks a sharp boundary of antisense transcription that stops at least 40 kb from the V_H genes [16•]. At the pre–pro-B cell stage and in T cells the two CTCF sites separate regions of active and inactive chromatin in the D_H and V_H regions, respectively (Figure 2c). Antisense transcription in this intergenic region occurs at high levels early in pre–pro-B cells (where the locus is undergoing D_H–J_H rearrangement) and is subsequently reduced in pro-B cells (where V_H–DJ_H recombination takes place). Antisense transcription is also observed in T cells where some D_H–J_H rearrangement of the *Igh* locus occurs. Featherstone *et al.* propose that the presence of these two conserved CTCF sites could prevent antisense transcripts from extending into the V_H region during D_H–J_H rearrangement. This could then act as a mechanism to ensure ordered rearrangement (D_H–J_H prior to V_H–DJ_H) in B cells and to

limit DJ_H recombination in T cells. However, the Feeney lab showed that although knocking down CTCF mildly increased transcription at DFL16.1, it did not extend antisense transcription into the proximal V_H regions [17]. Of course, an absence of CTCF is toxic to the cells so it is difficult to perform experiments in cells where CTCF is totally absent. Thus, the full effects of CTCF may not have been unmasked with this approach and this could explain why knockdown only has a mild effect on *Igh* locus contraction [17]. Also, there could be residual binding of CTCF to its target genes so once there it may be difficult to get rid of, at least in certain places.

CTCF sites regulate ordered, lineage specific rearrangement of the *Igh* locus

To circumvent these issues the Alt lab took a genetic approach and deleted the region containing the two conserved CTCF binding elements (CBE1/2) located adjacent to the DFL16.1 gene (discussed above) to investigate their role in regulating recombination [18••] (Figure 2c). The deleted 4.1 kb fragment (named IGCR1) encompasses both CBE sites alongside potential binding sites for other regulators (YY1 and PU.1). To be certain that phenotypic changes associated with deletion of this fragment could be attributed to the two CBE elements, they also scrambled these sequences to inhibit binding of CTCF. Guo *et al.* found a marked upregulation of proximal V_H7183 and V_HQ52 transcripts and an enrichment of active histone marks in RAG deficient IGCR1 mutant Abelson transformed pro-B cells that they generated. Increased accessibility/transcription of proximal V_H genes was linked to preferential rearrangement of V_H7183 and V_HQ52, at the expense of distal V_HJ558 gene rearrangement in recombination competent IGCR1 targeted cells isolated from mice. In addition, deleted IGCR1 and mutant CBE alleles could undergo V_H–D_H rearrangement prior to D_H–J_H rearrangement, indicating a role for these elements in regulating ordered recombination. Further, mutant *Igh* alleles could also undergo V_H–DJ_H rearrangement in developing thymocytes, in contrast to wild-type counterparts which normally only undergo D_H–J_H rearrangement. Thus, the two CTCF binding sites also have a role in regulating lineage specific V_H rearrangement. These results confirmed predictions made by the Corcoran lab about the insulating function of the two CBEs in regulating accessibility [16•]. Guo *et al.* propose that regulation of *Igh* could be mediated by chromatin loops that form between the CBEs and CTCF sites located downstream of the 3′ regulatory region (3′RR), which involve the intronic enhancer (Eμ). These loops, which were detected by both the Feeney and Alt labs, create a D_H–J_H–Eμ–C_H domain that is separated from the V_H domain during D_H–J_H rearrangement [17,18••] (Figure 2d, upper panel). The CTCF dependent nature of these loops was confirmed by knockdown experiments and analysis of IGCR1 mutant alleles in RAG deficient Abelson cells by the Feeney and Alt labs, respectively.

Eμ dependent and independent loops at the *Igh* locus

New insights into loop formation at the *Igh* locus and the factors involved come from studies performed by the Sen lab [19]. This group examined the entire locus and defined two forms of loops that alter the conformation of *Igh*. The first, which creates the D_H–J_H–Eμ–C_H domain that is found at the earliest stages of B cell development (discussed above), is Eμ dependent, while two other domains that form loops at the 5′ end of the locus are Eμ independent (Figure 2d).

Locus contraction, which brings mid and distal V_H gene segments together at the pro-B cell stage, is known to be dependent on YY1 although the mechanism for this has not been elucidated [20]. Using chromatin immunoprecipitation Guo *et al.* determined that YY1 and CTCF colocalize at the 3′RR and sites 5′ of DFL16.1. YY1 also binds Eμ, but CTCF is absent from this site [19]. Furthermore, Sen and co-workers find YY1 binds selectively to

the 5′7183 and 3′J558 gene segments but there is little enrichment on other V_H genes, which they argue could explain their selective interaction with Eμ. To determine whether CTCF is involved in looping they performed a CTCF ChIP loop assay and looked at CTCF bound regions that interact with V_H3 (within V_H7183 genes) and V_H10 (in distal V_HJ558 genes). V_H3 contacts other proximal V_H genes as well as the 5′ DFL16.1 site while V_H10 makes several contacts within the distal $V_HJ558/3609$ region. The two regions do not interact with each other indicating that they exist as two different chromatin domains (Figure 2d, lower panel). Interaction sites within the two regions bind CTCF but enrichment is higher in the V_H3 versus the V_H10 region. The V_H3 Eμ independent looped domain is brought into contact with the D_H–J_H–Eμ–C_H domain through an interaction with Eμ. They propose a model in which loops between the different Eμ dependent and independent domains come together and separate in a dynamic manner. It is interesting to note that although the V_H10 domain lies in the same region as the newly identified PAIRs [14•] none of the interaction sites within this region correspond to CTCF-binding sites within PAIR elements. Thus it is clear that there are more loops to be discovered.

As a result of their recent ChIP-seq analysis [21] the Murre lab has also been able to equate CTCF binding with loop formation [22]. Lucas *et al.* [22] propose that CTCF organizes the proximal V_H segments into rosettes with the inner cavity containing the D_H–J_H region associated in a recombination center. They suggest that these rosette-structures would be of a dynamic nature, with loops rapidly associating and dissociating, in order to allow equal access to all the V_H segments in the recombination center.

Interestingly, it has recently been reported that mb1-Cre mediated conditional deletion of *Ctcf* in pre–pro-B cells has little effect on *Igh* recombination [23]. These data do not reconcile with the results of CTCF knockdown experiments [17] that impact on the formation of CBE dependent loops [18••], which would be expected to have an effect on distal versus proximal V_H gene rearrangements [18••]. Contradictory data from the two systems highlight a need for further investigation.

Implications for CTCF in regulating *Igk* recombination

At the *Igk* locus CTCF is bound in both pro- and pre-B cells [10]. Degner *et al.* reported that fewer sites are bound on *Igk* relative to *Igh*, but binding to the CTCF sites in *Igk* is more stage-specific in pre-B cells than CTCF binding to the *Igh* locus in pro-B cells (Figure 3a). The only other CTCF site identified is located in the silencer (Sis) within the Vk–Jk intron (Figure 3a). This site is enriched for CTCF in pro- and pre-B cells as well as in T cells, with the highest binding found in pre-B cells. Binding of CTCF to this site was confirmed by the Garrard lab who deleted the entire Sis element [24]. Xiang *et al.* found that an absence of Sis skews the usage of Vk genes so that proximal genes are rearranged at the expense of distal Vk genes. In this respect the CTCF site within the *Igk* Sis imitates the function of the CBE sites located adjacent to the DFL16.1 gene in the *Igh* locus. However, an absence of Sis does not impact on lineage specific regulation, as *Igk* rearrangements are not detected in thymocytes from $Sis^{-/-}$ mice.

The Hendriks lab reports that mb1-Cre mediated conditional deletion of *Ctcf* in the presence of the V_H81X *Igh* transgene also promotes preferential proximal versus distal Vk usage [23]. This group reports more coverage of CTCF at *Igk* than Degner *et al.*, most probably due to the greater sensitivity of ChIP-seq versus ChIP-chip. They identified approximately 60 unevenly distributed binding sites throughout the locus, with regions of high (H1-5) and low (L1-4) CTCF-occupancy (Figure 3b). These include the major CTCF binding sites at the 5′ and 3′ boundaries of *Igk* as well as the Sis element (Figure 3b). Ribeiro de Almeida *et al.* find that proximal Vk genes that are rarely used in wild-type alleles lack CTCF occupancy,

while those Vk genes that bind CTCF are the most frequently used. To examine CTCF mediated interactions within *Igk* they performed 3C-seq using the Sis element as bait in targeted mice bred onto a RAG deficient background. They showed that Sis interacts with regions of high CTCF occupancy through-out the locus. These interactions are not affected by an absence of CTCF however, although interactions with the proximal Vk regions are increased in line with increased proximal Vk usage. These data are also consistent with the data published by Garrard and co-workers [24] showing that deletion of Sis increases proximal Vk usage at the expense of distal Vk genes but has no apparent effect on locus contraction as judged by DNA FISH analyses. Interestingly, in a 3C-seq assay the intronic and 3′ enhancer bait sequences give a similar spectrum of interactions as the Sis bait, but in contrast to Sis, deletion of CTCF decreases interaction of the enhancers (particularly the 3′ Ek) with the distal Vk genes. Loss of CTCF also alters interaction of the *Igk* enhancers with regions upstream and downstream of 3′ and 5′ boundaries of the *Igk* locus. In light of these data they propose a model in which Sis forms CTCF dependent loops that separate the enhancers (in one loop) from proximal Vk genes (in a second loop) (Figure 3b). Separation of the proximal Vk genes favors enhancer–promoter interactions on distal Vk genes which impacts on the choice of Vk genes that are rearranged.

Cohesin has a role in regulating *Tcra* locus conformation and V(D)J rearrangement

Rearrangement of *Tcra* takes place in non-cycling $CD4^+CD8^+$ double positive (DP) T cells so it is possible to deplete the cells of cohesin and monitor the effects on the *Tcra* locus independent of cell division. The Merkenschlager lab took advantage of this and conditionally deleted Rad21, a component of cohesin, using a CD4-Cre transgene [25•]. They showed that cohesin binds to the Tcra locus control region (LCR), the Eα enhancer, the Jα49 promoter, the TEA promoter, sites located between *Tcrd* and the first Vα segments and to Vα gene promoters (Figure 3c). In CD4-Cre $Rad21^{lox/lox}$ thymocytes, primary Jα rearrangements involving 5′ proximal Jα gene segments occur at normal levels, probably because some Rad21 molecules remain bound to these regions before complete depletion by CD4-Cre. By contrast, cohesin is depleted at distal 3′ Jα gene segments and this is linked to impaired secondary rearrangements, decreased transcription, decreased H3K4me3 deposition, RAG binding and a reduction in double strand DNA breaks within this region. An absence of cohesin also affects locus conformation resulting in significantly lowered interaction between the TEA and Eα as well as between Eα and the *Dad1* cohesin site, which is located within the *Tcra* LCR and is known to be a CTCF-dependent insulator (Figure 3c). The absence of Rad21 is linked to an increase in *Dad1* transcription, suggesting that the CTCF-insulator function is impaired. Cohesin also mediates interactions during sequential rearrangements, with Eα contacting the Jα49 and 37 promoters as well as Vα promoters. Finally, Seitan *et al.* show that thymocyte differentiation is impaired in CD4-Cre $Rad21^{lox/lox}$ mice with reduced diversity of TCRA molecules. However, functionally rearranged *Tcr* genes rescue differentiation indicating that this particular defect can be attributed to defects in the *Tcra* locus.

Conclusions

The combined data from the papers discussed here indicate a fundamental role for CTCF and its cofactor, cohesin, in regulating locus conformation and V(D)J rearrangement of the antigen receptor loci. It remains to be seen how other cofactors such as YY1 participate in this control. Moreover, additional genetic experiments targeting individual and/or multiple CTCF binding sites will determine which elements are the most functionally relevant within each of the loci. This will present the greatest challenge given the number of binding sites that are present within each locus.

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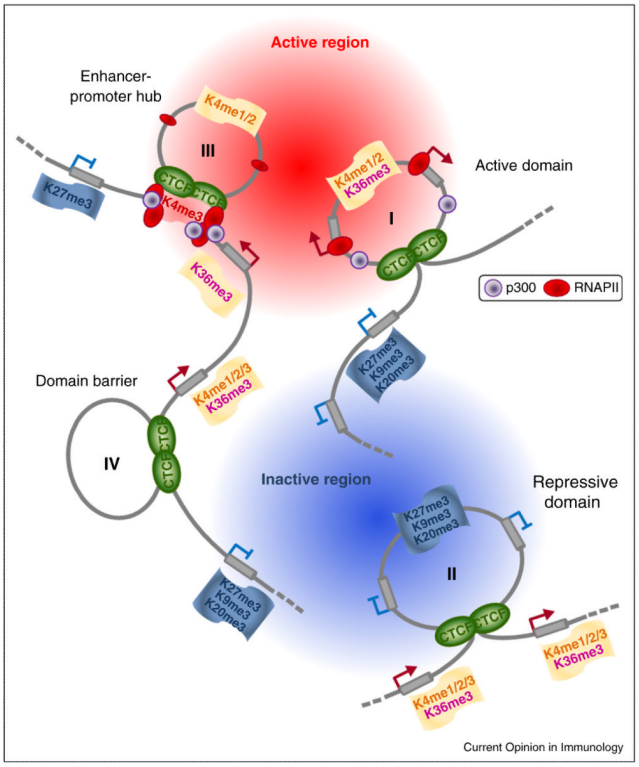

Figure 1.

CTCF as a genome organizer: 5 types of chromatin domains. CTCF has several functions in higher-order nuclear organization. It has a role in (i) creating active or repressive domains which contain coordinately regulated genes, (ii) insulator activity which prevents the spread of active or repressive chromatin into neighboring domains, and in (iii) the cross-talk between regulatory sequences and their targets. (I) Active chromatin domains enriched in H3K4me1/2, H3K36me3, RNA PolII and p300. (II) Repressive chromatin domains enriched in H3K27me3, H3K9me3, H4K20me3. (III) Enhancer–promoter hubs with loops enriched in H3K4me1/2 and boundaries particularly enriched in H3K4me3, RNA PolII and p300. (IV) Domain barriers physically separate active and repressive domains. (The type V is not represented as it does not show any specific chromatin and gene expression pattern).

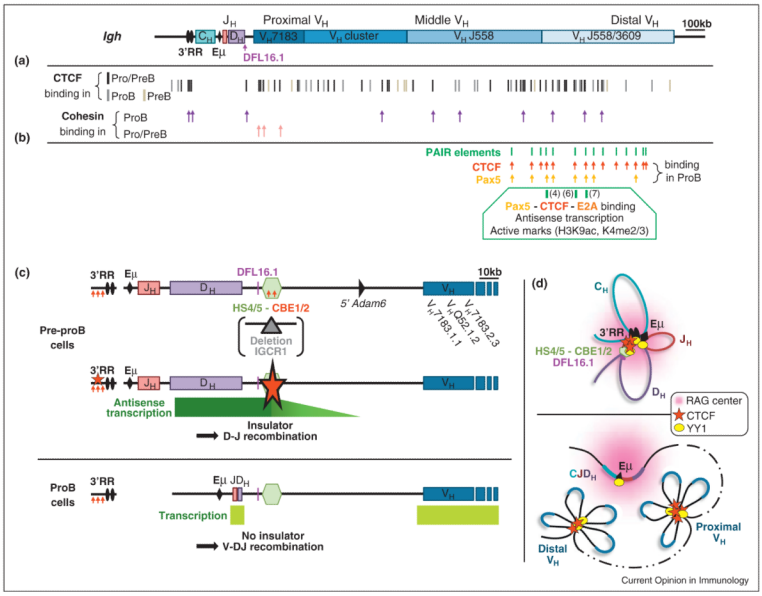

Figure 2.

CTCF and the architecture of the *Igh* locus. (a) Schematic presentation of the *Igh* locus with C_H, D_H, J_H and V_H regions as well as the 3′ regulatory region (3′RR) and the *Igh* enhancer (Eμ). CTCF- and cohesin-binding sites are shown below [10]. (**b**) Location of the 14 PAIR elements with CTCF- and Pax5-binding sites [14•]. The green box contains the details of epigenetic characteristics of 3 PAIR elements (4,6,7). (**c**) Detail of the 3′ region of the *Igh* locus from the 3′RR to the proximal V_H segments showing CTCF-binding sites: (i) the two hypersensitive sites (HS4/5) corresponding to the two CTCF-binding sites (CBE1/2) located upstream of DFL16.1 [16•,18••], and the IGCR1 deletion [18••] and (ii) the CTCF-binding outside of the 3′ RR. At the pre–pro-B cell stage, CTCF bound to CBE1/2 acts as an insulator, which stops antisense transcription and active chromatin from the D_H region spreading to the V_H region, thus limiting recombination to D_H–J_H. At the subsequent pro-B cell stage, the barrier is released and transcription and active chromatin can spread to the V_H region, allowing V_H–DJ_H recombination. (**d**) Model of the two-types of CTCF-mediated loops in the *Igh* locus [19]. At the early stage of B cell development, the Eμ-dependent loop involves the CTCF-sites at the 3′ RR and CBE1/2, the Eμ enhancer and the D_H and J_H segments (upper panel). Later, two types of Eμ-independent loops create two chromatin domains in the V_H segments, one with the proximal and one with the distal V_H genes (lower panel).

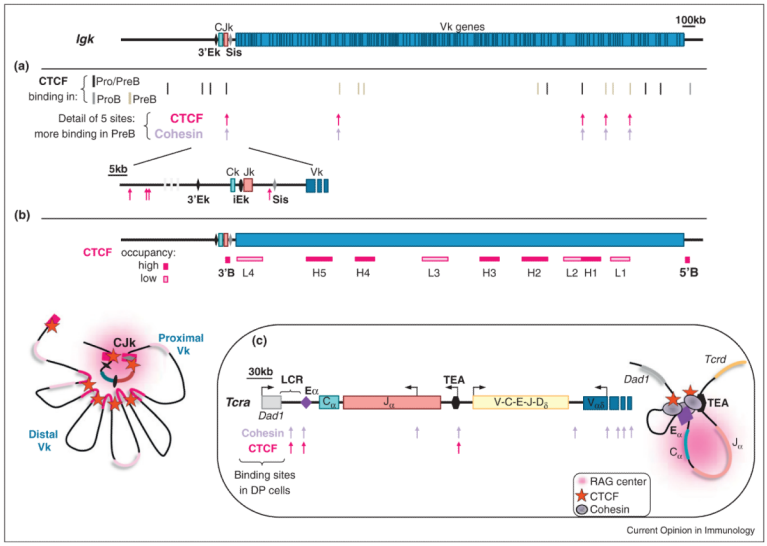

Figure 3.

CTCF/cohesin and the architecture of the *Igk* and *Tcra* loci. (**a**) Schematic presentation of the *Igk* locus with Ck, Jk and Vk regions as well as the 3′ *Igk* enhancer (3′Ek) and the *Igk* silencer (Sis). CTCF- and cohesin-binding sites identified by Degner *et al.* [10] are shown below. Detail of the 3′ region including the regulatory elements (3′Ek, intronic *Igk* enhancer (iEk) and Sis) with the CTCF-binding sites shown below [10,24]. (**b**) Regions of low (L1-4) and high (H1-5) CTCF-occupancy over the Vk region as well as the 3′ and 5′ CTCF-boundaries (3′B and 5′B) are shown below the *Igk* schematic [23]. Model of the CTCF-mediated loops: (i) separation of the enhancers from the proximal Vk genes through CTCF-binding to Sis, (ii) separation of the enhancers from the outside 3′ sequences, (iii) separation of the proximal and distal Vk gene segments, and (iv) interactions between the C-Jk region and distal Vk segments [23]. (**c**) Schematic presentation of the 3′ region of the *Tcra* locus, from the *Dad1* gene to the first Vα genes, including the *Tcra* locus control region (LCR), the *Tcra* enhancer (Eα), the C-Jα region, the T early α promoter (TEA), and the V–C–E–J and Dδ elements of *Tcrd*. Cohesin and CTCF-binding sites are shown below [25•]. Model of the cohesin/CTCF-mediated loops in the 3′ *Tcra* region that allow interactions of Eα with TEA and the LCR in order to insulate the C-Jα region from *Dad1* and *Tcrd*.