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

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The role of the transcription factor Yin Yang 1 (YY1) in development is largely unknown. Here we show that specific ablation of YY1 in mouse B cells caused a defect in somatic rearrangement in the immunoglobulin heavy-chain (*IgH*) locus and a block in the progenitor-B-to-precursor-B-cell transition, which was partially rescued by a prerrearranged *IgH* transgene. Three-dimensional DNA fluorescence in situ hybridization analysis revealed an important function for YY1 in *IgH* locus contraction, a process indispensable for distal V_H to D_HJ_H recombination. We provide evidence that YY1 binds the intronic Eip enhancer within the *IgH* locus, consistent with a direct role for YY1 in $V_HD_HJ_H$ recombination. These findings identified YY1 as a critical regulator of early B-cell development.

[**Keywords:** YY1; immunoglobulin gene; B-cell development; $V(D)J$ recombination]

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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, D_H to J_H rearrangement occurs before V_H to D_HJ_H recombination. Upon the generation of a productively rearranged $V_HD_HJ_H$ 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, $\lambda 5$ 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; Morshead 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 $V_HD_HJ_H$ recombination, remain to be determined. Previous studies have identified several *cis*-acting elements within the *IgH* locus that are important for $V_HD_HJ_H$ recombination, including the variable gene promoters, the intronic en-

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hancer (E μ), and the 3' enhancer (Sleckman et al. 1996). Studies using knockout mice confirmed an important role of the core intronic E μ in mediating V $_H$ D $_H$ J $_H$ recombination (Sakai et al. 1999; Perlot et al. 2005). However, it is unclear whether the E μ enhancer regulates V $_H$ D $_H$ J $_H$ recombination by controlling IgH locus nuclear repositioning, contraction, and/or chromosomal changes. The E μ 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; Atchison et al. 2003; Srinivasan and Atchison 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*). To investigate the role of YY1 in B-cell development, we took advantage of the novel *mb1-Cre* transgenic mouse (Hobeika et al. 2006), which recombines *LoxP*-flanked sequences in early B-cell progenitors. Phenotypic analyses of the B-cell-specific *yy1* knockout mice (*mb1-Cre yy1^{ff}*) 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 E μ 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 E μ enhancer.

Results

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

In order to study the role of YY1 in lineage development, we generated a conditional *yy1* knockout allele (*yy1^f*) by flanking the *yy1* promoter region and exon1 with *loxP*-sites (Fig. 1A; Affar et al. 2006). The *yy1^f* allele expresses normal levels of YY1 protein and Cre recombinase-mediated recombination yields a *yy1*-null allele (*yy1^Δ*) 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* 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 (Hobeika et al. 2006). PCR analysis failed to detect *loxP*-flanked *yy1^f* alleles in purified BM pro-B (CD19⁺CD43⁺sIgM⁻) and pre-B (CD19⁺CD43⁻sIgM⁻) cells of *mb1-Cre yy1^{ff}* (knockout/KO) and *mb1-Cre yy1^{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 (Srinivas 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/+}*, *yy1^{ff}*, *mb1-Cre*, and *mb1-Cre yy1^{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. In contrast, compared with control mice, KO mice contained twice as many pro-B cells (B220^{lo}CD19⁺CD43⁺cKit⁺CD25⁻sIgM⁻), but a markedly reduced number of pre-B cells (B220^{med}CD19⁺cKit⁻CD25⁺CD43⁻sIgM⁻) in the BM, and hardly any immature and mature B (CD19⁺sIgM⁺)

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 (*yy1*^f) 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* (*yy1*^Δ). The arrows indicate the location of the primers used for PCR detection of deletion efficiency, PCR genotyping, and RT-PCR to detect YY1 mRNA. Primers 1 and 2 detect both *yy1*⁺ (223 bp) and *yy1*^f (369 bp). Primers 1 and 4 detect *yy1*^Δ (292 bp). Primers 3 and 4 detect both *yy1*^f and *yy1*^Δ (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⁺slgM⁻) and pre-B (CD19⁺CD43⁺slgM⁻) cells from *mb1-Cre yy1*^{f/+} (HET) and *mb1-Cre yy1*^{f/f} (KO) mice. A sample of *YY1*^{f:Δ-1:1} was used to show the similar amplifying efficiency of the *yy1*^f and *yy1*^Δ alleles in the mixed primers of 1, 2, and 4. (C) PCR detection of deletion efficiency in sorted pro-B (CD19⁺CD43⁺slgM⁻) cells from *yy1*^{f/f} (cKO), HET, and KO mice. Primers 1 and 2 were used to detect the *yy1*^f allele in the top panel. Primers 1 and 4 were used to detect the *yy1*^Δ allele in the

middle panel. The bottom panel showed the total *yy1*^f and *yy1*^Δ allele to serve as control for equal loading. (D) RT-PCR detection of YY1 mRNA. Two different primer combinations, primers 5 and 8 (p5/8) and 6 and 7 (p6/7), were used to identify YY1 mRNA in both control and KO mice. Input cDNA was normalized using the HPRT mRNA.

cells in BM, spleen, and lymph nodes (LNs) (Fig. 2B,C), suggesting a critical role for YY1 during differentiation of pro-B cells to pre-B cells.

YY1-deficient pro-B cells exhibit impaired *V_H* to *D_HJ_H* recombination

Successful rearrangement of the IgH gene and subsequent expression of Igμ is essential for pro-B-cell differentiation (Jung and Alt 2004). As shown in Figure 3A, while ~35% of pro-B (B220⁺CD43⁺slgM⁻) and 90% of pre-B cells expressed intracellular μ (iμ) chain in the BM of control mice, the percentage of iμ⁺ cells was 8% and 14% in the BM of *yy1*^{-/-} pro-B and pre-B cells, respectively. To determine whether reduced iμ expression was due to a defect in *V_HD_HJ_H* recombination, we compared the recombination frequency of *D_H* to *J_H* and *V_H* to *D_HJ_H* in pro-B cells purified by cell sorting from control and *yy1*^{-/-} mice using degenerative PCR primers as described previously (Fuxa et al. 2004). We found that pro-B cells without YY1 underwent *D_H* to *J_H* recombination normally (Fig. 3B–D). In contrast, YY1-null pro-B cells had a gradually decreased frequency of *V_H* to *D_HJ_H* rearrangement, which was inversely proportional to the distance separating *V_H* families from the *D_HJ_H* region (Fig. 3B–D). The recombined *V_H* gene fragments from the most proximal *V_H* families *V_H*7183 and *V_H*DQ52 in the KO pro-B cells were 50%–100% of those in the control pro-B cells. The recombination frequency of the distal *V_H*3609 and *V_H*J558 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 recombined IgH alleles. These findings suggest that YY1 plays a critical role in *V_H* to *D_HJ_H* recombination, thus identifying a novel role for YY1 in pro-B-cell differentiation.

YY1 regulates *V_HD_HJ_H* 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 *V_HD_HJ_H* recombination (Kosak et al. 2002). Locus contraction brings the distal and mid *V_H* gene segments into close proximity with the *D_HJ_H* region. This enables RAG recombinase-mediated *V_H* to *D_HJ_H* 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 *V_H* to *D_HJ_H* 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⁺cKit⁺) 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

Figure 4. Loss of YY1 prevents IgH locus contraction. 3D DNA FISH was performed to show the subnuclear localization and contraction of the IgH locus in CTR and KO pro-B cells. (A) Schematic diagram indicating the probe positions in the IgH locus (not drawn to scale). (B) Confocal images of different IgH segments in pro-B nuclei. Pro-B cells were prepared from CTR and KO mice and sorted as CD19⁺cKit⁺ cells. The V_HJ558 probe was directly labeled with Cy5-dUTP (blue) and synthesized from BAC526A21. The V_H15 probe (BAC 243G9) and V_H7183 (BAC167C1) probe were directly labeled with Cy3-dUTP (red). The C_H probe (BAC C34H6) was directly labeled with FITC-dUTP (green). (C) Statistical analysis of the nuclear position of the V_HJ558 gene segments in CTR and KO pro-B cells. (D) Statistical analysis of the distance separating different IgH gene segments in CTR and KO pro-B cells. A distance of 0.3–0.5 μ m was defined as “apart,” and a distance of 0.5–1.5 μ m was referred to as “far apart.” (**) $P < 0.01$; (***) $P < 0.001$.

of the control pro-B cells, the distal V_H gene segments were colocalized with the proximal V_H or C_H region (Colocalization/Apart/Far apart percentage: V_HJ558/C_H 79.6/2.9/17.5, total 121 alleles; V_H15/C_H 83/3/14, total 61 alleles; V_HJ558/V_H7183 87/1/12, total 60 alleles) (Fig. 4D; Supplementary Table 1). In contrast, loss of YY1 resulted in a significant increase in alleles with separated distal and proximal IgH signals (Colocalization/Apart/Far apart percentage: V_HJ558/C_H 40/9/51, total 175 alleles; V_H15/C_H 35/11/54, total 98 alleles; V_HJ558/V_H7183 46/8/46, total 78 alleles, $p < 0.001$) (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_HJ_H recombination in the YY1 KO pro-B cells.

Loss of YY1 does not change RNA transcript levels of many molecules required for pro-B-cell differentiation and V_HD_HJ_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 (γ c), FLT3, and components of the pre-BCR, including Ig- α , Ig- β , λ 5, 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 I μ , C μ , V_H7183, and V_HJ558 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 E μ 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 μ E1 site of the IgH intronic enhancer (E μ) (Park and Atchison 1991). Using quantitative ChIP assays, we confirmed binding of YY1 to E μ 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 V_H region, but found no significant YY1 binding. (Fig. 5C). Since the selected regions only represent a small percentage of the total V_H regions, further systematic analysis (ChIP-chip) is necessary to determine whether YY1 binds to other V_H regions in addition to E μ .

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 V_HD_HJ_H recombination, we introduced a prerearranged V_HD_HJ_H 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 E μ 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 V_H gene family; (S107bp) the middle region of the V_H gene; (J588aP and J558aRSS) the proximal V_HJ588 genes, (J558cRSS) the distal regions of the V_HJ588 genes; (Intergenic D) intergenic regions between members of the V_HJ588 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, *yy1^f* 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

Figure 6. The prerrearranged IgH transgene (B1-8i) partially rescued the pro-B/pre-B-cell differentiation defect in the KO mice. (A) Visualization of Cre activities by detecting green-fluorescing cells at different stages of BM B-cell development with the *R26eYFP* Cre reporter. Results are representative of three experiments. (B) PCR detection of deletion efficiency in purified CD19⁺ BM B cells from HET/B1-8i and KO/B1-8i mice. A sample of *yy1^f: Δ 1 = 1:1* was used to show similar amplifying efficiency of the *yy1^f* and *yy1 Δ* alleles with the primer mixture of 1, 2, and 4. (C) Detection of μ -chain expression in pro-B and pre-B cells from CTR and KO mice. Results are representative of three experiments. (D) FACs analysis of CTR/B1-8i and KO/B1-8i mice. The numbers shown on the dot plot are an average percentage of each subpopulation in the total cells of BM, spleen, or LNs from five to 10 experiments. (E) Total cell number of each B-cell subpopulation in KO/B1-8i and CTR/B1-8i mice (Mean $\pm$ SE). Pro-B cells were identified as CD19⁺CD43⁺sIgM⁺, CD19⁺cKit⁺sIgM⁺, or CD19⁺CD25⁺sIgM⁺, and pre-B cells as CD19⁺CD43⁺sIgM⁺, CD19⁺cKit⁺sIgM⁺, or CD19⁺CD25⁺sIgM⁺. Cells from two femurs and two tibias were counted. A total of five to 10 mice were analyzed for each genotype.

among the CD19⁺sIgM⁺ B-cell population, indicating that YY1 was not required for the transcription of the recombined 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 V_H to D_HJ_H 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 prerecombined IgH transgene in the YY1-depleted pre-B cells, point to other functions for YY1 in pre-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 V_H to D_HJ_H 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 V_H, D_H and J_H gene segments are brought together by means of DNA looping and IgH locus contraction to allow for RAG recombination-mediated V_HD_HJ_H 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 (Pawlitzy et al. 2006; Zhang et al. 2006), suggesting that Pax5 may participate in DNA loop formation within the IgH locus. Here we show that loss of YY1 interfered with 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 E μ 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 E μ in V $_H$ D $_H$ J $_H$ recombination and its short distance to the D $_H$ J $_H$ 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 E μ knockout mice should provide insights into the role of E μ in IgH DNA looping and locus contraction. The E μ 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-binding site within the E μ may play distinct role in different aspects of V $_H$ D $_H$ J $_H$ 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 E μ may play an important role in V $_H$ to D $_H$ J $_H$ recombination (Fernex et al. 1995; Sakai et al. 1999; Perlot et al. 2005). This 38-bp *cis*-acting element contains the μ E1–YY1-binding sites and μ E5–E2A-binding sites. A recent study showed that expression of E2A and binding of E2A to μ E5 is not required for V $_H$ D $_H$ J $_H$ 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/ μ E1 interaction is functionally important for the V $_H$ to D $_H$ J $_H$ 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 and critical for IgH germline transcription (Chowdhury and Sen 2001; Johnson et al. 2003), is neither required for nor dependent on IgH locus contraction as suggested by studies on Pax5 and Stat5 knockout pro-B cells (Fuxa et al. 2004; Bertolino et al. 2005). The normal levels of IgH germline transcripts in the YY1^{-/-} pro-B cells suggests that loss of YY1 may not change the histone acetylation status of the IgH locus. The fact that Pax5 controls both loss of histone H3K9 methylation at the IgH locus and IgH locus contraction in pro-B cells (Fuxa et al. 2004; Johnson et al. 2004) suggests a possible link between methylation and IgH locus contraction. Consistent with this, EZH2-mediated methylation of histone H3 Lys 27 (K27) is believed to be important for V $_H$ to D $_H$ J $_H$ rear-

rangement and IgH locus contraction (Su et al. 2003; A. Tarakhovsky, pers. comm.). YY1 interacts with the EED/EZH2 PcG complex and was shown to be required for recruiting EZH2 to DNA during muscle differentiation (Satiijn et al. 2001; Caretti et al. 2004). Whether YY1 is required for recruiting EZH2 specifically to the IgH locus remains to be determined.

Both YY1KO and interleukin-7 receptor α (IL-7R α) KO pro-B cells have a more severe defect in distal than proximal V $_H$ to D $_H$ J $_H$ recombination (Corcoran et al. 1998). Although we cannot completely exclude an interplay between YY1 and IL-7-mediated signals, the loss of YY1 has no obvious effects on a number of IL-7-dependent processes, including expression of distal V $_H$ gene germline transcription and histone acetylation of the distal V $_H$ gene segments, as well as expression of Pax5 and its target genes (Corcoran et al. 1998; Chowdhury and Sen 2001).

Lastly, it is worth pointing out that our phenotypic analysis of the B-cell-specific YY1 KO mice suggests that YY1 plays roles in B-cell development in addition to pro-B-cell differentiation. In the YY1KO mice, ~10% of the pro-B cells expressed μ chain (Fig. 3A). If YY1 were dispensable for B-cell development beyond pro-B-cell differentiation, these μ -chain-expressing B cells would have differentiated into pre-B, immature, and mature B cells and accumulated in the peripheral lymphoid organs. In contrast, we observed an almost complete absence of immature and mature B cells in the YY1KO mice, suggesting that YY1 is also required at later stages of B-cell development following V $_H$ D $_H$ J $_H$ recombination and μ -chain expression. This hypothesis is further supported by the incomplete rescue of the YY1KO phenotype by a prerrearranged IgH transgene. The partial rescue was reflected by the significantly reduced pre-B-cell number, and the incomplete down-regulation of CD43 and up-regulation of CD25 upon expression of the prerrecombined IgH transgene in the YY1-deficient pre-B cells (Fig. 6D,E). A much lower percentage of pre-B cells from the YY1KO/B1-8i mice contain intracellular κ -chain expression compared with that of CTR/B1-8i mice, suggesting an additional role of YY1 in light-chain recombination (H. Liu and Y. Shi, unpubl.). Collectively, our results suggest that YY1 is likely to play critical roles at multiple stages of B-cell development.

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 $_H$ J $_H$ 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*^f) 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 B1-8i 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 LNs 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 (1D3), and PerCP-conjugated-streptavidin. The following antibodies were purchased from eBiosciences: PE- and APC-anti-cKit, 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 Technology) 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 B220^{lo}CD19⁺CD43⁺sIgM⁻ and pre-B cells were sorted as B220^{med}CD19⁺CD43⁺sIgM⁻. For 3D FISH, freshly prepared BM cell suspension was stained directly with anti-CD19-PE and anti-cKit-APC and sorted as CD19⁺cKit⁺ pro-B cells with an Aria (BD Biosciences). The purity of sorted cells was verified by re-sorting. Sorted cells for further experiments were at least 95% pure.

PCR detection of deletion efficiency and recombination efficiency

Sorted pro-B cells and pre-B cells or total thymocytes (1×10^5) from heterozygous *mb1-Cre yy1^{f/f}* and homozygous *mb1-Cre yy1^{f/f}* mice were dissolved in 80 μ L of 50 mM NaOH, heated for 5 min at 95°C, and vortexed to dissolve the cell pellets. NaOH was neutralized with 20 μ L of 1 M Tris.HCl (pH 6.8). The resulting DNA solution was serially diluted at a 1:5 ratio. About 1 μ L of solution was used for each PCR reaction to detect either the deletion efficiency of the floxed *yy1* allele in pro-B and pre-B cells or the $V_H(D)J_H$ 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 dis-

solved in DEPC-treated H₂O. cDNA was synthesized using the Reverselt 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 μ L 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).

Pro-B-cell culture

Total BM suspension was prepared from 2- to 3-wk-old pups and seeded on mitomycin C-treated S17 stromal cells at a density of 2–4 million per well of six-well tissue culture plates with Iscove's modified Dulbecco's medium, supplemented with 2% fetal bovine serum (FBS), 0.03% (w/v) Primatone RL (P8388, Sigma), 2.5% conditioned supernatant of rIL-7 (recombinant interleukin 7) secreting J558L cells, 1 mM glutamine, 50 μ M β mercaptoethanol, 100 U/mL penicillin, and 100 μ g/mL streptomycin. After 7–10 d, the culture contains >90% B220⁺CD43⁺sIgM⁻ pro-B cells, as shown by FACS analysis.

ChIP

ChIP assay was essentially conducted according to the protocol of Upstate Biotechnology with some modifications. About 5×10^6 to 10×10^6 cultured pro-B cells from wild-type animals were used for each immunoprecipitation and rabbit anti-mouse YY1 antibody (H414, Santa Cruz Biotechnology) was used at 1:50 dilution followed by protein G bead precipitation. Control for ChIP was conducted with a twofold amount of normal rabbit IgG. Following reverse linking and proteinase K digestion, DNA was purified with phenol-chloroform extraction and ethanol precipitation. DNA samples were analyzed by real-time PCR using the Roche 480 LightCycler and LightCycler 480 SYBR Green 1 Master (Roche) with primer sets specific for different regions of the mouse IgH gene. Results are presented as fold changes enriched by anti-YY1 antibody relative to normal rabbit IgG control, calculated as $2^{-(Ct \text{ CTR} - Ct \text{ YY1})}$, where Ct is the cycle threshold. The mouse actin-B promoter region serves as negative control, while the mouse RPL30 promoter region serves as positive control. Most primer information was described previously and is available in Supplementary Table 2 (Johnson 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- μ m 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_HJ558), 243G9 (V_H15), 167C1 (V_H7183), and C34H6 (C_H) 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 μ m was defined as colocalized, 0.3–0.5 μ m was defined as "apart," and a distance of 0.5–1.5 μ m was referred to as "far apart." Calculation of *P* value was performed by applying the χ^2 test to observed and expected frequencies (Supplementary Table 1).

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

Huifei Liu, Marc Schmidt-Suppran, Yujiang Shi, et al.

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