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CTCF Binding Elements Mediate Control of V(D)J Recombination

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Abstract

Immunoglobulin heavy chain (IgH) variable region exons are assembled from V_H, D and J_H gene segments in developing B lymphocytes. Within the 2.7 megabase (Mb) mouse IgH locus (*IgH*), V(D)J recombination is regulated to ensure specific and diverse antibody repertoires. Herein, we report a key *IgH* V(D)J recombination regulatory region, termed InterGenic Control Region-1 (IGCR1), that lies between the V_H and D clusters. Functionally, IGCR1 employs CTCF looping/insulator factor binding elements and, correspondingly, mediates *IgH* loops containing distant enhancers. IGCR1 promotes normal B cell development and balances antibody repertoires by inhibiting transcription and rearrangement of D_H-proximal V_Hs and promoting rearrangement of distal V_Hs. IGCR1 maintains ordered and lineage-specific $V_H(D)J_H$ recombination, respectively, by suppressing V_H joining to Ds not joined to J_Hs and V_H to DJ_H joins in thymocytes. IGCR1 also is required to allow feedback regulation and allelic exclusion of proximal V_H to DJ_H recombination. Our studies elucidate a long-sought *IgH* V(D)J recombination control region and implicate a new role for the generally expressed CTCF protein.

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The variable region exons of IgH, Ig light (IgL), and T cell receptor genes are assembled during B or T cell development from variable (V), diversity (D) and joining (J) gene segments[1]. The V(D)J recombination reaction is initiated by RAG endonuclease[1], which cleaves paired gene segments flanked by complementary recombination signals (RSs) referred to as 12RSs and 23RSs[1]. The cleaved segments are then fused via classical non-homologous end-joining (C-NHEJ)[2]. The mouse *IgH* contains hundreds of V_Hs within a several Mb region, followed downstream by a 100 kilobase (kb) "intergenic" region separating the most downstream V_H ($V_{H7183.a2.3}$, referred to as "V_{H81X}")[3] from $D_{FL16.1}$, the first of 13 clustered D_Hs. The most downstream D (D_{Q52}) lies upstream of 4 J_Hs[4]. V_Hs and J_Hs are flanked by 23RSs and Ds are flanked on both sides by 12RSs, ensuring that V_HDJ_H assembly involves joining V_Hs and J_Hs, respectively, to upstream and downstream sides of a D_H[4]. The *IgH* constant region exons (C_Hs) lie in the 200kb region downstream of the J_Hs; RNA splicing fuses productively assembled V_HDJ_H and C_H exons during IgH mRNA formation.

IgH V(D)J recombination in developing B cells is regulated to be highly "ordered" and "stage" specific; thus, D_H to J_H joining developmentally occurs first on both alleles in "pre"-progenitor (pro)-B cells followed by appendage of a V_H to a DJ_H complex in pro-B cells[4-6]. Direct joining of a V_H to an un-rearranged D_H does not occur, even though theoretically permitted by the 12/23 rule[3,7]. The V_H to DJ_H joining step also is regulated to achieve "lineage specificity"; thus, while developing T cells generate DJ_H joins, they do not form complete V_H DJ_H exons[7,8]. At the pro-B stage, V(D)J recombination is regulated in the context of "allelic exclusion", with a signal from a productive (*i.e.* μ IgH protein-encoding) V_HDJ_H rearrangement inhibiting V_H to DJ_H joining on the other *IgH* allele, if it is in the DJ_H configuration[5]. Expression of the μ chain also signals development to the precursor (pre)-B cell stage and *IgL* V(D)J recombination[9]. To generate such signals in pro-B cells, μ IgH chains must pair with surrogate IgL chains[10]. Subsequently, μ chains must pair with IgL chains in pre-B cells to mediate the pre-B to IgM^+ B cell transition. Finally, *IgH* V(D)J recombination is regulated to ensure utilization of V_Hs across the large V_H locus. However, proximal V_Hs, particularly V_{H81X}, are rearranged more frequently than distal V_Hs, leading to over-representation in primary V_HDJ_H repertoires[3]. Repertoire "normalization" for distal V_Hs in mature B cells relies on cellular selection[3,11], promoted, in part, by inability of certain proximal V_Hs, including V_{H81X}, to pair with surrogate IgL chains and IgL chains[12,13].

V(D)J recombination at all antigen receptor loci is effected by the common V(D)J recombinase comprised of RAG and C-NHEJ components. Regulation of *IgH* V(D)J recombination in the context of order/stage, lineage and allelic exclusion is achieved via modulation of substrate V, D, and J accessibility[14,15]. Correlates of such accessibility include transcription of un-rearranged gene segments and certain DNA and histone modifications[4,14-21]. *IgH* locus contraction and looping also may mediate higher order regulation of V(D)J recombination, for example by bringing distant V_Hs into proximity with the DJ_H[8,22-24]. Until now, *cis* elements that control order, lineage-specificity, allelic exclusion, and/or differential V_H utilization have been elusive[16,19]. The only known long-range *IgH* regulatory elements are a transcriptional enhancer (termed iEμ) in the intron

between the J_Hs and C_Hs and a set of long-range enhancers (termed 3′*IgH* regulatory region or 3′*IgH*RR) downstream of the C_Hs[18,25]. The iEμ is required for efficient *IgH* V(D)J recombination, particularly V_H to DJ_H joining[26-28], although mechanisms by which it influences this process are unknown[18]. To date, the 3′*IgH*RR has not been implicated in V(D)J recombination[25]. As most critical aspects of *IgH* V(D)J recombination are regulated at the V_H to DJ_H step[7, 8], relevant regulatory elements may reside in the 100 kb "intergenic" region separating V_Hs and D_Hs (See Supp. Discussion)[7,16,17,19,29,30].

Role in Normal B Cell Development

The region several kb upstream of $D_{FL16.1}$ harbors chromatin modifications[29,31,32] and two CTCF binding elements ("CBEs")[29,31-33] suggestive of a potential regulatory region (Supp. Fig. 1). CTCF is an 11-zinc finger nuclear protein implicated in transcriptional insulation, chromatin boundary formation, transcriptional activation/repression, and chromosome looping[34-36]. There are several other potential *cis*-elements closely linked to these CBEs including potential PU.1[31] and YY1-binding sites (based on JASPAR data base). We refer to this cluster of factor binding sites as IGCR1 (Fig. 1). To test for a role in *IgH* V(D)J recombination, we generated an "IGCR1-deleted" 129SV allele in which 4.1 kb DNA fragment that contains both CBEs and other binding sites was deleted in the mouse germline (Fig. 1a; Supp. Fig. 2). To test for specific roles of the CBEs, we generated mice in which both were replaced with scrambled sequences that do not bind CTCF (Supp. Figs. 1, 3). Mice heterozygous or homozygous for the IGCR1 deletion are referred to, respectively, as IGCR1$^{+/-}$ and IGCR1$^{-/-}$and mice heterozygous or homozygous for the dual CBE-mutation are referred to, respectively, as IGCR1/CBE$^{+/-}$ or IGCR1/CBE$^{-/-}$. Because generation of mutant alleles involved *loxP* insertion, we generated control lines heterozygous or homozygous for the *loxP* insertion referred to, respectively, as loxP$^{+/I}$ or loxP$^{I/I}$ (Fig. 1a). As wild-type (WT), loxP$^{+/I}$, and loxP$^{I/I}$ mice gave essentially identical results, we refer to them collectively as "controls". As a further control, we deleted an approximately 2 kb DNA fragment downstream of the D_H-proximal end of IGCR1 and found no obvious phenotype (Supp. Fig. 10).

129SV IGCR1/CBE$^{+/-}$ or IGCR1/CBE$^{-/-}$ mice had similar splenic IgM$^+$ B cell numbers as controls (Fig. 1b; Supp. Fig. 5a). However, IGCR1/CBE$^{+/-}$ and, more so, IGCR1/CBE$^{-/-}$ mice had a substantial diminution in bone marrow (BM) pre-B cell numbers (Fig. 1b). As the pro-B to pre-B transition is signaled by a productive V_HDJ_H in pro-B cells, this developmental defect suggests an *IgH* V(D)J recombination defect. As a more sensitive test for roles of IGCR1 in B cell development, we bred 129SV IGCR1/CBE$^{+/-}$ mice with C57BL/6 WT mice to generate F1 mice with a WT IgM^b allele and a CBE-mutated IgM^a allele and assayed B cells for surface IgM^a and IgM^b expression. Remarkably, while normal F1 mice, as expected, have roughly equal numbers of IgM^a or IgM^b B cells (but not both due to *IgH* allelic exclusion), most IgM$^+$ BM and splenic B cells in F1 mice carrying the IGCR1 CBE-mutant IgM^a allele express IgM^b (Fig. 1c; Supp. Fig. 5b). Thus, mutation of the IGCR1 CBEs renders an *IgH* allele ineffective in supporting B cell development when competing against a WT *IgH* allele. We found identical B cell developmental defects in IGCR1$^{+/-}$ and IGCR1$^{-/-}$ mice (Supp. Figs. 4b, 4c, 5c, 5d).

Mediation of Diverse *IgH* Repertoires

We employed a PCR approach (Supp. Fig. 6a) to assay for DJ_H and V_HDJ_H rearrangements in purified control, IGCR1/CBE$^{+/-}$, IGCR1/CBE$^{-/-}$, IGCR1$^{+/-}$, and IGCR1$^{-/-}$ BM pro-B and pre-B cells, and in splenic B cells. We assayed for rearrangements of the two most D_H-proximal V_H families (V_{H7183} and V_{HQ52}) and the most distal V_H family (V_{HJ558}). *IgL* Vκ to Jκ joins were assayed as a stage-specific control and the mouse *DLG5* gene as a loading control. Levels of DJ_H and VκJκ rearrangements did not vary markedly among different populations or genotypes; thus, V(D)J recombination in general was not affected by the mutations (Fig. 2a; Supp. Fig. 6). However, relative levels of proximal $V_{H7183}DJ_H$ rearrangements were dramatically increased and those of distal $V_{HJ558}DJ_H$ rearrangements dramatically reduced in IGCR1/CBE$^{-/-}$ and IGCR1$^{-/-}$ pro-B cells, with both being intermediate in IGCR1/CBE$^{+/-}$ and IGCR1$^{+/-}$ pro-B cells (Fig. 2a; Supp. Fig. 6). Within the two proximal V_H families, V_H usage was even more skewed towards the most D proximal members in IGCR1$^{-/-}$ pro-B cells (Supp. Fig. 6c). Together, these findings are consistent with IGCR1 mutations resulting in *cis*-acting increases and *cis*-acting decreases, respectively, in proximal and distal V_H rearrangement. Given that the proximal V_Hs contribute to a substantial fraction of V_HDJ_H rearrangements (about 40%) in normal pro-B cells[3,11], increased V_{H7183} joins in IGCR1/CBE$^{+/-}$ and IGCR1$^{+/-}$ pro-B cells indicates that the absolute level of V_H to DJ_H rearrangements on mutant alleles, while even more biased towards proximal V_Hs than normal, is not decreased. In the various IGCR1 mutant pre-B cells and splenic IgM$^+$ B cells repertoire bias remained; although the extent was progressively moderated (Supp. Fig. 6), likely due to cellular selection for V_H repertoire normalization.

Regulation of Germline V_H Transcription

To measure germline V_H transcripts, we generated RAG2-deficient A-MuLV-transformed WT, IGCR1$^{+/-}$ and IGCR1$^{-/-}$ pro-B lines. RAG2-deficient lines have unrearranged *IgH* alleles; thus, any detected V_H transcripts are germline. RNA was assayed via RT-PCR for V_H expression, utilizing one primer from the V_H leader sequence and another from downstream of the RS (Supp. Fig. 7a). Based on size, the PCR assay detects both unspliced germline V_H transcripts (sense or antisense) and slightly smaller, spliced sense germline V_H transcripts (Fig. 2b). RAG2$^{-/-}$ pro-B lines had robust D_H transcripts and spliced and un-spliced V_{HJ558} transcripts, but lacked readily detectable V_{HQ52} or V_{H7183} transcripts (Fig. 2b). However, RAG2$^{-/-}$IGCR1$^{+/-}$ and, more so, RAG2$^{-/-}$IGCR1$^{-/-}$ pro-B lines showed dramatic upregulation of spliced and unspliced V_{HQ52} and V_{H7183} transcripts with normal levels of V_{HJ558} and D_H transcripts (Fig. 2b; Supp. Fig. 7d). We even detected by Northern blotting an ~3.5 kb V_{H81X}-hybridizing transcript in RNA from RAG2$^{-/-}$IGCR1$^{-/-}$ lines, but not in WT RAG2$^{-/-}$ lines, (Supp. Fig. 7f). Primary RAG2$^{-/-}$IGCR1$^{-/-}$ pro-B cells also strongly up-regulated germline V_{H7183} transcripts (Supp. Fig. 7e). Finally, ChIP-seq and ChIP-qPCR analyses revealed that deletion of IGCR1 led to a dramatic increase in active histone marks over V_{H81X} ($V_{H7183.a2.3}$) and the adjacent $V_{HQ52.a2.4}$ germline gene segments (Fig. 2c; Supp. Figs. 7b, 7c). Thus, IGCR1 suppresses activation of germline V_Hs over distances of at least 100 kb.

Role in Order and Lineage-specificity

We assayed for V_{H81X} to germline D_{Q52} joins via PCR with a forward V_{H81X}-specific primer and a reverse primer from sequences between D_{Q52} and J_H1 (Supp. Fig. 8). While we did not detect direct V_{H81X} to D_{Q52} joins in control pro-B cells; we readily detected them in IGCR1/CBE$^{+/-}$, IGCR1/CBE$^{-/-}$, IGCR1$^{+/-}$ and IGCR1$^{-/-}$ pro-B cells (Fig. 2d; Supp. Fig. 8). Sequences of 133 independent direct $V_{H7183}D_{Q52}$ joins revealed that 120 involved V_{H81X}, 12 involved the downstream pseudo-V_{H7183}, and one involved the next V_{H7183} upstream of V_{H81X} (Supp. Table 2). Therefore, integrity of the IGCR1 CBEs is required for ordered *IgH* V(D)J recombination in pro-B cells, at least for proximal V_H segments.

To examine potential IGCR1 roles in lineage-specific *IgH* V(D)J recombination, we assayed for D to J_H, V_H to DJ_H and Vκ to Jκ rearrangements in DNA from CD4$^+$/CD8$^+$ ("DP") thymocytes from control and IGCR1/CBE$^{+/-}$, IGCR1/CBE$^{-/-}$, IGCR1$^{+/-}$ and IGCR1$^{-/-}$ mice (Fig. 3a; Supp. Fig. 9). We detected $D_{Q52}J_H$ rearrangements in all mice (Fig. 3a; Supp. Fig. 9). However, while there were no V_HDJ_H rearrangements in controls, we readily detected V_HDJ_H rearrangements of proximal V_{H7183} and V_{HQ52} segments, but not distal V_{HJ558} segments, in mutant DP thymocytes (Fig. 3a; Supp. Fig. 9). Lack of VκJκ rearrangements confirmed absence of B cell contamination. Cloning and sequencing of V_{H7183} and V_{HQ52} to DJ_H rearrangements from IGCR1$^{-/-}$ DP thymocytes revealed predominant utilization of the most proximal V_Hs (V_{H81X} and $V_{HQ52.a2.4}$; Supp. Table 3). We also assayed for direct V_{H81X} to germline D_{Q52} joins in DP thymocytes (Fig. 3b; Supp. Fig. 8). As expected, controls lacked detectable direct V_H to D joins; but such joins were readily apparent in mutant thymocytes (Fig. 3b; Supp. Fig. 8). Nucleotide sequencing of 32 V_HD joins revealed 29 utilized V_{H81X} and the rest utilized the downstream pseudo-V_{H7183} (Supp. Table 2). Thus, IGCR1 CBEs are required for lineage-specific *IgH* V_H to DJ_H recombination.

Role in Proximal V_H Feedback Regulation

Surface staining of splenic B cells heterozygous for the IGCR1-deleted IgM^a allele and a WT IgM^b allele did not reveal allelic inclusion (Supp. Fig. 4c). Likewise, no IgM^a/IgM^b double expressers were found in nearly 900 individual IGCR1$^{+/-}$ F1 splenic B cells by cytoplasmic staining (Supp. Fig. 11a). Hybridoma analyses showed that about 60% of WT B cells had a productive V_HDJ_H on one allele and a DJ_H on the other (*i.e.* $V_H(D)J_H^+/DJ_H$ configuration) and about 40% had $V_H(D)J_H$ rearrangements on both alleles (*i.e.* $V_H(D)J_H^+$ /$V_H(D)J_H^-$ configuration) (Fig. 4a). This "60/40" ratio reflects feedback regulation of V_H to DJ_H joining from productive rearrangements[5,6]. In IGCR1$^{+/-}$ B cells, this ratio inverted to 30/70, demonstrating that heterozygous IGCR1 deletion markedly increases B cells with $V_H(D)J_H$ joins on both alleles, despite allelic exclusion at the protein level. Analyses of 39 V_HDJ_H/V_HDJ_H IGCR1$^{+/-}$ B cell hybridomas revealed that most had a $V_H(D)J_H^+$ that utilized a distal V_H and a $V_H(D)J_H^-$ that used V_{H81X} or a nearby proximal V_H (Supp. Table 6). The skewed $V_H(D)J_H^+/V_H(D)J_H^-$ ratio in IGCR1$^{+/-}$ B cells can be explained by frequent early formation of $V_{H81X}DJ_H$ rearrangements on the mutant allele. Thus, $V_{H81X}DJ_H^+$ rearrangements would exclude rearrangement of the WT allele but would be lost developmentally; leading to most peripheral B cells deriving from progenitors that

formed productive $V_H(D)J_H$ rearrangements on the WT allele subsequent to $V_{H81X}(D)J_H^-$ rearrangements on the mutant allele (Supp. Fig. 11d).

The extremely high representation of proximal V_Hs (*e.g.* V_{H81X}) rearranged on the IGCR1-deleted allele might mask allelic inclusion because productive V_{H81X} rearrangements are selected against cellularly[10,12,13]. Therefore, to further examine potential effects of IGCR1-deletion on allelic exclusion, we assayed the $V_H(D)J_H^+/DJ_H$, versus $V_H(D)J_H^+/V_HDJ_H^-$ ratio of IGCR1$^{-/-}$ hybridomas. Because both *IgH* alleles would be similarly biased for proximal V_H rearrangements in IGCR1$^{-/-}$ B cells, one still would expect the 60/40 ratio if V_H to DJ_H recombination was feedback regulated (Supp. Fig. 11e). However, we found an inverted ratio of 20/80 in IGCR1$^{-/-}$ hybridomas (Fig. 4a), strongly suggesting that IGCR1 deleted alleles escape feedback regulation, at least for proximal V_Hs (Supp. Fig. 11e). Because of the ambiguities of cellular selection against V_{H81X} and the lack of allotypically marked IGCR1-deleted alleles, we tested for escape from feedback inhibition by assaying for endogenous rearrangements in peripheral B cells from mice with a productive $V_H(D)J_H$ knock-in *IgH* allele ("VB1-8 KI") that was IGCR1$^+$ and a second allele that was IGCR1$^+$ or IGCR1$^-$. Strikingly, IGCR1$^{+/-}$ VB1-8 KI B cells had a more than 20-fold increased level of V_{H7183} rearrangements compared to IGCR1$^{+/+}$ VB1-8 KI B cells, but little if any change in the very low level rearrangement of distal V_Hs (Fig. 4b). Moreover, most rearrangements in IGCR1$^{+/-}$ VB1-8 KI B cells were non-productive V_{H81X} rearrangements (Supp. Fig. 11f), consistent with lack of substantial allelic inclusion at the protein level in IGCR1$^{+/-}$ F1 splenic B cells resulting from selection against V_{H81X} expression (Supp. Figs. 4c, 11a). We conclude that IGCR1 is required to allow feedback regulation of the most proximal V_Hs.

IGCR1 mediates chromosomal *IgH* loops

We considered that IGCR1 might mediate *IgH* loops that would include iEμ and thereby modulate V(D)J recombination. The next CBEs downstream of IGCR1 are a set of 10, about 5 kb downstream of the 3′*IgH*RR ("3′*IgH*CBEs"). To test for interactions between the IGCR1 and 3′*IgH*CBEs, we performed quantitative chromosome conformation capture (3C) assays on 129SV RAG2$^{-/-}$IGCR1$^{+/+}$ and RAG2$^{-/-}$IGCR1$^{-/-}$ A-MuLV transformed pro-B lines. These analyses revealed interaction between the IGCR1 and 3′*IgH*CBE locales in RAG2$^{-/-}$IGCR1$^{+/+}$ pro-B lines (Fig. 5a; Supp. Fig. 12a), as found in another study[37]. We also found this interaction in DP thymocytes (Supp. Fig. 13). Notably, this interaction was eliminated in RAG2$^{-/-}$IGCR1$^{-/-}$ pro-B lines (Fig. 5a; Supp. Fig. 12a). We also found interactions between the iEμ locale and the IGCR1 and 3′*IgH*CBE locales in RAG2$^{-/-}$IGCR1$^{+/+}$ A-MuLV transformed pro-B cells that were diminished in RAG2$^{-/-}$IGCR1$^{-/-}$ pro-B lines (Fig. 5b; Supp. Fig. 12b). Finally, we found strong interactions between the iEμ and 3′*IgH*RR locales, as reported for mature B cells[38]; but these were not diminished by IGCR1 deletion (Fig. 5b). These studies demonstrate that IGCR1 mediates formation of 300 kb iEμ-containing *IgH* loops to the 3′*IgH*CBE locale in pro-B lines, with iEμ also being directly juxtaposed to the IGCR1 locale in an IGCR1-dependent manner, likely within the larger loop. As iEμ lacks CBEs, its interactions with the IGCR1 locale likely are mediated, at least in part, by factors other than CTCF.

Discussion

IGCR1, through its CBEs, mediates ordered and lineage-specific V_H to DJ_H recombination and balances proximal versus distal V_H rearrangement. Indeed, IGCR1 functions are required for an *IgH* allele to efficiently generate peripheral B cells. Notably, IGCR1 and its CBEs are not required for overall V_H to DJ_H recombination levels, but rather to decrease relative recombination of proximal V_Hs, particularly V_{H81X}. Inability of the dominant V_{H81X} to promote B cell development likely leads to developmental defects associated with IGCR1 mutations. Yet, the enigmatic V_{H81X} is strongly conserved across mouse strains[39] and, correspondingly, has been suggested to play important roles in early antibody repertoires[40]. Now, we find that IGCR1 plays a key role in regulating V_{H81X} rearrangement. IGCR1 also is required to allow feedback regulation of proximal V_H to DJ_H rearrangements, implicating IGCR1 as a critical element for the allelic exclusion of V_{H81X} and other very proximal V_Hs. Our findings indicate IGCR1 allows feedback by suppressing early, unordered proximal V_H rearrangement, providing the first evidence in support of long-standing hypothesis that ordered V_H to DJ_H joining provides a means of mediating allelic exclusion[5,6]. However, we found no evidence for loss of feedback regulation of distal V_Hs, in accord with the proposal that locus contraction mediates their allelic exclusion[24].

Our findings show that IGCR1-mediated promotion of the utilization of V_Hs up to several Mb distant does not involve alterations in distal V_H transcription. In pro-B cells, *IgH* contraction promotes distal V_H usage[23,24]. In the absence of certain transcription (*e.g.* Pax-5 or YY1) or chromatin modifying (*e.g.* Ezh2) factors, distal V_H transcription is unimpaired but *IgH* contraction does not occur, diminishing distal V_H rearrangement. In such factor-deficient pro-B cells, transcription and rearrangement of proximal V_Hs does not increase[8,41-43], in marked contrast to the dramatic increases in IGCR1$^{-/-}$ pro-B cells. This phenotypic difference is consistent with IGCR1 normalizing V_H repertoires via mechanisms other than *IgH* contraction. We suggest that IGCR1 promotes distal V_H usage indirectly by preventing premature proximal V_H rearrangement via insulating functions prior to contraction, thereby, preserving DJ_H substrates for distal V_H rearrangement. The location of CBEs throughout the V_H portion of *IgH* led to the notion that recruitment of V_Hs into DJ_H recombination centers[44] subsequent to contraction is promoted via interaction of V_H and IGCR1 CBEs[45]. Due to the dominance of proximal V_H rearrangements on IGCR1-mutant alleles, assays for such putative IGCR1 functions require additional model systems.

IGCR1 CBEs suppress inappropriate transcription and rearrangement of proximal V_Hs 100kb or more upstream. These suppressive functions are consistent with enhancer insulating functions of CBEs *in vitro*[29,35], which may relate to loop formation[35]. We propose that IGCR1 CBEs mediate loops with downstream 3′*IgH*CBEs that segregate the D/J_H and V_H portions of *IgH* into separate regulatory domains during the D to J_H rearrangement stage of B cell development, blocking activity of iEμ or other elements beyond IGCR1[17,19] (Supp. Fig. 14a). Thus, inactivation of the IGCR1 CBEs allows transcriptional enhancing activity to extend to the proximal V_Hs promoting their premature rearrangement (Supp. Fig. 14b). Notably, such activity does not appear to extend beyond the most proximal V_Hs, which may result from formation of new CBE-mediated loops to upstream V_H CBEs in the absence of IGCR1. In DJ_H-containing pro-B cells, IGCR1-

insulating functions that prevent V_H to D_H rearrangements must be neutralized to allow V_H to DJ_H joining (Supp. Fig. 14c). As CTCF binding to *IgH*CBEs does not vary with B cell stage[32,33], other factors must modulate activity of bound CTCF within IGCR1 to allow for *IgH*-specific functions. Such factors might include CTCF modifications, interacting proteins such as cohesin[32,33], or CBE sequence context[35] and orientation[46,47]. In addition, other putative binding elements within IGCR1 may recruit proteins, such as YY1, that have been implicated in modulating CTCF function[35].

Methods Summary

Mice

The targeting strategy and analysis of IGCR1-deleted and CBE-mutated ES cells is diagrammed in Supp. Figs. 2a and 3a (see Full Methods for details). The Institutional Animal Care and Use Committee of Children's Hospital (Boston, MA) approved all animal work.

V(D)J rearrangement assays

PCR assays for D to J_H or V_H to DJ_H rearrangements were performed as described[30] (see Supp. Table 1 for primers). Generation of B cell hybridomas and VDJ recombination analyses was performed as described[48].

RT-PCR and Northern blot

RT-PCR and Northern blotting assays for germline transcripts of *IgH* gene segments were performed as described[30] (primers for RT-PCR and Northern blot probes are in Supp. Table 1).

Chromosomal conformation capture assay (3C)

3C assays were performed as described[49].

ChIP-seq/ChIP-qPCR assays

Assays were done as described[32].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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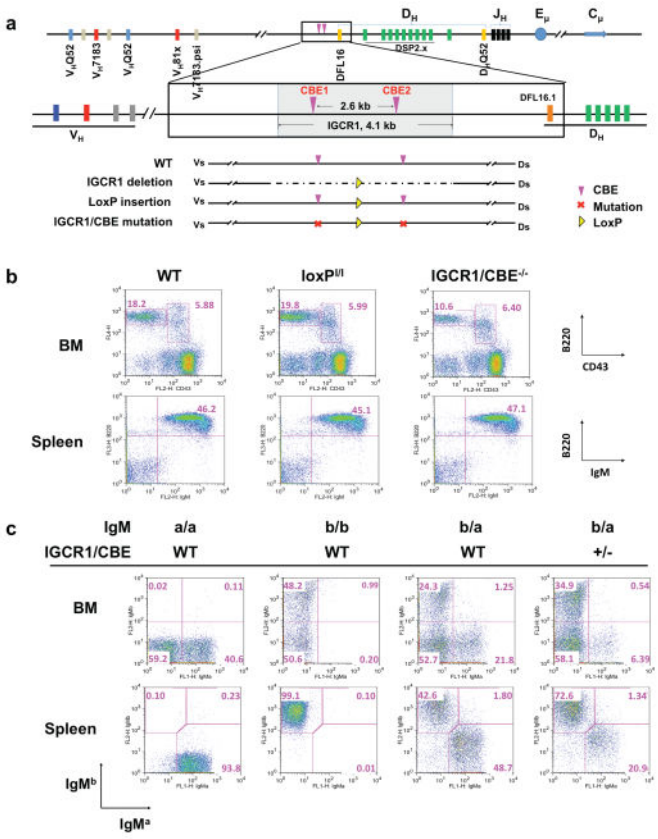

Figure 1. Mutation of IGCR1 CBEs impairs B cell development

(a) Murine 129SV *IgH* locus (accession number: AJ851868) schematic showing 4.1 kb IGCR1 region in WT compared to IGCR1 deleted, loxP inserted, or CBE mutated configuration. **(b)** Flow cytometry analysis of IgM^{-} bone marrow (BM) and IgM^{+} splenic B cell populations in WT, loxP$^{I/I}$, and IGCR1/CBE$^{-/-}$ mice. In BM the B220^{int}CD43^{+} pro-B and B220^{+}CD43^{-} pre-B cell populations are indicated. **(c)** Expression of IgM^{a} and IgM^{b} allotypic markers in BM and spleen from WT IgM^{a}/IgM^{a} (pure 129SV), WT IgM^{b}/IgM^{b} (pure C57BL/6), WT F1 (IgM^{a}/IgM^{b}), and heterozygous mutant IGCR1/CBE^{-} IgM^{a}/ WT IgM^{b} mice.

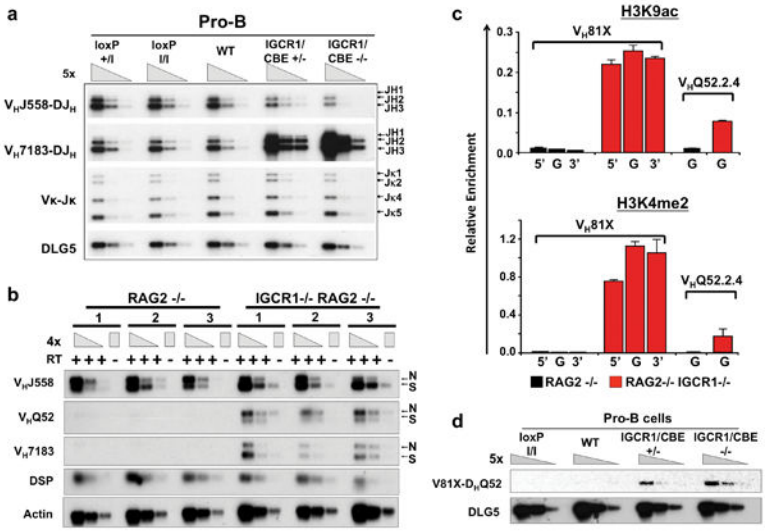

Figure 2. IGCR1 mutations alter V_H usage, germline transcription and rearrangement order

(a) PCR analyses of indicated V_H family rearrangements in pro-B cells from indicated mice compared to a *DLG5* loading control. Results are typical of four experiments. Bands corresponding to rearrangements to various J_Hs are indicated on right. **(b**) RT-PCR analysis of indicated germline V_H transcripts in three independent WT and IGCR1$^{-/-}$ A-MuLV virus transformed RAG2$^{-/-}$ pro-B cell lines. N=nonspliced sense/antisense and S=spliced sense. **(c)** ChIP-qPCR analyses of H3K4me2 and H3K9ac histone modifications at indicated V_Hs in 129SV Rag2$^{-/-}$ (black) and Rag2$^{-/-}$ IGCR1$^{-/-}$ (red) A-MuLV-transformed pro-B lines. The 5′ region (5′), body (G) and 3′ region (3′) of V_{H81X} and body (G) of $V_{HQ52.2.4}$ were analyzed. Average values and standard deviations of three experiments with one line shown are representative of results from both. **(d)** Semi-quantitative PCR analyses of direct V_H-D rearrangements in sorted pro-B cells from indicated mice. The PCR assays utilized for panels a, b, and d are diagrammed in Supp. Figs. 6a, 7a, and 8a.

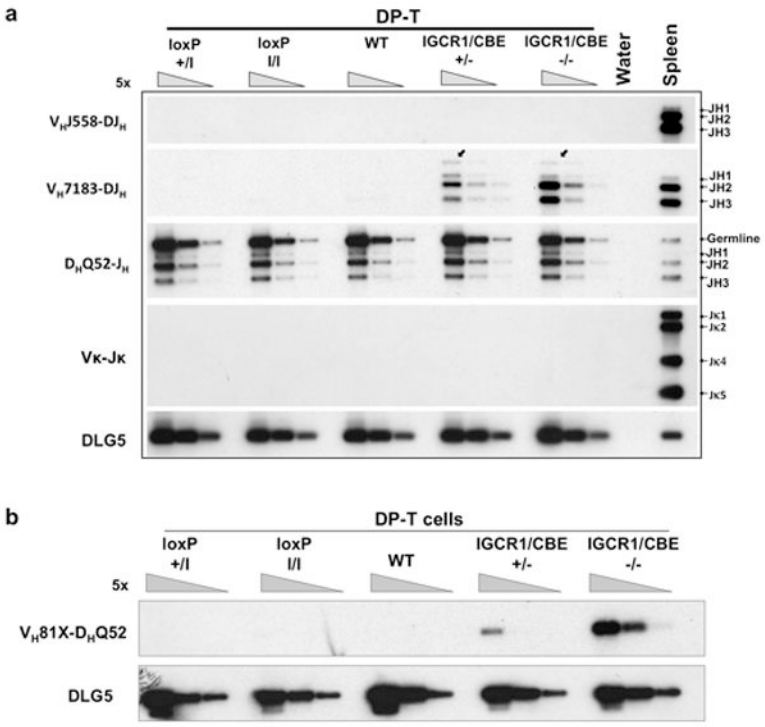

Figure 3. IGCR1/CBE Mutations lead to V_HDJ_H and V_HD rearrangements in thymocytes

(a) PCR analyses of V_H family rearrangements in sorted DP thymocytes and total splenic B cells from indicated mice with *DLG5* as a loading control. Bands corresponding to rearrangements to various J_Hs are indicated on right. *Igκ* rearrangement (VκJκ) served as a control for B cell contamination. **(b)** Semi-quantitative PCR analyses of direct V_H-D rearrangements in sorted DP-T cells from indicated mice. Assays are diagrammed in Supp. Figs. 6a and 8a.

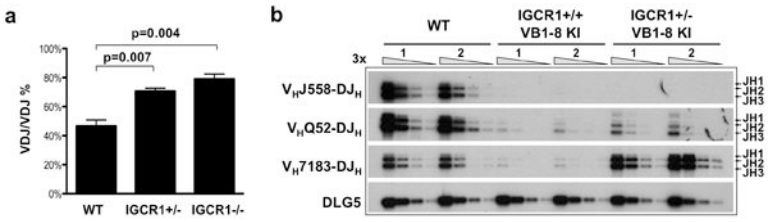

Figure 4. IGCR1 is required to allow feedback regulation of proximal V_H to DJ_H recombination

(a) Mean percentage of splenic B cells with V_HDJ_H rearrangements on both *IgH* alleles as determined by analyses of hybridomas from three independent sets of WT, IGCR1$^{+/-}$, and IGCR1$^{-/-}$ mice (Supp. Table 5). Error bars represent standard deviation. *p*-values were calculated by student t-test. **(b)** *IgH* V_HDJ_H rearrangements in splenic B cells from two independent WT and VB1-8 knock-in mice carrying either a WT (IGCR1$^{+/+}$ VB1-8 KI) or an IGCR1 deleted (IGCR1$^{+/-}$ VB1-8 KI) second allele. Bands corresponding to rearrangements to various J_Hs are indicated on right. *DLG5* is the loading control.

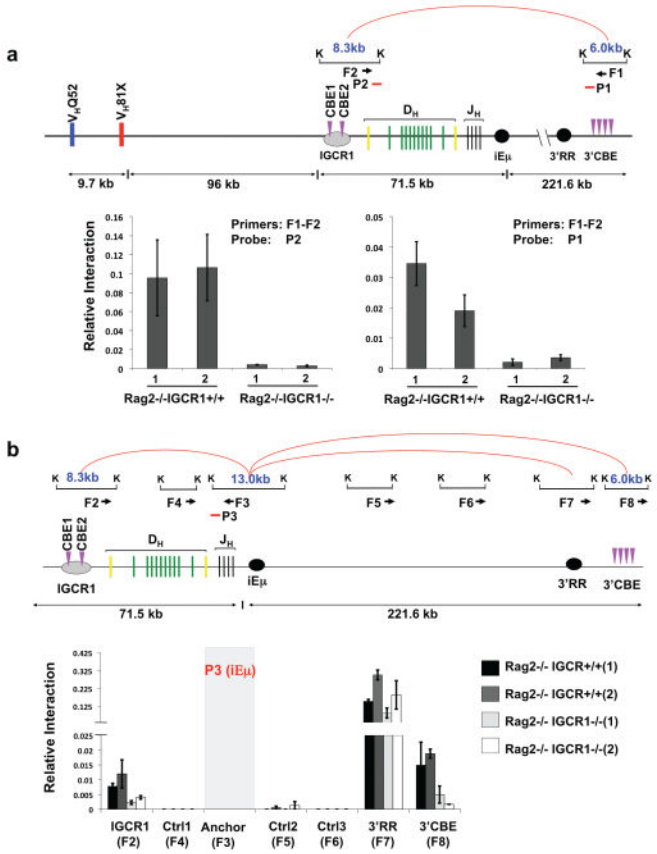

Figure 5. IGCR1 mediates long distance *IgH* chromosomal loops

(a) Schematic of chromosome interactions between IGCR1-containing and 3′*IgHC*BE-containing KpnI restriction fragments in 3C assays. Interactions between IGCR1 and 3′*IgH*CBE locales in 129SV RAG2$^{-/-}$ and RAG2$^{-/-}$IGCR1$^{-/-}$ A-MuLV transformed pro-B cells were quantified by real time PCR (Taqman) using probe (P2) (left) and a probe (P1) (right). **(b)** Schematic of chromosome interactions between iEμ-containing KpnI restriction fragment and indicated KpnI restriction fragments in other *IgH* locales. Interactions between iEμ and IGCR1, iEμ and 3′*IgH*RR locales, iEμ and 3′*IgH*CBE locales in RAG2$^{-/-}$ and RAG2$^{-/-}$IGCR1$^{-/-}$ A-MuLV transformed pro-B cells were quantified by real time PCR using a probe (P3) from the iEμ locale. F1-F8 indicate primers used for PCR. K indicates *Kpn*I sites. Red arcs indicate interactions detected in RAG2$^{-/-}$ cells. The average association frequency of three independent 3C experiments with 2 independent A-MuLV transformed lines from each genotype is shown with standard deviation indicated.