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PAX5/BSAP Transactivates RAG-mediated Immunoglobulin Gene Recombination

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Abstract

Generation of diversified immunoglobulin (Ig) and T cell receptor (TCR) repertoires through V(D)J recombination is the foundation of adaptive immunity. For immunoglobulin heavy chain (IgH) gene, the step of V_H to DJ_H rearrangement occurs exclusively in B lineage cells. Accumulating studies indicate that Pax5 (BSAP), a B lineage specific transcription factor, plays an important role in the regulation of B lineage specific V_H to DJ_H recombination. In *Pax5*$^{-/-}$ mice, the recombination of upstream V_H558 genes is severely impeded. Conversely, ectopic expression of Pax5 transgenes in early thymocytes initiates V_H7183 to DJ_H recombination. Pax5 is essential for IgH locus contraction and histone hypo-methylation across the IgH locus prior to V(D)J recombination. We found that Pax5 associates with the V_H gene coding regions and interacts with the RAG1 and RAG2 protein complexes to enhance RAG-mediated V_H to DJ_H recombination. Based on our results, we proposed a novel experimental model that Pax5 directly transactivates RAG-mediated V_H to DJ_H recombination.

Keywords: B cell, Pax5/BSAP, immunoglobulin gene, RAG, V(D)J recombination.

1. Generation of a diversified immunoglobulin gene repertoire through RAG-mediated V(D)J recombination

The function of the adaptive immune system is dependent on the tremendously diversified repertoires of immunoglobulin (Ig) and T cell antigen receptor (TCR). The coding exons of the variable domains of Ig and TCR genes are generated through somatic rearrangement of previously separated variable (V), diversity (D), and joining (J) gene segments in developing B and T lineage cells, respectively [1-6]. The recombination process is catalyzed by a pair of recombination activating gene products, RAG1 and RAG2 [7-9]. Both proteins are required for rearrangement of Ig and TCR genes, and disruption of either gene in mice results in a complete block of B and T cell generation [10,11]. For Ig and TCR genes, specific recombination is directed by the recombination signal sequence (RSS) flanking each V, D, or J gene segment [12]. The RSS is composed of two highly conserved motifs, a heptamer (5'-CACAGTG-3') and a nonamer (5'-ACAAAAACC-3'), separated by a non-conserved spacer with either 12-bp or 23-bp in length [12]. The heptamer and nonamer are important for RAG complexes binding and cleavage of the RSS [13,14], while the length of the spacer region specifies the recombination of different gene fragments [12]. Known as the 12/23 rule, recombination preferentially occurs between a pair of 12-bp and 23-bp RSS tagged gene segments [12].

2. V(D)J recombination is tightly regulated at multiple steps

To ensure the successful assembly of Ig or TCR genes and mean time, to prevent unwanted cleavage of genomic DNA, V(D)J recombination is tightly controlled at different levels. First, recombinations of Ig and TCR genes are restricted in early developing B and T lineage cells, respectively [5,6]. This developmental stage specific regulation is mainly achieved through regulation of the *RAG1* and *RAG2* gene expression and the differential accessibilities to the rearranging gene loci. *RAG1* and *RAG2* gene expression is initiated in the common lymphoid progenitor cells and continued in early B and T lineage cells [15]. In B lineage cells, RAG proteins are expressed in pro B cell stage to catalyze IgH gene D_H to J_H recombination and then V_H to DJ_H recombination. After generation of functional μ heavy chains to form the pre-B cell receptors (pre-BCR), *RAG1* and *RAG2* gene expression will be temporarily down modulated in large pre B cells [16,17], to prevent additional DNA cleavage during cell proliferation. After expansion of the pre B cells, *RAG* genes are reexpressed in the small pre B cells to catalyze immunoglobulin light chain (IgL) gene recombination [16,17]. Functionally rearranged IgL genes will produce conventional light chains to form the B cell receptors (BCR). *RAG* gene expression can be reinduced in immature B cells to edit IgH and IgL genes encoding self reactive BCRs [18,19] and then, will be shut down in mature B cells.

To prevent unwanted DNA damage during cell proliferation, the V(D)J recombinase activity is strictly confined at the G1 phase of cell cycle through regulation of the RAG2 protein level [20-22]. At the G2/M phases, cell cycle-dependent phosphorylation at Thr490 of RAG2 targets RAG2 to the proteosome-dependent degradation process [20-22]. Overexpression of $p27^{kip}$ blocks cell cycle progression and results in accumulation of RAG2 protein. Mutation of the Thr490 to Ala on RAG2 disrupts its cell cycle dependent degradation and leads to increased aberrant signal joint formation in transgenic mice [23,24].

3. The locus accessibility theory for regulation of V(D)J recombination

To ensure the expression of correct antigen receptors on appropriate cells, V(D)J recombination is tightly controlled in a lineage specific manner [2,5,6]. In developing B and T lymphocytes, there are total seven antigen receptor gene loci, including IgH, Igκ, Igλ, TCRα, TCRβ, TCRδ, and TCRγ, that are subjected to RAG-mediated recombination [2,5,6]. Ig genes are only rearranged in B lineage progenitor cells to generate Ig (BCR); while TCR genes are only assembled in early T lineage cells to produce TCR [2,5,6]. Such lineage specific regulation of V(D)J recombination has been recognized from the very beginning, however, the underlying molecular mechanisms are still unclear.

The current understanding of the developmental stage- and lineage- specific regulation of V(D)J recombination is based on the accessibility theory [25-27], which was originally proposed to explain the observation that Ig gene germline transcription precedes V(D)J recombination in progenitor B cells [25,28]. Germline transcript was thus considered as the first indicator for the accessibilities to Ig or TCR loci. The accessibilities to the Igκ or TCRα loci were directly examined in the *in vitro* cleavage assays using purified RAG proteins and intact nuclei substrates prepared from B cells, T cells, or fibroblasts [29]. RAG-mediated cleavage at the J_H RSS only occurs in B lineage nuclei, but not in T lineage and fibroblast nuclei. Conversely, RAG-mediated cleavage at the TCRδ RSS only occurs in T lineage nuclei, but not in other lineage nuclei [29]. These results provided the first evidence that the accessibilities to IgH and TCRδ loci are differentially regulated in B and T lineage cells.

In mammalian cells, DNA is packed into chromatin structure, which prevents most cellular reactions using DNA as templates or substrates, including transcription, V(D)J recombination, and DNA repair [30]. Modification of chromatin structure thus becomes an important mechanism to regulate these reactions. It has long been recognized that the core histone tails can be reversibly acetylated by histone acetyltransferases, such as CBP, p300, PCAF, and GCN5 at multiple lysine residues [31-34]. The chromatin structure can also be remodeled by chromatin remodeling complexes, such as the SWI/SNF complexes [35,36]. The mammalian SWI/SNF complex is a giant 2 mega Dalton complex that contains either the Brg1 or Brm ATPase, with a variable composition of subunits, known as the Brg1-associated factors (BAFs) [35,36]. It has been well documented that histone acetylation and chromatin remodeling are important activators in transcriptional regulation.

The requirement for histone acetylation and chromatin remodeling by SWI/SNF complex during RAG-mediated V(D)J recombination has also been well recognized. In B and T lineage cells, the histone acetylation patterns within the IgH and TCRα loci correlate with their recombination status, respectively [37,38], and thus serve as indicators for locus accessibility. In the *in vitro* cleavage assays, naked DNA substrates with either 12-bp or 23-bp RSS can be efficiently cleaved by purified RAG1/RAG2 proteins regardless of their Ig or TCR origins [39,40]. However, when the same RSS substrates were reconstituted into nucleosomal structures with purified core histone components, RAG-mediated cleavage was completely blocked [41-43]. Artificial modification of the histone tails by partial trypsin digestion, hyperacetylation, and supplementation with the chromatin remodeling SWI/SNF complexes enhance RAG-mediated cleavage of the chromatinized RSS templates [41,43,44]. The ATPase component of the SWI/SNF chromatin remodeling complex, Brg1, has been found to associate with the antigen receptor gene loci poised for V(D)J recombination, implying that SWI/SNF is involved in remodeling the chromatin structure for V(D)J recombination *in vivo* [45]. Clearly, histone acetyltransferase and chromatin remodeling complexes are important regulators in RAG-mediated recombination. However, how these generalized chromatin modification factors are specifically recruited to different rearranging genes is not clear.

4. Regulation of Ig gene recombination

Ig gene rearrangement occurs in a step-wised fashion during early stages of B lymphopoiesis. Normally, D_H to J_H rearrangement occurs first on one of the two IgH alleles, followed by V_H to DJ_H recombination and then light chain gene rearrangements [46-48]. D_H to J_H rearrangement marks the onset of B lineage differentiation, but this step of recombination is not B lineage specific. D_H-J_H joints can be detected in thymocytes [49]. The step of V_H to DJ_H rearrangement is B lineage specific. Functionally rearranged IgH genes will produce μ heavy chains (μHC) to form the pre B cell receptors (pre BCR) that define the pre B cell phenotype. Understanding how the B lineage specific V_H to DJ_H recombination is regulated remains an important topic in immunology.

B lineage specific transcription of the IgH gene is controlled by three regulatory elements, the V_H gene promoter, the intronic μ enhancer (Eμ), and the 3' IgH enhancer [50]. A combination of the V_H promoter and the Eμ enhancer can successfully direct B lineage specific expression of foreign transgenes. The important role of the Eμ enhancer in the regulation of B lineage specific V_H to DJ_H recombination has been studied extensively in different experimental systems [51-53]. First, deletion of the Eμ enhancer on one of the IgH alleles significantly affects the V_H to DJ_H recombination, but with only mild effect on D_H to J_H recombination [51,52]. Detailed mapping of the Eμ enhancer showed that the core region without the flanking matrix attachment regions is sufficient to promote normal V(D)J recombination [53]. Second, when placed downstream of a mini TCR locus, the Eμ enhancer can direct specific recombination of this artificial construct in B lineage cells in a transgenic mouse [54]. The function of the V_H gene promoter in the regulation of V_H to DJ_H recombination has not been fully studied. Early reports using a chicken Igλ mini locus showed that the promoter was important for the efficient recombination of that construct [55]. Recent studies using the mini TCRα locus showed that the important function of the TCRα promoter is to enhance the accessibility to the recombination constructs rather than to initiate the transcription of the Vα gene, as the promoter can be placed in either orientations relative to the Vα gene [54].

E2A, EBF, and Pax5 are important transcriptional regulators for early B lineage cell development and also play important roles in regulation of Ig gene recombination [56,57]. E2A was originally identified as an Ig enhancer binding factor [58]. The *E2A* gene encodes two proteins, E12 and E47, through differential RNA splicing. E12 and E47 are involved in the regulation of many B lineage specific genes expression, including *RAG1*, *RAG2*, *λ5*, *VpreB*, *Pax5*, and *EBF* [59,60]. In mice deficient of E2A activity either by targeted disruption of the E2A gene or by forced expression of the E2A inhibitor Id1 protein, B lineage cell development was completely blocked before the stage of D_H to J_H recombination [61-63]. EBF is a transcriptional regulator that is expressed in various tissues, including olfactory neurons, adipocytes, and B lineage cells [64]. EBF regulates the expression of many B lineage specific genes, including *mb-1*, *B29*, *λ5*, *VpreB*, *RAG1*, and *Pax5* [57]. In EBF deficient mice, B cell development is arrested at the pro B cell stage before D_H to J_H recombination [65]. These results indicated that E2A and EBF are dispensable for the initiation of IgH recombination. The activation function of E2A and EBF in IgH recombination is likely through regulating the locus accessibility. Indeed, previous studies have shown that forced expression of E47 activates IgH germline transcription and TdT gene expression in fibroblasts [66] and forced expression of E2A in a pre T cell line initiates IgH D to J_H rearrangement [67]. Recent studies have shown that forced expression of *E2A* or *EBF* together with the *RAG1* and *RAG2* genes in human embryonic kidney BOSC cells initiates endogenous IgH D_H to J_H, Vκ to Jκ, and TCRγ Vγ to Jγ gene rearrangements [68]. These results further confirmed the important function of E2A and EBF in V(D)J recombination. The failures to complete V_H to D_HJ_H recombination and to maintain the lineage specific pattern of rearrangement suggest that additional factors are required for the precise regulation of V(D)J recombination.

5. Pax5 (BSAP) is a B lineage specific transcription factor

Pax5 is an important regulator for B lineage cell development and function [56,57]. Pax5 belongs to the PAX family of transcriptional regulators, which contain the conserved paired DNA binding domain [69]. Pax5 also has a partial homeodomain and transcriptional activation and inhibitory domains [70-72]. Pax5 is mainly expressed in B lineage cells and in developing midbrain [69,73]. In B lineage cells, Pax5 expression is initiated in the common lymphoid progenitor cells and extinguished in terminally differentiated plasma cells [69]. In *Pax5*$^{-/-}$ mice, B lineage cell development is blocked at pro B cell stage [74,75]. Pax5 controls the transcription of many B lineage specific genes, including *Cd19*, *Blk, Mb-1, and Blnk,* through binding to their promoters [76]. A detailed comparison of the gene expression profiles between the wild type and the *Pax5*$^{-/-}$ pro B cells confirmed many previously identified Pax5 target genes and also revealed several new ones [76]. Forced expression of IgH and Igκ transgenes fail to advance *Pax5*$^{-/-}$ pro B cell development (69). *Blnk* is another Pax5 target gene, which encodes an important adaptor protein to transduce signals from the pre-BCR and BCR. Loss of Pax5 results in no Blnk expression and defective pre-BCR signaling (70). Reconstitution of Blnk expression in *Pax5*$^{-/-}$ B cells restored the pre-BCR-mediated signaling, but was not enough to rescue B cell development [77]. These results revealed the sequential requirement for Pax5 and its target genes at different stages of B cell development. However, these results can not fully explain the severe blockade of B cell development in *Pax5*$^{-/-}$ mice.

Pax5 also acts as a restriction factor to specify the B lineage cell differentiation pathway through suppression the expressions of lineage or differentiation stage non-appropriate genes [78,79]. Without such restrictions, *Pax5*$^{-/-}$ pro B cells can differentiate into myeloid, T, and dendritic cells [78,80]; and loss of Pax5 in mature B cells also promotes plasma cell differentiation [79,81].

To exert its multiple biological functions, Pax5 interacts with a diverse array of cellular proteins.

Pax5 interacts with Ets-1 through its N-terminal region [82,83]. Through this interaction, Ets-1 recruits Pax5 to the *Mb-1* gene promoter region, where they bind to a complex Ets-1/Pax5 binding site to enhance transcription [84]. A recent study showed that Pax5 interacts with the Ada2 adaptor protein, GCN5 histone acetyltransferase, and the SWI/SNF chromatin remodelling complex [85]. Through this functional interaction, co-expression of Ada2 and GCN5 dramatically enhances Pax5-mediated transcriptional activation function.

6. Pax5 is required for efficient IgH V_H to DJ_H recombination

An important function of Pax5 in the regulation of IgH V_H to DJ_H recombination was first indicated by the defective V_H to DJ_H recombination in *Pax5*$^{-/-}$ pro B cells with normal D_H to J_H recombination [75]. Detailed analyses of the recombination status of different V_H genes in *Pax5*$^{-/-}$ pro B cells revealed that the recombinations of the D_H distal V_HJ558 genes are more severely compromised than that of the D_H proximal V_H7183 genes [86]. Surprisingly, the germline transcriptions of V_HJ558 and V_H7183 genes and the global histone acetylation pattern across the IgH locus, two of the general indicators of locus accessibility, are not affected in *Pax5*$^{-/-}$ pro B cells [86]. However, without Pax5, the accessible V_H genes can not be efficiently rearranged. When the rearranged V_H7183 genes in the *Pax5*$^{-/-}$ pro B cells were sequenced, it becomes clear that even within the V_H7183 family, only a few V_H genes (including the V_H 81X) that are close to the D_H locus can be rearranged at normal frequency [87]. The recombinations of upstream V_H7183 genes are also defective in *Pax5*$^{-/-}$ pro B cells [87]. These results suggested that Pax5 is required for the recombination of most V_H genes.

Conversely, forced expression of Pax5 in early T lineage cells induced ectopic V_H to DJ_H recombination [88,89]. Interestingly, in these experiments, Pax5 only induces the rearrangement of some D_H proximal V_H7183 genes but not the upstream V_HJ558 genes [88,89]. These results confirmed the important function of Pax5 in the regulation of the V_H to DJ_H recombination and also suggested that additional regulators are required to fully activate B linage specific IgH gene recombination.

Recent studies using 3-dimensional FISH techniques showed that the IgH locus is contracted in normal pro B cells prior to recombination. However, such contraction was not seen in *Pax5*$^{-/-}$ pro B cells [88]. Pax5 might facilitate the recombination of distal V_H genes by contraction the IgH locus to bring upstream V_H genes close to the DJ_H region [87,88]. The IgH locus contraction occurs at an earlier step before D_H to J_H recombination, because contraction of the IgH locus can be seen in *RAG*$^{-/-}$ pro B cells and defective IgH locus contraction has also been observed in *E2A*$^{-/-}$ pro B cells [88,90].

7. Pax5 acts as a transactivator for RAG-mediated V_H to DJ_H recombination

Our recent studies revealed a novel function of Pax5 as a direct activator for RAG-mediated V_H to DJ_H recombination. As a B lineage specific transcription factor, Pax5 exerts most of its biological functions through binding to the cognate DNA binding sites within the promoters or regulatory regions of its target genes [56]. To explore the potential mechanism for how Pax5 regulates V_H to DJ_H recombination, we searched for potential Pax5 binding sites within the V_H gene promoters, V_H gene coding regions, and 3' flanking regions of human and mouse V_H genes using computer based transcription factor binding site search programs (TESS). Interestingly, our initial sequence analysis identified clustered Pax5 binding sites within the V_H gene coding regions of most human and mouse V_H genes, but not in the V_H gene promoter or the 3' flanking regions [91]. The Pax5 binding capacities of these V_H gene derived Pax5 binding sites were first confirmed by electrophoresis mobility shift assays (EMSA). Pax5 protein in the crude nuclear extracts prepared from B lineage cells, purified his-tagged Pax5 proteins, and purified PRD peptides containing the Pax5 DNA binding domain all bind to individual Pax5 binding sites derived from representative V_H genes of human and mouse origins [91]. Although the individual Pax5 binding sites from V_H genes display a lower Pax5 binding affinity comparing to the Pax5 binding site from the CD19 gene promoter region, the full length V_H gene coding regions have a comparable Pax5 binding affinity to that of the CD19 promoter region containing two high affinity Pax5 binding sites [91].

To determine if Pax5 associates with the V_H gene coding regions in developing B lineage cells, we performed chromatin immunoprecipitation (ChIP) assays. Human EU12 cells express endogenous *Pax5*, *RAG1*, *RAG2*, and *CD19* genes with ongoing V(D)J recombination and were used in our ChIP assays. Enrichment of the V_{H1} and V_{H3} coding regions was found in anti-Pax5 antibody precipitated chromatin samples in real time PCR based analyses [91]. These results confirmed that Pax5 associates with the V_H gene coding regions in the EU12 cells. Using pro B cells from the μMT mice, enrichment of the V1, V11, V_HJ558, and V_H7183 genes was also found in anti-Pax5 antibody precipitated chromatin samples [91], indicating that Pax5 associates with the V_H coding regions in mouse early B lineage cells.

The potential function of Pax5 in regulating RAG-mediated recombination was first suggested by the co-immunoprecipitation studies. Using anti-RAG1 or anti-RAG2 antibodies, we can immunoprecipitate Pax5 from nuclear extracts prepared from human EU12 cells, which contains endogenous Pax5,

RAG1, and RAG2 proteins [91]. Conversely, using anti-Pax5 antibodies, we can immunoprecipitate RAG1 and RAG2 proteins from the same nuclear extracts [91]. The interaction between Pax5 and the RAG1 and RAG2 protein complex was further confirmed by GST-fusion protein pull-down assays. Using co-purified GST-RAG1 and GST-RAG2 core proteins, we can efficiently pull down [^{35}S] Met labelled full length Pax5 proteins generated from the *in vitro* transcription and translation coupled system (TNT system, Promega) [91]. Individually purified GST-RAG1 or GST-RAG2 protein only interacts weakly with Pax5. Analyses using different Pax5 truncation constructs further showed that the N-terminal of Pax5 is required for interaction with the RAG1 and RAG2 complexes [91]. The interaction between Pax5 and RAG1, RAG2 has been further confirmed in mammalian one-hybridization assays [91].

To directly test the potential function of Pax5 in RAG-mediated recombination, we performed *in vitro* cleavage assays using the full length V_{H1-8} gene coding region as substrates. Co-purified RAG1 and RAG2 core proteins cleave the V_{H1-8} gene substrates and generate coding and signal end products. The addition of purified Pax5 protein into the *in vitro* cleavage reaction enhances RAG-mediated cleavage at the 23-bp RSS [91].

The activation function of Pax5 in RAG-mediated V_H to DJ_H recombination was further tested in fibroblast based recombination assays using the pJH289-V_{H1-8} construct containing the V_{H1-8} gene coding region with three identified Pax5 binding sites. Overexpression of Pax5 enhances RAG-mediated recombination of the pJH289-V_{H1-8} substrates as determined by PCR amplification of either the coding joints or the signal joints [91]. The Pax5-mediated enhancement of V_H to DJ_H recombination depends on the integrity of the full length Pax5 protein. Deletion of either the N-terminal region or the C-terminal region of Pax5 affects Pax5-mediated activation function [91]. Importantly, the Pax5-mediated activation of V_H to DJ_H recombination is dependent on the Pax5 binding sites within the V_H gene coding region. Mutation of the three Pax5 binding sites within the V_{H1-8} gene almost completely abolished Pax5-mediated activation of recombination in either NIH3T3 fibroblasts or EU12 cells [91].

These results led us to propose the current experimental model that Pax5 acts as a transactivator for the B lineage specific IgH V_H to DJ_H recombination (Fig. 1). This novel action of Pax5 parallels closely with its function in transcriptional regulation, in which Pax5 binds to the promoter regions of its target genes and recruits the basal transcriptional machinery and additional co-activators, such as the histone acetyltransferase p300 and the chromatin remodelling SWI/SNF complexes. We speculate that Pax5 binding to the V_H gene coding regions could also recruit additional co-activators, such as p300 and the SWI/SNF complexes, to facilitate individual V_H gene recombination. This mode of action also predicts that the Pax5 binding capacity to an individual V_H gene could directly determine its recombination efficiency and thus influence its usage in the primary IgH repertoire. On the other hand, negative regulators for Pax5 could indirectly regulate V_H to DJ_H recombination and thus provide additional levels of regulation for IgH recombination.

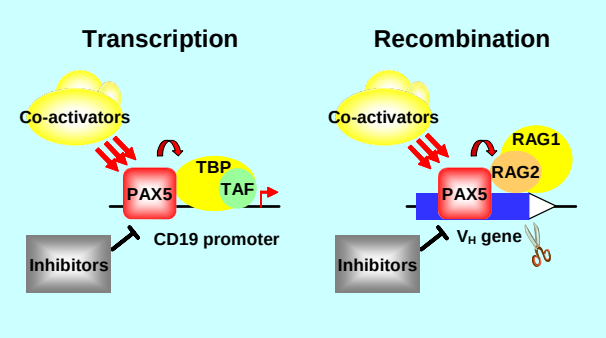

Figure 1. Diagram shows the transactivation functions of Pax5 in transcription and recombination with hypothetic co-activators and inhibitors.

8. Discussion

Collectively, the essential function of Pax5 in regulation of B lineage specific V_H to DJ_H recombination has been well recognized. Currently, two potential mechanisms have been proposed to explain the regulation function of Pax5 in RAG-mediated V_H to DJ_H recombination. Based on the 3-D FISH results, it has been proposed that Pax5 activates IgH gene recombination through contracting the IgH locus. This model provides a suitable explanation for the defective recombination of distal V_HJ558 genes in *Pax5*$^{-/-}$ pro B cells, in that locus contraction could bring the upstream V_HJ558 genes closer to the DJ_H regions and facilitate their recombinations. However, this model can not explain the defective recombination of the upstream V_H7183 genes in *Pax5*$^{-/-}$ pro B cells and the induction of D_H proximal V_H7183 gene recombination in early T lineage cells expressing *Pax5* transgenes.

We proposed the second model that Pax5 acts as a direct transactivator for RAG-mediated V_H to DJ_H recombination. Pax5 binds to the coding regions of individual V_H genes and recruits the RAG complexes to enhance recombination, which parallels closely to the activation function of Pax5 in transcriptional regulation. The activation function of Pax5 is required for almost all the V_H genes, because most of them have potential Pax5 binding sites. For a few D_H proximal V_H genes, their recombinations are likely independent of Pax5 in B lineage cells, presumably due to their close proximity to the DJ_H region and the Eμ enhancer. However, forced expression of Pax5 induces their

recombinations in T lineage cells, suggesting that Pax5 also has activation function for these genes.

Thus, despite the different working models, it is reasonable to conclude that the B lineage specific IgH V_H to DJ_H recombination is controlled by the B lineage specific transcription factor, Pax5 (BSAP). These findings will have significant implications for our future studies of the lineage specific regulation of RAG-mediated recombination of other antigen receptor gene loci in developing B and T lineage cells.

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