Yin Yang 1 is a critical regulator of B-cell development

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B lymphocyte development proceeds through the progenitor (pro-B), the precursor (pre-B), immature, and mature B-cell stages (Hardy 1989). Each stage is defined by the recombination status and expression pattern of the immunoglobulin heavy- and light-chain (IgH and IgL) genes and cell surface markers (Hardy et al. 2000; Hardy and Hayakawa 2001; Cancro 2004; Jung and Alt 2004). Ig genes (also known as B-cell receptor or antibody genes) are assembled by nonhomologous rearrangement from Variable [V], Diversity [D] (H only), and Joining [J] gene segments, mediated by the recombination-activating gene (RAG) recombinase (Jung and Alt 2004). Rearrangement of Ig loci is an ordered process (Alt et al. 1984; Chowdhury and Sen 2004). In pro-B cells, DH to JH rearrangement occurs before VH to DJH recombination. Upon the generation of a productively rearranged VHJH fragment and the expression of a μ chain, pro-B cells proceed to the pre-B-cell stage of development. The expressed μ chain, in combination with the surrogate light chain, λ5 and VpreB, and the Ig-α/β heterodimers, forms the pre-B-cell receptor (pre-BCR), which signals pre-B-cell expansion and subsequent light-chain gene rearrangement (Fleming and Paige 2001). Upon completion of light-chain rearrangement, two identical heavy chains and two identical light chains, together with the Ig-α/β heterodimers, form the B-cell receptor and pre-B cells transit to immature B cells, which exit the bone marrow (BM) to become mature peripheral B cells.

The lineage and developmental stage specificity of V(D)/J recombination relies on the local accessibility of the gene loci to the common RAG recombinase, which functions in both B- and T-cell receptor gene rearrangements (Chowdhury and Sen 2004; Jung and Alt 2004). The IgH locus becomes accessible to RAG recombinase in pro-B cells, accompanied by a series of changes including a periphery-to-center nuclear repositioning, locus contraction mediated by DNA looping, germline transcript expression, and covalent modifications of histones at specific sites (Yancopoulos and Alt 1985; Chowdhury and Sen 2001; Kosak et al. 2002; Morshow et al. 2003; Su et al. 2003; Bolland et al. 2004; Fuxa et al. 2004; Johnson et al. 2004; Roldan et al. 2005; Sayegh et al. 2005). The relationship, if any, among the multiple changes occurring at the IgH locus, and their exact roles in VHJH recombination, remain to be determined. Previous studies have identified several cis-acting elements within the IgH locus that are important for VHJH recombination, including the variable gene promoters, the intronic en-
hancer [Eiµ], and the 3’ enhancer [Sleckman et al. 1996]. Studies using knockout mice confirmed an important role of the core intronic Eiµ in mediating V_{H}D_{H}J_{H} recombination [Sakai et al. 1999; Perlot et al. 2005]. However, it is unclear whether the Eiµ enhancer regulates V_{H}D_{H}J_{H} recombination by controlling IgH locus nuclear repositioning, contraction, and/or chromosomal changes. The Eiµ enhancer contains binding sites for multiple transcription factors including YY1 [Ernst and Smale 1995; Sleckman et al. 1996]. The role of these proteins in V_{H}D_{H}J_{H} recombination is largely unknown.

YY1 is a zinc finger protein that functions as a transcriptional activator, repressor, or transcription-initiator element-binding protein, depending on the promoter context [Liu and Shi 2005]. Recently, YY1 has also been shown to regulate p53 stability independent of its transcriptional activity [Gronroos et al. 2004; Sui et al. 2004]. YY1 is evolutionarily conserved from Drosophila to human and has been suggested to function as a Polycomb Group [PcG] protein during development [Brown et al. 1998, 2003; Athkinson et al. 2003; Sirinivas and Athison 2004]. Animal studies indicate a role for YY1 in embryogenesis and in neuronal development [Donohoe et al. 1999; Satijn et al. 2001; Kwon and Chung 2003; Morgan et al. 2004]. In vitro biochemical and cell-based analyses suggest that YY1 may play important roles in a number of biological and pathological processes, including B-cell development and function [Thomas and Seto 1999; Gordon et al. 2003; Patrone et al. 2004; Su et al. 2004; Liu and Shi 2005]. However, the early embryonic lethality of YY1 knockout mice precluded the investigation of YY1 in specific developmental pathways in vivo.

To address the role of YY1 during later stage development, we generated mice carrying conditional yy1 alleles (yy1^{f/f}). To investigate the role of YY1 in B-cell development, we took advantage of the novel mb1-Cre transgenic mouse [Hobin et al. 1999; Affar et al. 2006], which recombinates loxP-flanked sequences in early B-cell progenitors. Phenoypic analyses of the B-cell-specific yy1 null allele (yy1^{f/f}) demonstrated that YY1 plays a critical role in controlling the pro-B-to-pre-B-cell transition. Analysis of recombination events in the IgH locus of YY1-deficient pro-B cells revealed normal D_{H} to J_{H}, but impaired V_{H} to D_{H}J_{H} recombination. A prearranged IgH transgene inserted into the IgH locus partially rescued the pro-B to pre-B block caused by loss of YY1. This indicates that YY1-dependent V_{H} to D_{H}J_{H} recombination is important for pro-B-cell differentiation and that YY1 also plays additional roles in the pro-B-to-pre-B-cell transition. Three-dimensional DNA fluorescence in situ hybridization (3D FISH) showed a significantly increased pro-B population unable to undergo IgH locus contraction upon loss of YY1. Chromatin immunoprecipitation (ChIP) showed YY1 binding to the Eiµ enhancer within the IgH locus. Taken together, our study identifies a novel function for YY1 in early B-cell development by controlling IgH locus contraction and V_{H}D_{H}J_{H} recombination, possibly through direct interaction with the IgH Eiµ enhancer.

## Results

### B-cell-specific deletion of yy1 with the mb1-Cre transgenic mouse

In order to study the role of YY1 in lineage development, we generated a conditional yy1 knockout allele (yy1^{f/f}) by flanking the yy1 promoter region and exon1 with loxP-sites [Fig. 1A; Affar et al. 2006]. The yy1^{f/f} allele expresses normal levels of YY1 protein and Cre recombinase-mediated recombination yields a yy1-null allele (yy1^{f/f}) similar to the constitutive null allele described before [Fig. 1A; Donohoe et al. 1999; Affar et al. 2006; data not shown]. To achieve B-cell-specific ablation of YY1, we intercrossed yy1^{f/f} mice with mice carrying the mb1-Cre transgene, which facilitates deletion of loxP-flanked sequences at the earliest stages of B-cell development with high efficiency [Hobin et al. 2006]. PCR analysis failed to detect loxP-flanked yy1^{f/f} alleles in purified BM pro-B (CD19^{+}CD43^{−}slgM^{+}) and pre-B (CD19^{+}CD43^{−}slgM^{−}) cells of mb1-Cre yy1^{f/f} (knockout/KO) and mb1-Cre yy1^{f/f} (heterozygous/HET) mice (Fig. 1B,C). In addition, YY1 mRNA was essentially undetectable by RT–PCR in pro-B cells purified from the KO mice (Fig. 1D), indicating almost complete ablation of YY1 expression in early B cells.

To identify cells in which Cre-mediated recombination occurred by flow cytometric analysis (FACS), we used the Rosa26-eYFP (R26-eYFP) allele (Sirinivas et al. 2001). Cells carrying this allele fluoresce green light upon Cre-mediated excision of a loxP-flanked stop cassette. Therefore, deletion efficiency of the R26eYFP allele as reflected by the percentage of green fluorescent cells serves as an indirect measurement of recombination efficiency of other loxP-flanked alleles in the same cell population. The B220^{+}CD19^{+} population in the BM, comprising the earliest B-cell progenitors, contained a relatively low percentage of green fluorescent cells (5%–6%) (Fig. 2A). In contrast, >95% BM CD19^{+} B cells were green in mb1-Cre/R26eYFP, HET/R26eYFP, and KO/R26eYFP mice, which is consistent with the deletion efficiency detected by PCR and RT–PCR. These results confirmed successful generation of a B-cell-specific yy1 knockout mouse.

### Loss of YY1 blocks pro-B-cell differentiation

Mice genotyped as yy1^{f/f}, yy1^{f/f}, mb1-Cre, and mb1-Cre yy1^{f/f} (HET) were indistinguishable from wild-type mice and were subsequently grouped as controls (CTR), suggesting that a single yy1 allele is sufficient to support B-cell development. Consistent with the very low percentage of eYFP+ cells at the earliest B220^{+}CD19^{+} stage, no significant difference was detected between control and KO mice at this stage of B-cell development.

## Conclusion

In summary, YY1 plays an essential role in B-cell development by controlling the pro-B-to-pre-B transition. Loss of YY1 blocks pro-B-cell differentiation, possibly through direct interaction with the IgH Eiµ enhancer.
YY1 and B-cell development

**Figure 1.** B-cell-specific deletion of yy1 with the mb1-Cre transgenic mice. (A) Schematic diagram of the yy1 locus. The wild-type yy1 allele (yy1*) contains five exons. The conditional yy1 allele (yy1f) was constructed by inserting a pair of LoxP sites flanking the exon1 and the promoter region, which will be excised in the presence of Cre recombinase, thereby generating a null allele of yy1 (yy1f/f). The arrows indicate the location of the primers used for PCR detection of deletion efficiency, PCR genotyping, and RT–PCR to detect YY1mRNA. Primers 1 and 2 detect both yy1f (233 bp) and yy1f (369 bp). Primers 1 and 4 detect yy1f (292 bp). Primers 3 and 4 detect both yy1f and yy1f (138 bp). Primers 5 and 8 detect a 480-bp YY1 mRNA, and primers 6 and 7 detect a 205-bp YY1 mRNA. (B) PCR detection of deletion efficiency in sorted pro-B (CD19+CD43+sIgM) and pre-B (CD19+CD43+sIgM) from mb1-Cre yy1f/f [HET] and mb1-Cre yy1f/f [KO] mice. A sample of YY1 A-i-1 was used to show the similar amplifying efficiency of the yy1f and yy1f alleles in the mixed primers of 1, 2, and 4. (C) PCR detection of deletion efficiency in sorted pro-B (CD19+CD43+sIgM) cells from yy1f/f [HET], KO, and KO mice. Primers 1 and 2 were used to detect the yy1f allele in the top panel. Primers 1 and 4 were used to detect the yy1f allele in the bottom panel. The middle panel showed the total yy1f and yy1f allele to serve as control for equal loading. (D) RT–PCR detection of YY1 mRNA. Two different primer combinations, primers 5 and 8 (pp5/8) and 6 and 7 (pp6/7), were used to identify YY1 mRNA in both control and KO mice. Input cDNA was normalized using the HPRT mRNA.

**YY1-deficient pro-B cells exhibit impaired Vμ to DμH recombination**

Successful rearrangement of the IgH gene and subsequent expression of Iγμ is essential for pro-B-cell differentiation (Jung and Alt 2004). As shown in Figure 3A, while ~35% of pro-B (B220+CD43+sIgM) and 90% of pro-B cells expressed intracellular μ (μi) chain in the BM of control mice, the percentage of μi+ cells was 8% and 14% in the BM of yy1f/f pro-B and pre-B cells, respectively. To determine whether reduced μi expression was due to a defect in VμDμH recombination, we compared the recombination frequency of Hμ from JH to DμH in pro-B cells purified by cell sorting from control and yy1f/f mice using degenerative PCR primers as described previously (Fuxa et al. 2004). We found that pro-B cells without YY1 underwent DμH to JH recombination normally [Fig. 3B–D]. In contrast, YY1-null pro-B cells had a gradually decreased frequency of Vμ to DμH rearrangement, which was inversely proportional to the distance separating Vμ families from the DμH region [Fig. 3B–D]. The recombined Vμ gene fragments from the most proximal Vμ families Vμ7183 and VμDQ52 in the KO pro-B cells were 50%–100% of those in the control pro-B cells. The recombination frequency of the distal Vμ3609 and VμJ558 segment in the KO pro-B cells was 6%–20% of that in the control pro-B cells. Consistently, RT–PCR showed that the expression of IgH μ mRNA corresponded to the genomic DNA recombination frequencies [Fig. 3D], indicating that loss of YY1 did not prevent transcription of the recombinated IgH alleles. These findings suggest that YY1 plays a critical role in Vμ to DμH recombination, thus identifying a novel role for YY1 in pro-B-cell differentiation.

**YY1 regulates VμDμH recombination by controlling IgH locus contraction**

The murine IgH locus spans ~3 Mb and the V gene region occupies the 5’ 2.5 Mb (Johnston et al. 2006). The IgH locus relocates from the periphery to the center of the nucleus in pro-B cells, a process believed to facilitate VμDμH recombination (Kosak et al. 2002). Locus contraction brings the distal and mid Vμ gene segments into close proximity with the DμH region. This enables RAG recombinase-mediated Vμ to DμH recombination (Kosak et al. 2002; Roldan et al. 2005; Sayegh et al. 2005). Locus contraction, mediated by DNA looping, has been observed by three-color 3D DNA FISH experiments (Roldan et al. 2005; Sayegh et al. 2005) to investigate mechanisms by which YY1 regulates VμDμH recombination, and to specifically address the question of whether YY1 plays a role in IgH locus relocation and/or contraction, we performed 3D DNA FISH with ex vivo purified pro-B cells [CD19+Kit+] from control and KO mice. The nuclear location of different IgH gene segments in three-dimensionally preserved pro-B cells was detected with three differentially labeled locus-specific probes. Gene segments were scored as colocalized if the two fluorescence signals were overlapping or separated by a distance of <0.3 μm. In contrast, if the two signals were separated by 0.3–0.5 or 0.5–1.5 μm, they were scored as apart or far apart, respectively. Figure 4A shows the position and color of the three probes in the IgH...
locus and the approximate distance (in base pairs) separating the probes. As shown in Figure 4C, the percentage of pro-B cells with a centrally localized VHJ558 signal in the YY1 KO mice was increased compared with that of the control (YY1KO 63% and CTR 45%, \( p < 0.01 \)), suggesting that loss of YY1 did not prevent relocation of the distal IgH locus from the periphery to the center of the nucleus.

We next analyzed the relative distance separating the distal \( V_H \) probes (\( V_H \)J558 and \( V_H \)15) from the proximal \( V_H \) probe (\( V_H \)7183) or the constant region probe \( C_H \). Consistent with the linear distance separating the gene segments along the chromosome, we found that the signals of the two distal \( V_H \) gene families, \( V_H \)J558 and \( V_H \)15, were colocalized, as were the signals of the proximal \( V_H \) gene family \( V_H \)7183 and the \( C_H \) signal (Fig. 4B,D). While the colocalization of the distal \( V_H \) probes and the \( C_H \) probes can be a consequence of either IgH locus contraction or \( V_H \)-DHJH recombination due to the deletion of DNA segments between the recombined \( V_H \) and \( D_H \)JH genes, only IgH locus contraction will result in the convergence of the distal \( V_H \)J558 and the proximal \( V_H \)7183 probes. If recombination occurs 5′ of \( V_H \)7183 genes, no \( V_H \)7183 signal will be detected in pro-B cells due to the deletion of \( V_H \)7183 gene segments. If recombination occurs within the \( V_H \)7183 to \( D_H \)JH region, it will not change the linear distance between distal \( V_H \)J558 and proximal \( V_H \)7183 segments. In the majority

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**Figure 2.** Loss of YY1 caused a pro-B to pre-B differentiation block. (A) Detection of Cre-mediated recombination by FACS analysis of green-fluorescing cells at different stages of BM B-cell development with the R26eYFP Cre reporter. The mb1-Cre mice were used as a negative control for the eYFP signal. A total of three mice from each group were analyzed. (B) FACS analysis of B-cell development in mb1-Cre yy1\( ^{fl/fl} \) knockout mice [KO]. Pro-B cells were characterized as B220\(^+\)CD19\(^+\)CD43\(^+\)cKit\(^−\)CD25\(^−\)sIgM\(^−\), and pre-B cells were characterized as B220\(^+\)CD19\(^+\)CD43\(^−\)cKit\(^−\)CD25\(^+\)sIgM\(^−\). In general, KO mice had a two- to threefold increase in pro-B percentage and a significant decrease of pre-B percentage. The numbers shown on the dot plot are the average percentage of each subpopulation in the total cells from BM, spleen, or LNs. A total of three to six experiments were performed. (C) Total cell number of each B-cell subpopulation in KO and control (CTR) mice. Pro-B cells were identified as CD19\(^+\)CD43\(^+\)sIgM\(^−\) and pre-B cells were identified as CD19\(^+\)CD43\(^−\)sIgM\(^−\). BM immature [MB] and mature B [MB] were CD19\(^+\)sIgM\(^−\). Cells from two femurs and two tibias were counted. Spleen B cells were defined as CD19\(^+\). Bars indicate the average number of six to seven experiments.

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**Figure 3.** Loss of YY1 caused a defect in \( V_H \) to \( D_H \)JH, but not \( D_H \) to \( J_H \) recombination. (A) Detection of \( \mu \)-chain expression in pro-B and pre-B cells from CTR and KO mice. Results are representative of three experiments. (B) Schematic drawing of the IgH locus to show the relative location of the different \( V_H \) gene clusters. Only the \( V_H \) gene families analyzed are shown. The most distal \( V_H \) family is J558 and the most proximal \( V_H \) family is 7183. (C) PCR detection of \( D_H \)-\( J_H \) and \( V_H \)-\( D_H \)JH recombination in sorted HET and KO pro-B cells. Input DNA was normalized by PCR amplification of the Cre DNA from the Ig-\( H9251 \) locus. Five-fold serial dilution was used for PCR. Degenerative forward primers are located in the respective V regions and the reverse primer was in the JH3 segment. (D) Quantitation of \( D_H \)-\( J_H \) and \( V_H \)-\( D_H \)JH recombination in sorted pro-B cells. The average recombination frequency with its standard errors is shown as the relative percentage of YY1KO compared with control pro-B cells. Six independent experiments were conducted. (E) RT–PCR to detect the mRNA levels of the \( \mu \) transcripts. Same forward primers used as in C, and the reverse primer was located in the first exon of the \( \mu \)-constant region. Equal loading of input cDNA was shown by the amplification of the HPRT mRNA.
of the control pro-B cells, the distal V_{H} gene segments were colocalized with the proximal V_{H} or C_{H} region (Co-localization/Apart/Far apart percentage: V_{H}J558/C_{H} 79.6/2.9/17.5, total 121 alleles; V_{H}15/C_{H} 83/3/14, total 61 alleles; V_{H}J558/V_{H}7183 87/1/12, total 60 alleles) [Fig. 4D; Supplementary Table 1]. This indicated that loss of YY1 adversely affected IgH locus contraction, providing a mechanistic explanation for the observed defects in V_{H}-D_{H}-J_{H} recombination in the YY1 KO pro-B cells.

Supplementary Table 1. This indicated that loss of YY1 adversely affected IgH locus contraction, providing a mechanistic explanation for the observed defects in V_{H}-D_{H}-J_{H} recombination.

Loss of YY1 does not change RNA transcript levels of many molecules required for pro-B-cell differentiation and V_{H}-D_{H}-J_{H} recombination

What is the mechanism by which YY1 regulates IgH locus contraction? To address this issue, we first asked
whether loss of YY1 affected transcription of genes whose products are known to be important for IgH locus contraction. Pax5 and EZH2 are known to play a role in IgH locus contraction (Fuxa et al. 2004; A. Tarakhovsky, pers. comm.). As shown in Figure 5A, loss of YY1 did not change the mRNA level of Pax5 and EZH2. We also examined the mRNA levels of other molecules known to be important for V(D)J recombination and/or pro-B-cell differentiation. RT–PCR confirmed that loss of YY1 did not alter the mRNA levels of the main components of the V(D)J recombination machinery, including RAG1 and RAG2, intranuclear terminal deoxynucleotidyl transferase (TdT), the DNA-dependent protein kinase c (PKC), and Ku70/Ku80 [Fig. 5A; Jung and Alt 2004]. We also examined the mRNA levels of several transcription factors previously shown to be critical for early B-cell development, including E2A, EBF, PU.1, spIB, EZH1/2, IKAROS, and surface receptors including Interleukin-7 (IL-7) receptor α, the common γ chain (γc), FLT3, and components of the pre-BCR, including Ig-α, Ig-β, λ5, and VpreB (Fleming and Paige 2002; Busslinger 2004; Corcoran et al. 2005). With the exception of the Ig-α [reduced by 50%] and FLT3 [two- to threefold increase], none of them appeared to be affected by the loss of YY1 [Fig. 5A]. Finally, KO pro-B cells expressed normal to higher levels of Iµ, Cµ, VH7183, and VHJ558 germline transcripts [Fig. 5B], which are markers for an accessible IgH locus (Yancopoulos and Alt 1985), indicating that loss of YY1 did not interfere with the initial chromatin opening of the IgH locus. Taken together, loss of YY1 did not appear to affect the expression of genes known to be important for IgH locus contraction, suggesting that YY1 may play a direct role in this process.

YY1 binds to Eiµ within the IgH locus

The direct model predicts YY1 binding at the IgH locus. Previous in vitro studies identified a potential YY1-binding site at the µE1 site of the IgH intronic enhancer [Eiµ] (Park and Atchison 1991). Using quantitative ChIP assays, we confirmed binding of YY1 to Eiµ in ex vivo cultured pro-B cells [Fig. 5C], consistent with a potentially important role of this YY1 site. We also tested a few selected areas in the Vµ region, but found no significant YY1 binding. [Fig. 5C]. Since the selected regions only represent a small percentage of the total Vµ regions, further systematic analysis (ChIP–chip) is necessary to determine whether YY1 binds to other Vµ regions in addition to Eiµ.

A prerecombined IgH transgene partially rescues pro-B-cell differentiation defect in the yy1 KO mice

To further investigate whether YY1 controls pro-B-cell differentiation mainly through regulation of VµDµJµ recombination, we introduced a prerearranged VµDµJµ segment [B1-8i] into the YY1 B-cell-specific mb1-Cre YY1 KO mice. The B1-8i IgH transgene is inserted into the endogenous IgH locus (Sonoda et al. 1997). As shown

Figure 5. YY1 binds to IgH locus directly. (A) RT–PCR to detect the mRNA levels of molecules involved in early B-cell development in sorted pro-B cells from heterozygous and KO mice. (B) RT–PCR to detect the IgH germline transcript. (A,B) Input cDNA was normalized by the amplification of the HPRT mRNA [Fig. 3D]. [C] Quantitative ChIP–PCR. ChIP assays showing YY1 binding to the Eiµ enhancer, but not several other selective regions of the IgH locus. Experiments were performed with rabbit polyclonal anti-YY1 or normal rabbit IgG as control. Real-time PCR was carried out using the Roche 480 LightCycler. Data are presented as “fold enrichment” of anti-YY1 signal relative to that of IgG. [JH and DFL16.1] J and D gene regions; [7183aRSS] the most proximal Vµ family; [S107bp] the middle region of the Vµ gene; [J588aP] and [J588aRSS] the proximal Vµ,J588 genes; [J558cRSS] the distal regions of the Vµ,J588 genes; [Intergenic D] intergenic regions between members of the Vµ,J588 family. RPL30 and Actin-B are positive and negative controls, respectively. Each DNA sample was repeated two to three times by real-time PCR for each primer pair, and a total of eight to 12 independent ChIP preparations were performed.
in Figure 6A,B, yy1f alleles were deleted efficiently in CD19+ BM B cells from both HET/B1-8i and KO/B1-8i mice, as reflected by the percentage of eYFP+ cells and PCR analysis. As shown in Figure 6C, both CTR/B1-8i and KO/B1-8i mice had a similar percentage of µ-chain-positive cells among the CD19+sIgM+ B-cell population, indicating that YY1 was not required for the transcription of the recombed IgH gene. We found very few cKit+CD19+ pro-B cells in both CTR/B1-8i and KO/B1-8i BM [Fig. 6D,E], indicating that expression of the B1-8i transgene bypassed the requirement for YY1 and successfully transduced the necessary signal to down-regulate surface expression of c-Kit to facilitate the transition of pro-B to pre-B cell. Compared with the yy1 KO mice, yy1 KO/B1-8i mice displayed reduced pro-B-cell number, and increased numbers of pre-B, immature and mature B cells in BM, spleen, and LNs [Fig. 6D,E], indicating that regulation of VH to DJH recombination is an important function of YY1 in pro-B-cell differentiation. However, the pre-B, immature, and mature B-cell populations were still significantly reduced in yy1 KO/B1-8i compared with those in CTR/B1-8i mice [Fig. 6D,E]. In addition, the incomplete down-regulation of CD43 and up-regulation of CD25 upon expression of the prerecombed IgH transgene in the YY1-depleted pre-B cells, point to other functions for YY1 in pro-B-cell expansion and differentiation.

**Discussion**

Pro-B-cell differentiation requires successful recombination and expression of a single IgH allele. In this report, we have demonstrated that the ubiquitously expressed YY1 transcription factor is important for VH to D JH recombination and pro-B-cell differentiation. Furthermore, we have provided evidence supporting a role for YY1 in regulating V(D)J recombination by controlling IgH locus contraction.

In pro-B cells, the spatially separated VH, D H and J H gene segments are brought together by means of DNA looping and IgH locus contraction to allow for RAG recombinase-mediated VH D JH recombination (Kosak et al. 2002; Roldan et al. 2005; Sayegh et al. 2005). Pax5 is the first protein identified to play a role in IgH locus contraction (Fuxa et al. 2004). Pax5-binding sites within the IgH locus and direct interaction between Pax5 and IgH locus were described in recent reports (Pawlitzky et al. 2002; Roldan et al. 2005; Sayegh et al. 2005). Pax5 is the first protein identified to play a role in IgH locus contraction, but did not affect IgH locus relocation. The normal expression of Pax5 in the pro-B cells lacking YY1 gene was reduced by means of DNA looping and IgH locus contraction to allow for RAG recombinase-mediated VH D JH recombination (Kosak et al. 2002; Roldan et al. 2005; Sayegh et al. 2005). Pax5-binding sites within the IgH locus and direct interaction between Pax5 and IgH locus were described in recent reports (Pawlitzky et al. 2002; Roldan et al. 2005; Sayegh et al. 2005). Pax5 is the first protein identified to play a role in IgH locus contraction, but did not affect IgH locus relocation. The normal expression of Pax5 in the pro-B cells lacking YY1 (Fig. 5A) indicates that YY1 does not control IgH locus contraction by regulating transcription of Pax5. It is likely that YY1 and Pax5 play nonredundant roles in IgH locus contraction. For instance, YY1 and Pax5 may require each other for efficient binding to the IgH locus and DNA loop formation. YY1 and Pax5 may also work together to induce histone modifications and alterations in chromatin structures that may facilitate IgH locus contraction and recombination.

The direct interaction between YY1 and Eiµ as shown by ChIP, together with a lack of requirement for YY1 in the transcription of a number of genes whose products are important for IgH recombination [Fig. 5A], support a direct involvement of YY1 at the IgH locus in regulating
IgH locus contraction. Thus far, cis-elements required for IgH DNA looping and locus contraction remain unknown. The importance of the core iµ in V_{H}D_{JH} recombination and its short distance to the D_{JH} region makes it a good candidate as a site that participates in the regulation of DNA looping [Sakai et al. 1999; Perlot et al. 2005]. Future studies on the status of IgH locus contraction with pro-B cells derived from Eµ knockout mice should provide insights into the role of iµ in IgH DNA looping and locus contraction. The Eµ enhancer contains binding sites for multiple transcription factors including YY1, E2A, and Pu.1 [Park and Atchison 1991; Ernst and Smale 1995]. Transgenic mutant mice studies suggested that each individual transcription factor-bind-
ing site within the Eµ may play distinct role in different aspects of V_{H}D_{JH} recombination [Fernex et al. 1994, 1995]. Our analysis of the published data suggests that a 38-base-pair (bp) fragment in the most 5′ region of the core iµ may play an important role in V_{H} to D_{JH} recombination [Fernex et al. 1995; Sakai et al. 1999; Perlot et al. 2005]. This 38-bp cis-acting element contains the µE1–YY1-binding sites and µE5–E2A-binding sites. A recent study showed that expression of E2A and binding of E2A to µE5 is not required for V_{H}D_{JH} recombination if a sufficient amount of the B-cell-specific EBF protein is present in the pro-B cells [Seet et al. 2004], suggesting that the YY1/µE1 interaction is functionally important for the V_{H} to D_{JH} recombination. DNA looping was originally suggested to be a mechanism of transcriptional regulation mediated by long-range cis-elements, such as distal enhancers and locus control regions within the same or different chromosomes [Tolhuis et al. 2002; de Laat and Grosveld 2003; Spilianakis and Flavell 2004; Spilianakis et al. 2005]. Therefore, understanding YY1-mediated IgH locus contraction will have important implications for understanding mechanisms that control communications among noncontiguous chromosomal DNA elements regulating both transcription and recombination.

An alternative, but not mutually exclusive possibility is that YY1 regulates IgH locus contraction indirectly by recruiting histone modifiers to change local histone modifications and chromatin structure, which, in turn, could influence the local chromatin accessibility for proteins directly involved in DNA loop formation. Histone acetylation, while a marker for an accessible IgH locus, could influence the local chromatin accessibility for pro-

In summary, our study has identified a novel lineage-specific role for YY1 in early B-cell development. Our findings not only provide new insights into the molecular mechanisms underlying V_{H}D_{JH} recombination and locus contraction, but also shed significant light on the role and mechanism of action of YY1 in living organisms. The findings here also highlight YY1 as a potential regulator, which may facilitate communications among noncontiguous DNA elements in the genome.

Materials and methods

Generation of the floxed YY1 conditional knockout mice

The generation of the loxp-flanked yy1 allele (yy1/) was described previously [Affar et al. 2006]. Generation of the B-cell-
specific mb1-Cre transgenic mice will be described elsewhere (Hobeika et al. 2006). The Rosa26EYFP cre reporter mice were kindly provided by Dr. Frank Costantini [Srinivas et al. 2001]. Generation of the IgH transgenic B2.1-s mice was described previously (Sonoda et al. 1997). All mice were bred and maintained under specific pathogen-free conditions at the animal facility of Harvard Medical School. All mouse protocols were approved by the Harvard Medical School IACUC. Mice are maintained on a mixed background of 129SvEvXc57BL/6. Analyzed animals range from 2 to 14 wk old, including both males and females. The observed phenotype is consistent at different ages and both sexes in the KO mice. Mutant mice were genotyped by PCR (for primer sequences, see Supplementary Table 2).

FACS analysis and cell sorting

Single-cell suspension prepared from BM, spleen, and LN were stained with antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), or biotin. For FACS analysis, the following antibodies were purchased from BD biosciences: PerCP- or APC-conjugated anti-B220 (RA3-6B2), FITC- or PE-conjugated anti-CD43 (S7), PE- or APC-conjugated anti-CD19 (CD19-I3D5), and PerCP-conjugated-streptavidin. The following antibodies were purchased from eBioscience: PE- and APC-anti-c-kit, and PE- and APC-anti-CD25. FITC- or biotin-conjugated anti-µ (M41) were prepared from the corresponding hybridoma. Flow cytometric acquisition was conducted with a FACScalibur (BD Biosciences) and data was analyzed with WinMDI2.8. For biochemical analysis, CD19+ B lymphocytes were enriched with the EasySep B-cell enrichment kit (StemCell Technologies) from freshly prepared BM cell suspension and purified according to the manufacturer’s protocol. Enriched cells were then stained with anti-B220, anti-CD43, and anti-µ antibodies. Pro-B cells were sorted as B220loCD19+CD43+sIgM’ from freshly prepared BM cell suspension and enriched with the EasySep B-cell enrichment kit (StemCell Technologies) from freshly prepared BM cell suspension and purified according to the manufacturer’s protocol. Enriched cells were then stained with anti-B220, anti-CD43, and anti-µ antibodies. Pro-B cells were sorted as B220loCD19+CD43+sIgM’ and pre-B cells were sorted as B220hiCD19+CD43+sIgM’.

PCR detection of deletion efficiency and recombination efficiency

Sorted pro-B cells and pre-B cells or total thymocytes (1 × 10^6) from heterozygous mb1-Cre yy+/− and homozygous mb1-Cre yy/yy mice were dispensed in 80 µL of 50 mM NaOH, heated for 5 min at 95°C, and vortexed to dissolve the cell pellets. NaOH was neutralized with 20 µL of 1 M Tris.HCl (pH 6.8). The resulting DNA solution was serially diluted at a 1:5 ratio. About 1 µL of solution was used for each PCR reaction to detect either the deletion efficiency of the floxed yy allele in pro-B and pre-B cells or the V_{H} [D]_{H} recombination efficiency in pro-B cells using primers described previously [Fuxa et al. 2004]. PCR products were separated on 2% agarose gel and visualized by ethidium-bromide staining. For primer sequence, see Supplementary Table 2.

RT–PCR analysis

Total RNA from 0.5 × 10^5 to 1 × 10^5 sorted pro-B cells were extracted with Trizol (Invitrogen) followed by RNase-free DNase (Promega) digestion for 1 h at 37°C. RNA was then purified with phenol-chloroform-ethanol precipitation and dissolved in DEPC-treated H_2O. cDNA was synthesized using the RevertAse first-strand synthesis kit according to manufacturer’s protocol [ABGene] using oligo-dT primer, random hexamer, or gene-specific primers. Total cDNA were serially diluted at a 1:5 ratio and ~1 µL of diluted cDNA was used for each PCR reaction. Most primer sequences for RT–PCR analysis were described previously and are provided in Supplementary Table 2 [DeKoter et al. 2002; Bolland et al. 2004, Fuxa et al. 2004].

3D DNA FISH and confocal analysis

Three-color 3D DNA FISH was carried out using sorted CD19+cKit’ pro-B cells from control and YY1KO mice as previously described in detail [Kosak et al. 2002, Fuxa et al. 2004]. Cells were analyzed by confocal microscopy on a Leica SP2 AOBS (Acoustica Optical Beam Splitter) system. Optical Z sections were collected at 0.3-µm steps through individual nuclei. Only cells containing signals of both IgH alleles were evaluated. The locus-specific DNA probes were prepared from the bacterial artificial chromosomes [BACs] 526A21 (V_{H}J558), 243G9 (V_{H}15), 167C1 (V_{H}7183), and C34H6 (C_{H}1] by nick translation and directly labeled with dUTP Cy5, dUTP Cy3, and dUTP fluorogreen [Amersham Pharmacia]. The distance separating the signals of the different IgH gene probes in the nucleus was measured on individual confocal images. A distance of <0.3 µm was defined as colocalized, 0.3–0.5 µm was defined as “apart,” and a distance of 0.5–1.5 µm was referred to as “far apart.” Calculation of P value was performed by applying the $\chi^2$ test to observed and expected frequencies [Supplementary Table 1].
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