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YY1 controls Igκ repertoire and B-cell development, and localizes with condensin on the Igκ locus

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Conditional knock-out (KO) of Polycomb Group (PcG) protein YY1 results in pro-B cell arrest and reduced immunoglobulin locus contraction needed for distal variable gene rearrangement. The mechanisms that control these crucial functions are unknown. We deleted the 25 amino-acid YY1 REPO domain necessary for YY1 PcG function, and used this mutant (YY1ΔREPO), to transduce bone marrow from YY1 conditional KO mice. While wild-type YY1 rescued B-cell development, YY1ΔREPO failed to rescue the B-cell lineage yielding reduced numbers of B lineage cells. Although the IgH rearrangement pattern was normal, there was a selective impact at the Igκ locus that showed a dramatic skewing of the expressed Igκ repertoire. We found that the REPO domain interacts with proteins from the condensin and cohesin complexes, and that YY1, EZH2 and condensin proteins co-localize at numerous sites across the Ig kappa locus. Knock-down of a condensin subunit protein or YY1 reduced rearrangement of Igκ Vκ genes suggesting a direct role for YY1-condensin complexes in Igκ locus structure and rearrangement.

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Introduction

Immunoglobulin heavy and light chain genes are produced during early antigen-independent B-cell development by a somatic rearrangement process that links together either V, D, and J segments (heavy chain), or V and J segments (light chain) to produce functional Ig genes (Fugmann *et al*, 2000; Cobb *et al*, 2006; Jung *et al*, 2006; Subrahmanyam and Sen, 2010). V(D)J recombination is regulated in the context of lineage and developmental stage specificity. Although the same trans-acting factors and indistinguishable cis-acting elements are involved in Ig heavy and light chain rearrangement events, heavy and light chain rearrangements are largely restricted to pro- and pre-B cells, respectively.

The Ig loci are huge (2.4–3.2 Mb), and for rearrangement of distal variable region genes to occur the loci must undergo a physical contraction process. While Ig_H D-J and proximal V_H to D and Vκ to Jκ rearrangements can occur without contraction, the distal V genes require locus contraction and looping for rearrangement (Kosak *et al*, 2002; Hesslein *et al*, 2003; Fuxa *et al*, 2004; Sayegh *et al*, 2005; Jhunjhunwala *et al*, 2009). However, the mechanism that controls this contraction process is unknown. Current data suggest that the Ig loci are organized as loops into rosette-like structures separated by spacer DNA (Jhunjhunwala *et al*, 2008, 2009; Lucas *et al*, 2010; Guo *et al*, 2011a). A number of domains have been identified at the IgH locus that adopt various conformations during development (Jhunjhunwala *et al*, 2008, 2009; Lucas *et al*, 2010; Guo *et al*, 2011a). At the pre-pro-B cell stage, these rosette domains are in an extended conformation, but in pro-B cells the structure changes such that each V region domain is repositioned with all V_H regions approximately equidistant to the D_H and J_H regions, thus affording roughly equal access for recombination (Jhunjhunwala *et al*, 2008; Lucas *et al*, 2010). Similar structures are believed to exist at the Igκ locus. The factors that regulate these domains are only now being identified. Pax5 binds to a repeat sequence in the distal region of the IgH locus and is believed to participate in rearrangement of distal V_H genes (Ebert *et al*, 2011). Polycomb Group (PcG) protein EZH2 knock-out (KO) results in arrest at the pro-B cell stage and impaired distal V_H to D_HJ_H rearrangement (Su *et al*, 2003). A similar phenotype results from conditional KO of YY1 in the B-cell lineage (Liu *et al*, 2007). CTCF and cohesin have been argued to regulate Ig locus structure and to control interactions of D_H and J_H regions with proximal V_H segments and Jκ regions with proximal Vκ segments (Degner *et al*, 2009, 2011; Seitan *et al*, 2011; Guo *et al*, 2011b; Feeney and Verma-Gaur, 2012).

YY1 has long been believed to play some role in immunoglobulin gene regulation and B-cell biology because it associates with multiple Ig enhancer elements including the heavy chain intron and 3′ enhancers, as well as the Igκ 3′ enhancer (Park and Atchison, 1991; Gordon *et al*, 2003). Conditional KO of YY1 in the B-cell lineage results in arrest at the pro-B cell stage, with cells exhibiting normal D_H-J_H recombination but reduced V_H-D_HJ_H recombination, with the defect being most

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severe for distal V_H genes (Liu *et al*, 2007). These YY1 KO pro-B cells also show a defect in Ig locus contraction (Liu *et al*, 2007). Despite the fact that proximal VDJ recombination occurs, very few mature B cells are generated in conditional KOs, and introduction of a rearranged heavy chain gene only partially rescues the conditional KO phenotype (Liu *et al*, 2007). This suggests that YY1 mediates additional roles in early B-cell development. Since the YY1 conditional KO arrests at the pro-B cell stage (Liu *et al*, 2007), YY1 function at the pre-B cell stage and later has never been addressed.

We previously showed that YY1 is a PcG protein, and is the only known mammalian PcG protein with DNA binding site specificity (Atchison *et al*, 2003; Srinivasan and Atchison, 2004; Srinivasan *et al*, 2005). PcG proteins comprise a family of proteins involved in haematopoietic development, epigenetic chromosomal condensation, stable transcriptional repression, control of cell proliferation, long distance interactions between DNA sequences, and stem cell self-renewal (van der Lugt *et al*, 1994; Alkema *et al*, 1995; Schumacher *et al*, 1996; Akasaka *et al*, 1997; Core *et al*, 1997; Lessard *et al*, 1999; Lessard and Sauvageau, 2003; Park *et al*, 2003; Lanzuolo *et al*, 2007). We found that YY1 can repress transcription in a PcG-dependent fashion and can recruit other PcG proteins to specific DNA sequences (Atchison *et al*, 2003; Srinivasan and Atchison, 2004). Human YY1 is the mammal homologue to *Drosophila* PcG protein Pleohomeotic (PHO) and YY1 can also correct mutant phenotypes in PHO mutant flies (Atchison *et al*, 2003). The mechanisms responsible for targeting mammalian PcG proteins to specific DNA regions have long been enigmatic because other known PcG proteins do not individually bind to specific DNA sequences, yet the PcG complexes must associate with specific DNA regions to function. Our demonstration that YY1 is a mammalian PcG protein with high affinity sequence-specific DNA binding activity suggests that YY1 is a crucial factor for targeting PcG proteins to specific DNA sequences. PcG proteins are known to contribute to B-cell biology, and the PcG protein EZH2, like YY1, is required for B-cell development (Su *et al*, 2003; Liu *et al*, 2007). Nucleation of PcG proteins to specific target DNA sites by YY1 could provide a mechanism for Ig locus contraction and Ig gene rearrangement but this connection has never been demonstrated at the Ig loci.

To study YY1 PcG function in B-cell development, we assessed the importance of the 25 amino-acid REPO domain (amino-acid residues 201–226) that we previously showed is necessary and sufficient for PcG-dependent transcriptional repression, and for recruitment of PcG proteins to DNA (Wilkinson *et al*, 2006). The YY1 REPO domain deletion mutant can mediate all other known YY1 functions such as DNA binding, transcriptional activation, transient transcriptional repression, and interaction with HDAC proteins, but fails to carry out YY1 PcG functions (Wilkinson *et al*, 2006). We used a REPO domain mutant (YY1ΔREPO) to explore the mechanism of YY1 PcG function in B-cell development. We found that the YY1ΔREPO mutant failed to rescue B-cell development in YY1 conditional KO bone marrow B cells. While the Ig heavy chain rearrangement pattern was largely normal, the expressed Ig kappa chain repertoire was severely altered suggesting that the REPO domain may have a direct role in Igκ VJ rearrangement. Interestingly, we found that the YY1 REPO domain can physically interact with condensin and cohesin complex proteins. Using computational approaches, we identified multiple YY1 binding site clusters across the Igκ locus, and found that YY1, EZH2, and condensin complex proteins SMC4, SMC2, and BRRN1 all co-localize at these sites. Knock-down of a condensin subunit protein or YY1 reduced Vκ-Jκ rearrangement to a subset of Vκ genes. Our findings provide specific molecular details to key functions that regulate B-cell development and for the first time implicate condensin complex proteins in Ig rearrangement.

Results

Conditional KO of YY1 or EZH2 in the B-cell lineage results in similar phenotypes: an arrest at the pro-B cell stage and impaired distal V_H heavy chain rearrangements (Su *et al*, 2003; Liu *et al*, 2007). Introducing a pre-rearranged Ig heavy chain into YY1 conditional KO mice only partially rescues the B-cell developmental defect, suggesting that YY1 plays roles in addition to stimulating distal V_H gene rearrangement (Liu *et al*, 2007). The similarity between YY1 and EZH2 conditional KO phenotypes suggested that PcG function might be involved in B-cell development. We had available a YY1 mutant that specifically ablates YY1 PcG function (YY1ΔREPO) while maintaining all other known YY1 functions (Wilkinson *et al*, 2006). In order to assess the importance of YY1 PcG function on B-cell development, we expressed either wild-type YY1 or YY1ΔREPO in a YY1 conditional KO background. For these studies, we transduced *yy1$^{f/f}$ mb1-CRE* bone marrow cells with retroviral vector alone (MigR1), a retrovirus expressing Flag-tagged wild-type YY1 (MigRI-FlagYY1) or a Flag-tagged YY1ΔREPO mutant (MigR1-FlagYY1ΔREPO). In this system, the endogenous *yy1* gene is deleted at the early pro-B cell stage by the action of CRE recombinase on flox sites flanking the first exon of the *yy1* gene (Liu *et al*, 2007). Thus, in this system, YY1 function past the early pro-B cell stage is completely dependent upon the exogenous YY1 constructs transduced into the cells. The MigR1 vector also allows us to track YY1 expression by monitoring green fluorescent protein (GFP) expression from a bicistronic RNA containing an internal ribosomal entry site, and level of GFP expression correlates with YY1 expression levels (Figure 1A).

The YY1 REPO domain and PcG function are needed for B-cell development

Bone marrow cells from *yy1$^{f/f}$ mb1-CRE* mice were transduced with MigR1-FlagYY1, MigR1-FlagYY1ΔREPO or MigR1 vector and transplanted into lethally irradiated C57BL/6 mice. Reconstituted mice were subject to analyses 14 weeks post injection. MigR1, MigR1-FlagYY1 and MigR1-FlagYY1ΔREPO transduced bone marrow cells yielded efficient reconstitution at 14 weeks post injection as evidenced by the high percentage of GFP-positive cells in bone marrow and spleen (Figure 1B; Supplementary Figure S1A, right panels). Bone marrow cells were examined fluorocytometrically by first gating for GFP expression, then further gated for bone marrow pro-B ($B220^+CD43^+CD19^+AA4.1^+$), pre-B ($B220^+CD43^-$ $IgM^-AA4.1^+$), immature B ($B220^+$ $CD43^-IgM^+AA4.1^+$) and mature B cells ($B220^+CD43^-$ $IgM^+AA4.1^-$) (Figure 1B, panels 1–3). Total numbers of pro-B, pre-B, immature B, and mature B cells in each fraction

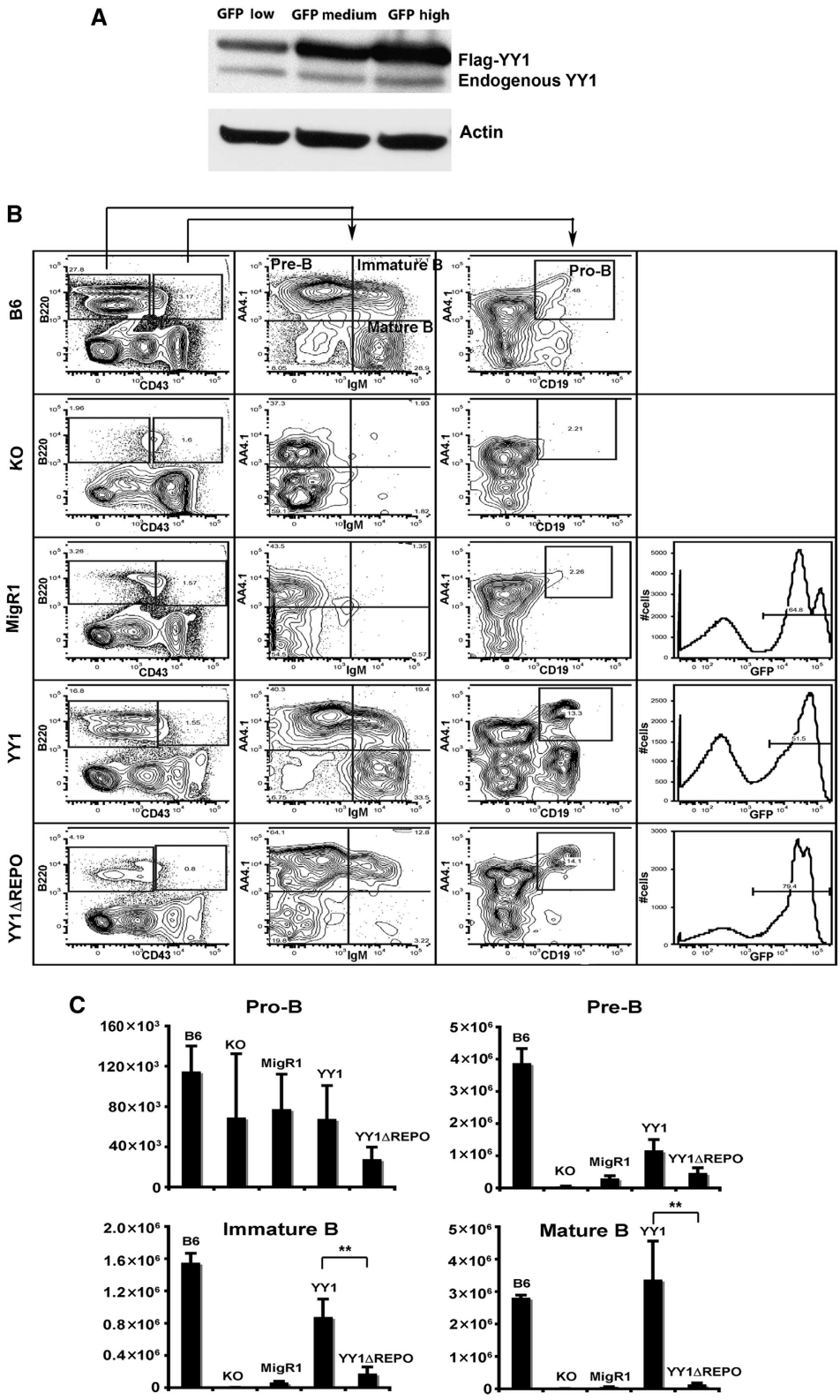

Figure 1 The YY1 REPO domain is needed for bone marrow B-cell development. (**A**) GFP levels correlate with YY1 expression. 38B9 cells were transduced with MigR1-FlagYY1 and 4 days later cells were sorted into low, middle, and high GFP-expressing populations. Cell lysates were evaluated by western blot with anti-YY1 antibody, and blots showed that YY1 expression correlates with the level of GFP expression. (**B**, **C**) MigR1, MigR1-FlagYY1, and MigR1-FlagYY1ΔREPO transduced *yy1*$^{f/f}$ *mb1CRE* bone marrow was injected into recipient irradiated C57BL/6 mice and bone marrow from reconstituted mice was subject to analyses at 14 weeks post transplantation. (**B**) FACS analyses of bone marrow B cells. Within the GFP$^+$ cell population, pro-B cells were characterized as B220$^+$CD43$^+$CD19$^+$AA4.1$^+$, pre-B cells as B220$^+$CD43$^-$ IgM$^-$AA4.1$^+$, immature B cells as B220$^+$CD43$^-$IgM$^+$AA4.1$^+$, and mature B cells as B220$^+$CD43$^-$IgM$^+$AA4.1$^-$. (**C**) YY1ΔREPO reconstituted mice show decreased pro-B, pre-B, immature B, and mature B sub-populations. Total cell numbers in each bone marrow B-cell sub-population are shown. Mean and standard error of the mean of 4 experiments with 10 mice per cohort are shown. Double asterisks indicate significant differences between YY1 and YY1ΔREPO reconstituted mice at $P<0.01$. Source data for this figure is available on the online supplementary information page.

are presented in Figure 1C. Compared with C57BL/6 wild-type mice, *yy1*$^{f/f}$ *mb1-CRE* KO mice had very few pre-B, immature B, and mature B cells (Figures 1B and C) as described previously (Liu *et al*, 2007). MigR1 vector control reconstituted mice shared a similar phenotype as *yy1*$^{f/f}$ *mb1-CRE* mice with very few pre-B, immature B, and mature B cells (Figures 1B and C). However, full-length YY1 was able to effectively rescue the B-cell developmental defect of *yy1*$^{f/f}$ *mb1-CRE* mice (Figure 1B) with increased numbers of pre-B, immature B, and mature B cells compared with *yy1*$^{f/f}$ *mb1-CRE* mice, although numbers of pre-B cells were still lower than wild type (Figure 1C). Conversely, YY1ΔREPO reconstituted mice were not able to rescue the *yy1*$^{f/f}$ *mb1-CRE* conditional mutant phenotype and showed reduced cell numbers of pro-B, pre-B, immature B, and mature B populations (Figures 1B and C). The drops in immature and mature B cells were statistically significant, and the reduction in pro-B and pre-B cells was reproducible. The drop in pro-B cells with the YY1ΔREPO mutant compared to MigR1 vector alone suggests a possible dominant-negative effect of this protein. Alternatively, the YY1ΔREPO protein may reduce cell growth or increase apoptosis. We previously observed that YY1 overexpression can reduce B-cell growth and can induce apoptosis (Pan *et al*, 2012). However, we observed little difference in growth properties or apoptosis of pro-B or pre-B cell lines transduced with either wild-type YY1 or YY1ΔREPO (Supplementary Figures S2A and B). Together, our results indicate that the YY1 REPO domain is crucial for normal B-cell development.

We also evaluated mature B-cell phenotypes in reconstituted spleen. Fourteen weeks post bone marrow reconstitution, splenic GFP-positive cells were evaluated fluorocytometrically for B2 ($CD19^+CD43^-$), mature B ($CD19^+CD43^-B220^+AA4.1^-$), transitional B ($CD19^+CD43^-B220^+AA4.1^+$), follicular B ($CD19^+CD43^-B220^+AA4.1^-CD23^{hi}CD21/35^+$), marginal zone B ($CD19^+CD43^-B220^+AA4.1^-CD23^{low}CD21/35^{high}$), T1 ($CD19^+CD43^-B220^+AA4.1^+IgM^+CD23^-$), T2 ($CD19^+CD43^-B220^+AA4.1^+IgM^+CD23^+$), T3 ($CD19^+CD43^-B220^+AA4.1^+IgM^-CD23^-$), B1 ($CD19^+CD43^+$), B1a ($CD19^+CD43^+CD5^+$), and B1b ($CD19^+CD43^+CD5^-$) cells (Supplementary Figure S1A). Total cell numbers of each cell type are shown in Supplementary Figure S1B. Consistent with our bone marrow phenotype (Figure 1), full-length YY1 reconstituted mice showed a dramatic rescue effect in each splenic B-cell population with complete restoration of mature B, follicular B, marginal zone B, and B1 populations. In contrast, YY1ΔREPO transduced *yy1*$^{f/f}$ *mb1-CRE* cells were not able to rescue B-cell developmental defects caused by deletion of the endogenous *yy1* gene yielding significantly reduced cell numbers in each B-cell population (Supplementary Figure S1B). Thus, the YY1 REPO domain is crucial for B lymphocyte development both in bone marrow and in spleen. As the YY1ΔREPO mutation specifically ablates YY1 PcG function but retains all other known YY1 functions (Wilkinson *et al*, 2006), our experiments demonstrate that YY1 PcG function is needed for normal B-cell development.

To assess YY1ΔREPO expression *in vivo*, we isolated bone marrow GFP-positive pre-B cells ($B220^+CD43^-IgM^-AA4.1^+$) by FACS from MigR1-FlagYY1 and MigR1-FlagYY1ΔREPO reconstituted mice and compared YY1 transcripts to the levels in wild-type C57BL/6 mice. As the endogenous *yy1* gene is efficiently deleted at the early pro-B cell stage in our reconstituted mice (Liu *et al*, 2007), we were able to use quantitative real-time PCR experiments to compare exogenous Flag-YY1 and Flag-YY1ΔREPO expression with YY1 levels in wild-type mice. We found that exogenous FlagYY1 or FlagYY1ΔREPO transcripts were expressed 2.5- to 2-fold higher compared to endogenous YY1 (Supplementary Figure S3A). To demonstrate that the YY1ΔREPO protein was expressed, we isolated GFP^+ lymphoid cells (mixed B and T cells) by FACS from the blood of MigR1-FlagYY1ΔREPO reconstituted mice, and found the exogenous Flag-tagged YY1ΔREPO protein was expressed 1.4-fold higher compared with endogenous YY1 (Supplementary Figure S3B). Thus, the FlagYY1ΔREPO protein is well expressed in our reconstituted mice.

Deletion of the YY1 REPO domain alters Igκ, but not IgH, rearrangement

YY1 is a critical regulator of distal heavy chain rearrangement as conditional deletion of YY1 in mouse early pro-B cells causes a defect in distal V_H to D_HJ_H recombination and a blockage at the pro-B cell stage (Liu *et al*, 2007). Reduced B-cell development in our YY1ΔREPO reconstituted mice suggested that YY1 might also impact Igκ gene rearrangement. Therefore, we evaluated IgH and Igκ rearrangement in YY1 and YY1ΔREPO reconstituted chimeric mice. GFP^+ $B220^+CD19^+$ $CD3^-$ B cells were isolated from MigR1, YY1, and YY1ΔREPO reconstituted mice, and genomic DNA from these sorted cells was subjected to PCR with primers that detect V_H rearrangement, with amplification of the Cκ region serving as a loading control (Fuxa *et al*, 2004). Both YY1 and YY1ΔREPO reconstituted mice showed normal D_H to J_H recombination patterns (Figure 2A). Similarly, recombination patterns of the most proximal V_H families V_H7183 andV_HQ52 were equal in YY1 and YY1ΔREPO mice. Rearrangement of the distal V_H558 families was only slightly lower in YY1ΔREPO reconstituted mice compared to wild-type YY1. Therefore, the YY1 REPO domain and PcG function appear to be largely dispensable for heavy chain rearrangements. However, a distinct result was obtained when assaying Vκ-Jκ rearrangement in pre-B cells.

Preliminary Igκ Vκ gene rearrangement assays suggested that the Vκ repertoire in YY1 and YY1ΔREPO reconstituted mice differed greatly. YY1 reconstituted pre-B cells generated a Vκ repertoire that appeared to utilize a large number of Vκ genes, whereas the YY1ΔREPO repertoire appeared highly skewed. To gain a more full appreciation of the Vκ repertoires in our reconstituted mice, we isolated pre-B cells from YY1 and YY1ΔREPO reconstituted mice and amplified all Igκ rearranged transcripts and performed transcriptome sequencing. The percentage of specific Vκ transcripts relative to all Vκ rearrangements in each sample was plotted (Figures 2B–D). The representation of the various Vκ rearrangements in YY1 reconstituted mice spanned the entire Igκ locus with numerous Vκ genes utilized. Although Vκ gene usage spanned the entire Vκ locus similar to wild-type C57BL/6 mice, the precise Vκ usage was not identical to that observed in C57BL/6 mice, perhaps due to retroviral YY1 expression in the bone marrow transplants which may not completely recapitulate normal endogenous YY1 expression (Figures 2B and C). Nonetheless, the patterns were similar in that many Vκ genes were rearranged that spanned the full

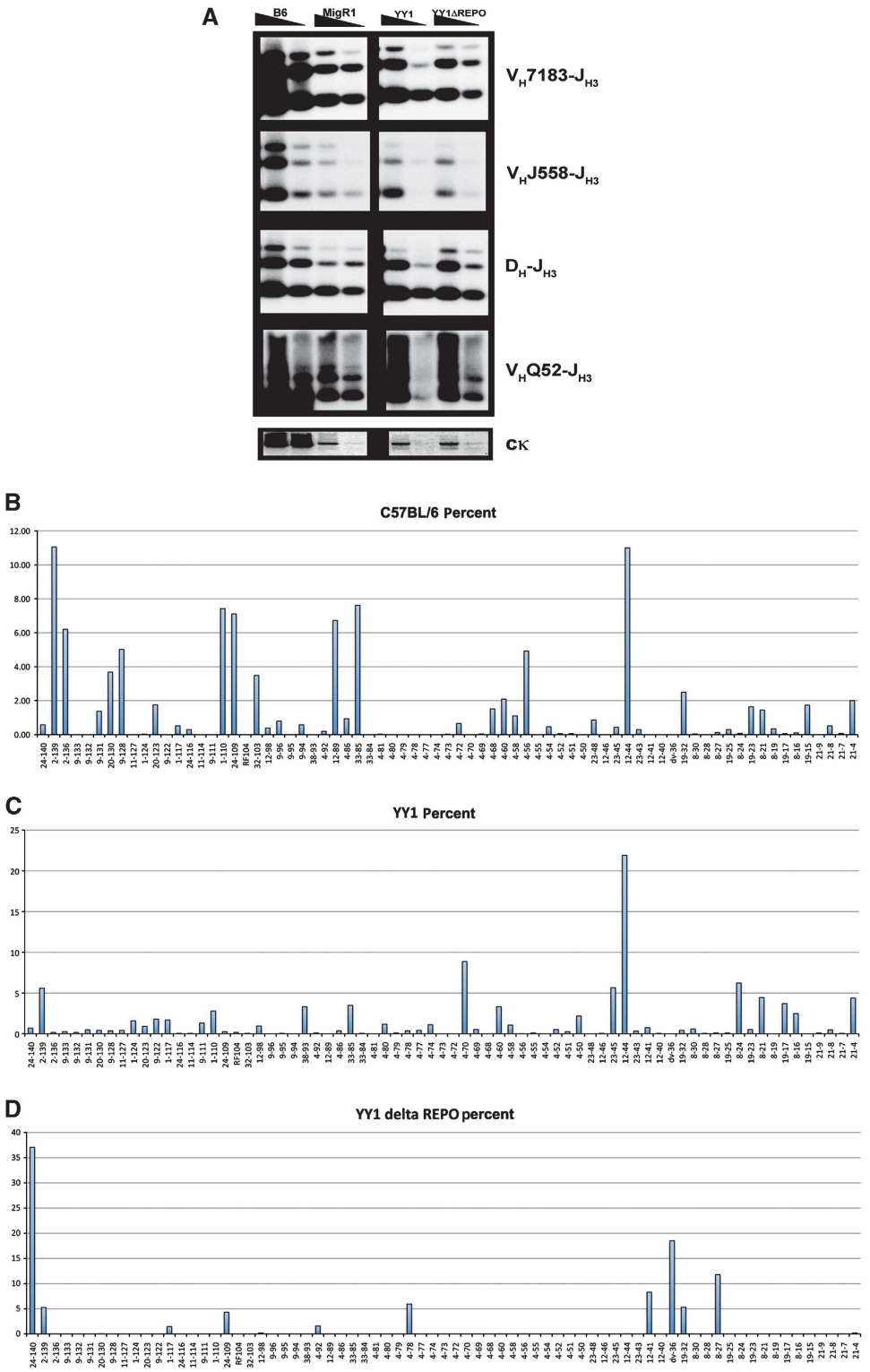

Figure 2 Deletion of the YY1 REPO domain results in skewed Igκ, but not Ig heavy chain, rearrangement patterns. (**A**) IgH rearrangement in GFP^+ splenic B cells from wild-type C57BL/6 and MigR1, YY1, and YY1ΔREPO reconstituted mice. PCR with V family-specific degenerate primers was performed on two-fold serially diluted DNA samples. Results are representative of three experiments. (**B–D**) Deletion of the YY1 REPO domain results in altered Igκ V gene rearrangement pattern. RNA isolated from pre-B cells from C57BL/6 (**B**), YY1 (**C**), and YY1ΔREPO (**D**) mice was subjected to Vκ transcritome sequence analysis. The x axis shows the individual Vκ genes from the most distal (left) to most proximal (right). Percentage of total transcripts representative of each listed Vκ gene is shown on the y axis. The analysis of YY1 mice shows a full representation of numerous Vκ genes that span the Igκ locus, whereas the YY1ΔREPO mice show a dramatically skewed repertoire with reduced Vκ gene usage. Source data for this figure is available on the online supplementary information page.

breadth of the Igκ locus. Forty-five different Vκ genes were observed in wild-type mice (from 17 613 sequences) whereas the YY1 reconstituted mice showed rearrangement to 55 different Vκ genes (from 16 358 sequences). In striking contrast, the YY1ΔREPO samples showed a highly restricted repertoire. Only 15 different Vκ genes were observed (from 54 170 sequences) with over 40% of rearrangements to the two most distal Vκ genes (Figure 2D). Other rearrangements were observed in several regions, the most significant cluster being within the Vκ12-41 to Vκ8-27 region, regions actually underrepresented in wild-type and YY1 reconstituted mice.

To determine if this skewed repertoire was reproducible, we assayed for Vκ rearrangements in multiple individual mice with selected Vκ primers representing genes either similarly or differentially rearranged in YY1 versus YY1ΔREPO mice. Consistent with our transcriptome analysis, Vκ38-93, Vκ12-44, and Vκ21-4 rearrangements (Brekke and Garrard, 2004) were easily detected in wild-type YY1, but not in YY1ΔREPO reconstituted mice (Figure 3A). Again, consistent with our transcriptome analysis, Vκ2-139 was observed at similar frequencies in YY1 and YY1ΔREPO mice (Figure 3B). Similarly, most YY1ΔREPO mice showed greatly reduced rearrangements to Vκ9-122, Vκ4-52, Vκ19-15, and Vκ21-5 compared to wild-type YY1 mice (Figure 3B). Therefore, the skewed repertoire in YY1ΔREPO mice showed considerable consistency in individual mice.

Igκ Vκ genes can rearrange either by deletional recombination or by inversion (Lewis *et al*, 1982; Van Ness *et al*, 1982; Nemazee and Weigert, 2000). We found both types of rearrangement occurring in YY1ΔREPO mice. The Igκ locus also frequently undergoes receptor editing in which an upstream Vκ gene recombines to a downstream Jκ sequence to delete a previously rearranged autoreactive Vκ gene (Nemazee and Weigert, 2000; Segal and Melamed, 2002; Edry and Melamed, 2004; Schlissel, 2004; Verkoczy *et al*, 2004). Such rearrangements are biased towards downstream Jκ sequences (Nemazee and Weigert, 2000; Segal and Melamed, 2002; Edry and Melamed, 2004; Schlissel, 2004; Verkoczy *et al*, 2004). However, we saw no evidence of increased Jκ5 usage, and in fact, observed elevated Jκ1 and Jκ2 usage, and reduced Jκ5 usage (Figure 3C), suggesting that receptor editing is reduced in YY1ΔREPO mice. In all, 60% of YY1ΔREPO Vκ rearrangements used 2 or more J genes compared to 62% of YY1 mice. Our results indicate an important requirement for YY1 PcG function in regulating Igκ V gene repertoire.

The YY1 REPO domain physically interacts with condensin and cohesin proteins

To mechanistically study YY1 REPO domain function, we searched for REPO domain-interacting proteins. We transfected 293 cells with GALREPO or GAL alone expression plasmids, and nuclear extracts were incubated with anti-GAL antibody beads and bound proteins were fractionated by SDS–PAGE (Supplementary Figure S4). Bands specific for GALREPO binding were excised from the gel and their identities were determined by MALDI-TOF mass spec analyses. These studies showed that the YY1 REPO domain physically interacted with a number of proteins including structural maintenance of chromosome proteins 1 and 4 (SMC1 and SMC4) (Supplementary Table SI). The locations of specific SMC1 and SMC4 peptides identified are shown in Figure 4A.

REPO domain interaction with SMC1 and SMC4 was particularly intriguing. SMC1 and SMC4 are components of the cohesin and condensin complexes, respectively, that are involved in mitotic chromosome contraction and higher order chromosome organization and dynamics (Lehmann, 2005; Losada and Hirano, 2005). Condensin and cohesin complex proteins are also implicated in long-range promoter–enhancer interactions and mutations can lead to developmental defects (Losada and Hirano, 2005). Roles in transcriptional repression have also been noted (Lupo *et al*, 2001; Dej *et al*, 2004; Machin *et al*, 2004) and some condensin proteins are believed to interact with epigenetic machinery (Geiman *et al*, 2004). In addition to mitotic contraction and gene regulation functions, cohesin complex proteins have been indicated to play important roles in lymphocyte development (Gosling *et al*, 2007; Degner *et al*, 2009, 2011; Seitan *et al*, 2011; Feeney and Verma-Gaur, 2012). YY1 may thus regulate B-cell development by interacting with cohesin and condensin complex proteins.

To confirm the physical interaction of YY1 and condensin proteins, we performed co-immunoprecipitation experiments. We transduced MigR1 vector or MigR1-FlagYY1 into 38B9 pro-B cells and immunoprecipitated extracts with Flag antibody. Western blot with antibody for condensin subunit BRRN1 showed co-immunoprecipitation with Flag-YY1, but not with control vector (Figure 4B). To confirm association of endogenous proteins, lysates from bone marrow IL-7 cultures (90% $B220^+CD43^+IgM^-$) were immunoprecipitated with anti-YY1 antibody and precipitates were immunoblotted for BRRN1, SMC4, EZH2, and SuZ12 (the latter two are PcG proteins). We found that YY1 interacted with all proteins (Figure 4C, top four panels), but not with lamin B1 or β-actin (lowermost panels). Lysates from primary pro-B cells isolated from $rag^{-/-}$ mice immunoprecipitated with anti-SMC4 showed co-immunoprecipitation with YY1 (Figure 4C, fifth panel), but not with lamin B1 or β-actin (lowermost panels).

Identification of YY1 binding sites across the Igκ locus

Based on our results showing that the YY1 REPO domain is important for B-cell development and is needed for rearrangement of many Igκ genes, and further physically interacts with condensin proteins, we reasoned that YY1 might bind to sites across the Igκ locus and serve to nucleate the binding of PcG and condensin complexes, both of which can mediate long-distance DNA interactions (Lanzuolo *et al*, 2007; Haeusler *et al*, 2008). As the YY1 binding site sequence is degenerate, we used a consensus binding site based on YY1 binding site selection assays (GCCATNTT) (Hyde-DeRuyscher *et al*, 1995). Based on this YY1 binding consensus matrix, and using the position-specific propensity model (PSPM) (Wang and Hannenhalli, 2006) we scanned the entire mouse Igκ locus by a sliding-window approach with 1 bp per step. We recorded all hits with window scores greater than the threshold scores (P-value < 0.001), and further narrowed the search by only considering modules that contained at least two YY1 binding sites that were conserved between human and mouse. Binding sites with a conservation P-value of < 0.05 were retained. These YY1 binding site clusters were

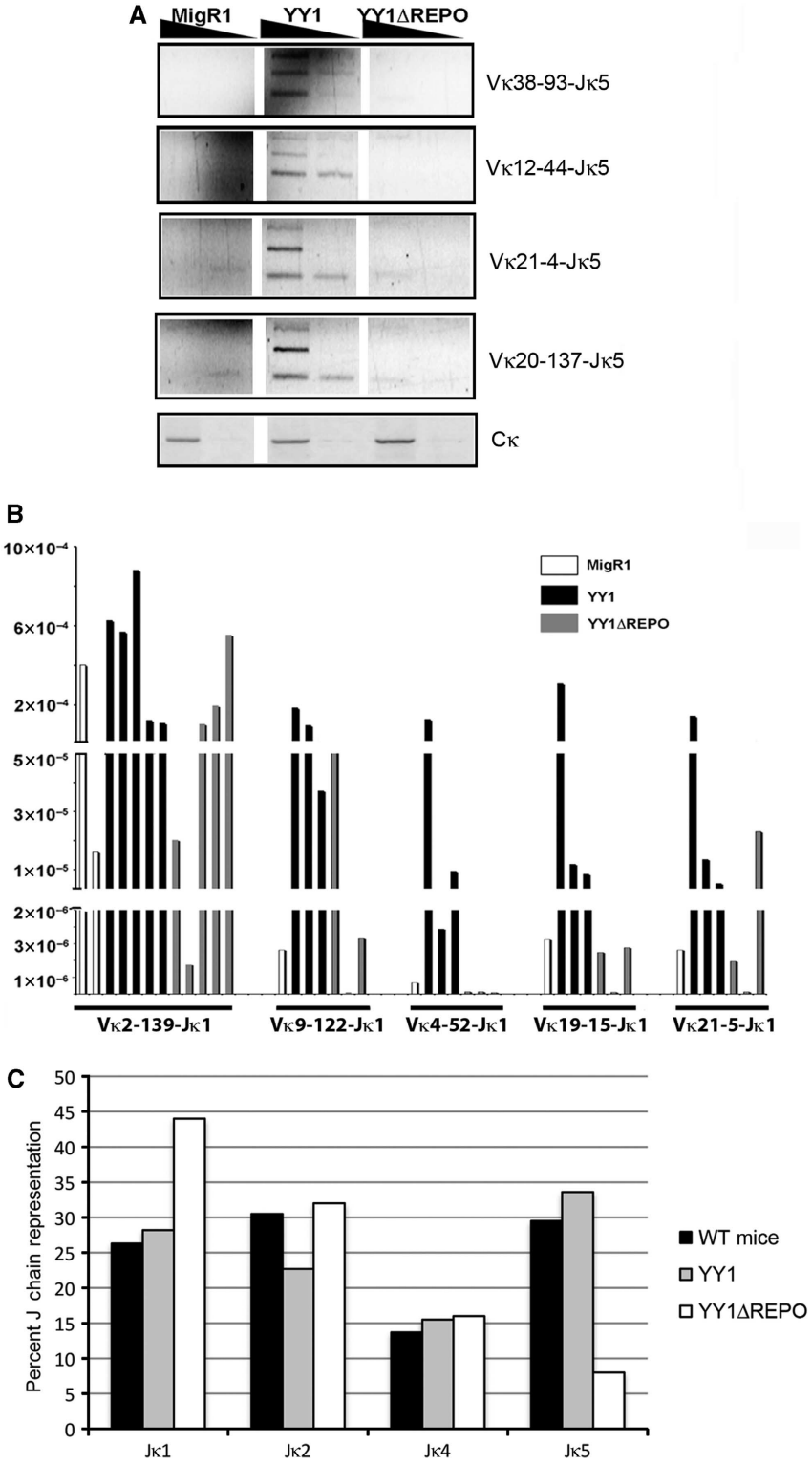

Figure 3 The skewed Vκ repertoire in YY1ΔREPO mice is reproducible. (**A**) PCR detection of specific Igκ chain rearrangements using DNA from sorted GFP$^+$ splenic B cells from MigR1, YY1, and YY1ΔREPO reconstituted mice. Input DNA was normalized by PCR amplification with primers flanking the Cκ region. Two-fold serially diluted DNA samples were used for PCRs. (**B**) Real-time PCR was performed to detect specific Igκ chain rearrangements in sorted GFP$^+$ splenic B cells from MigR1, YY1, and YY1ΔREPO reconstituted mice. GAPDH was used as an internal control for normalization and quantitation was determined by the ΔΔCT method. Each bar represents one mouse. (**C**) Jκ chain gene usage in YY1ΔREPO mice favours Jκ1 and Jκ2, whereas Jκ5 usage is reduced. The percentage of each Jκ segment used in C57BL/6, YY1, and YY1ΔREPO mice is shown.

compared to the position of Pax5 binding sites identified by a similar computational approach because Pax5 is implicated in regulating V gene rearrangement (Zhang *et al*, 2006). This approach enabled us to identify 21 potential YY1 binding modules across the Igκ locus (summarized in Figure 6; see below).

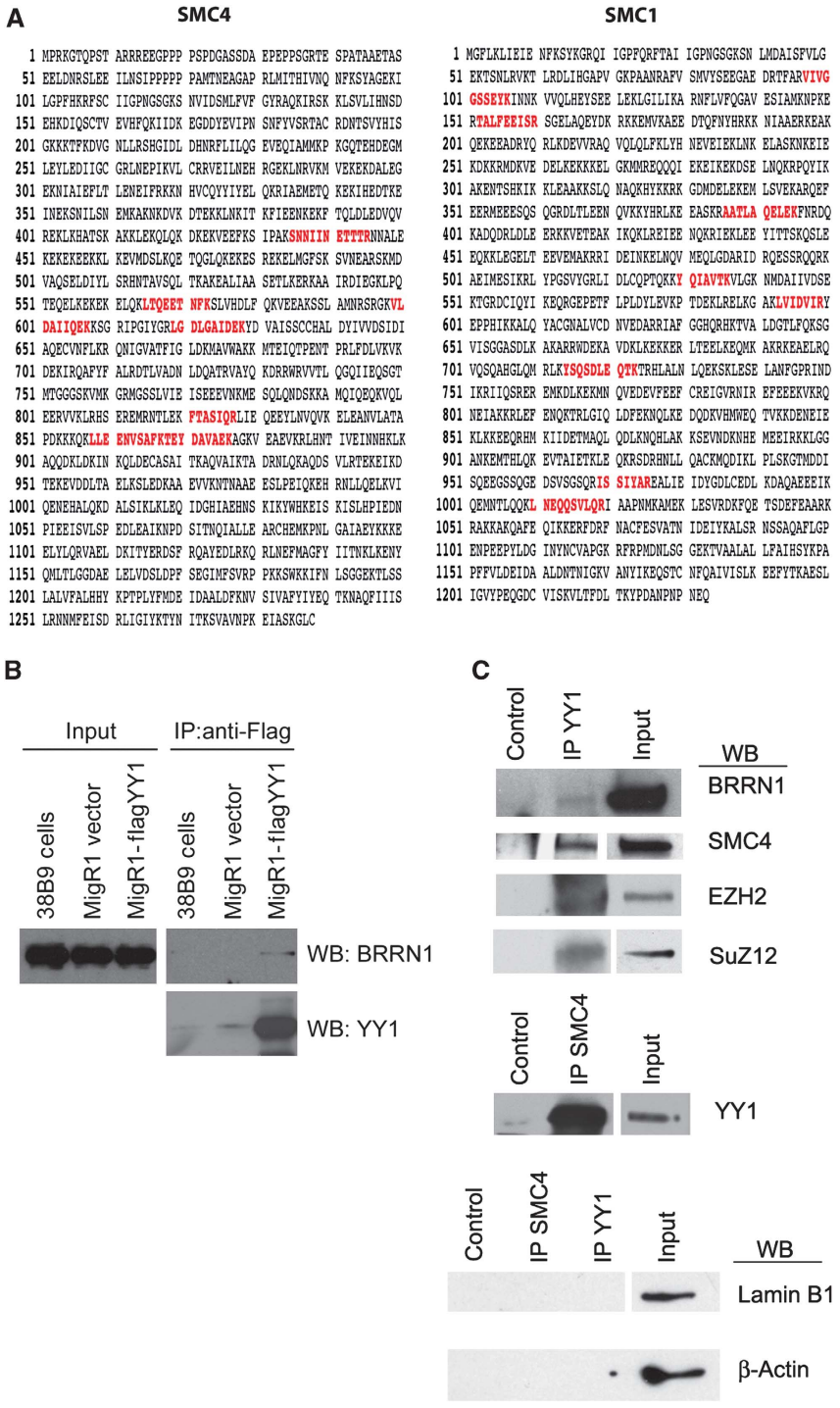

Figure 4 The YY1 REPO domain physically interacts with SMC4 and SMC1. (**A**) Sequences in red represent peptides identified by MALDI-TOF mass spec analysis of proteins that physically interact with the YY1 REPO domain in co-immunoprecipitation experiments. (**B**) YY1 interacts with condensin complex protein BRRN1. Retroviral vector or MigR1-FlagYY1 was transduced into 38B9 cells. Lysates were immunoprecipitated with Flag antibody and subjected to western with anti-BRRN1 or anti-YY1 antibodies. (**C**) YY1 interacts with condensin and PcG proteins. IL-7 cultured B-cell lysates were immunoprecipitated with YY1 antibody or rabbit Ig control. Precipitants then were blotted for BRRN1, SMC4, EZH2, SuZ12, Lamin B1, or β-actin using specific antibodies. Alternatively, lysates from purified primary pro-B cells were precipitated with anti-SMC4 followed by western blot for YY1, Lamin B1, or β-actin with specific antibodies.

YY1, PcG, and condensin proteins co-localize at sites across the Igκ locus

To confirm that the predicted YY1 binding sites identified above truly bind to YY1, chromatin immunoprecipitation (ChIP) assays were performed using IL-7 cultured bone marrow B cells. Chromatin was immunoprecipitated with YY1 antibody and bound DNA was amplified by either traditional PCR or real-time quantitative PCRs with primer sets flanking the 21 identified regions across the Igκ locus. YY1 bound to all of the predicted binding sites (representative data

are shown in Figures 5A and D). Similarly, ChIP assays showed that PcG protein EZH2 was associated with the majority of the YY1 binding sites described above (representative data shown in Figure 5A). We also performed ChIP assays to determine whether condensin proteins SMC4, SMC2, or BRRN1 bound to the same DNA sites. Indeed, we detected strong binding of SMC4 at all of the YY1 binding sites using chromatin from IL-7 cultured bone marrow cells, but no binding using chromatin from NIH3T3 fibroblasts (Figures 5B and D). Similarly, condensin subunit proteins SMC2 and BRRN1 co-localized at the same sites (Figures 5C and D). While YY1 bound to the housekeeping RP-L30 promoter in IL-7 cultured B cells, there was no co-localization of SMC4 or BRRN at this site (Figure 5D). Thus, YY1, PcG protein EZH2, and condensin complex proteins SMC4, SMC2, and BRRN1 all co-localize to the same DNA sites within the Igκ locus.

We selected five Igκ region sequences that failed to bind to YY1 and explored binding by EZH2 and SMC4 (Figure 5E). In four out of five cases, there was no binding to SMC4 (Figure 5E, lanes 8, 13, 18, and 23). In one case, we detected very weak binding (Figure 5E, lane 28). Similarly, in most cases EZH2 failed to bind at these sites (Figure 5E, lanes 12, 17, and 27). One site bound EZH2 fairly strongly in the absence of YY1 (lane 7) and another bound very weakly (lane 22). In general, there was a strong correspondence of YY1, EZH2, and condensin binding sites. All these data are summarized in Figure 6.

Knock-down of SMC4 or YY1 interferes with Vκ-Jκ rearrangement

If condensin complex proteins are involved in Igκ gene rearrangement, then knock-down of condensin subunits should reduce Vκ-Jκ recombination. We treated IL-7 bone

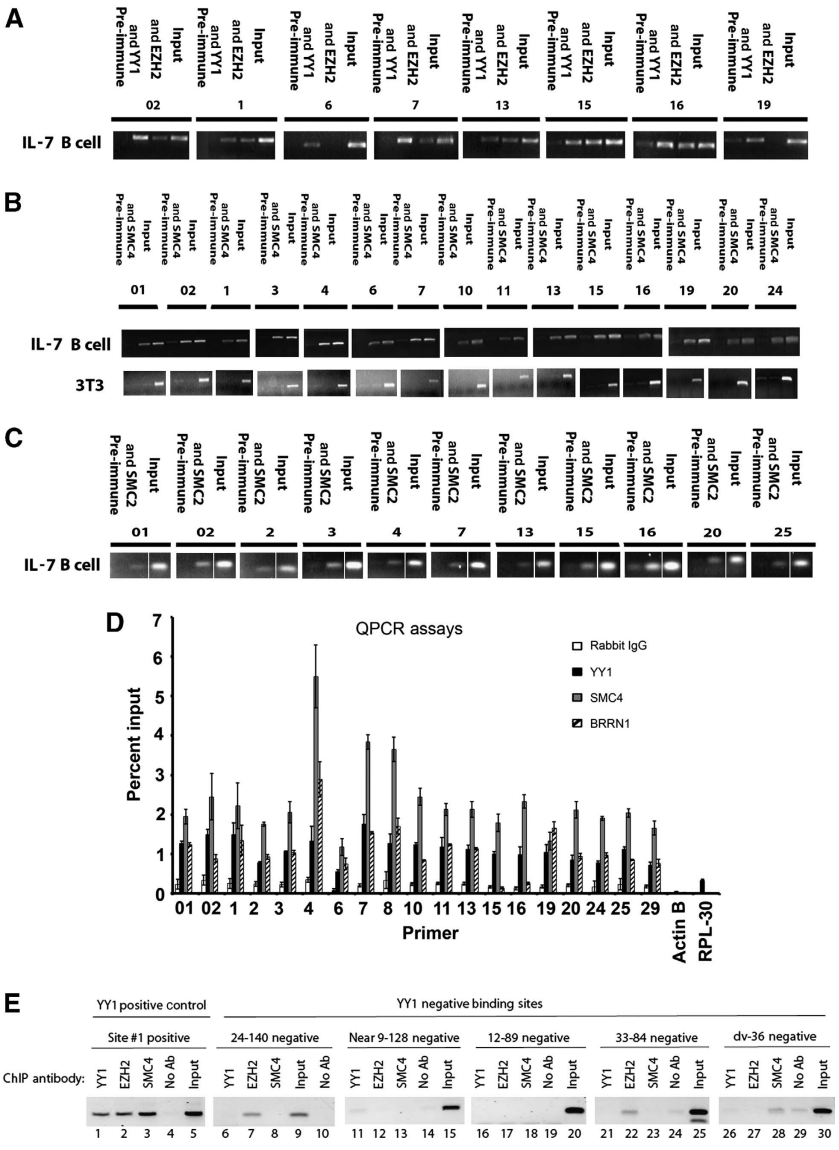

Figure 5 YY1, EZH2, and condensin proteins co-localize across the Vκ locus. (**A**–**D**) Representative ChIP analyses from IL-7 bone marrow cultures or NIH3T3 cells at YY1 binding sites identified computationally across the Igκ locus for (**A**) YY1 and EZH2, (**B**), SMC4, (**C**) SMC2, (**D**) YY1, SMC4, and BRRN1. Data in (**D**) are real-time PCR data presented as percent input. The RP-L30 promoter was used as a positive control for YY1 binding, and actin B was used as a negative control for YY1 binding. The mean and standard error of the mean are shown. (**E**) ChIP analysis of YY1-negative regions within the Igκ locus showing general loss of EZH2 and SMC4. Primers and antibodies are shown above each lane. Source data for this figure is available on the online supplementary information page.

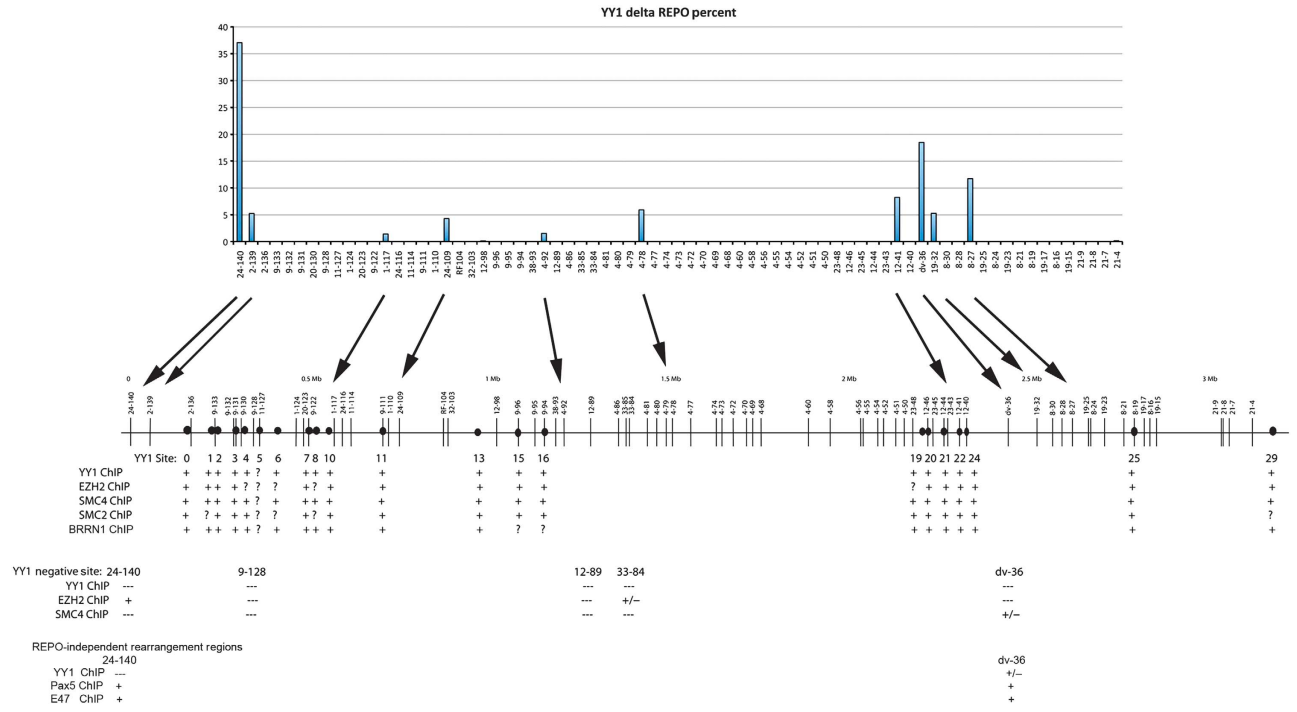

Figure 6 Location of YY1 binding site clusters within the Igκ locus. Summary of YY1, EZH2, SMC4, SMC2, and BRRN1 (CAP-H) ChIP data. The top panel shows the Vκ rearrangement data for YY1ΔREPO mice. The bottom panel shows the Igκ locus drawn to scale with each Vκ gene represented as a vertical line. YY1, EZH2, and condensin binding sites are shown as black circles on the map. The Vκ genes that continue to rearrange in the YY1ΔREPO background (top panel) are matched to their relative locations on the locus map by arrows. Binding of specific proteins are listed below the map for each site. The YY1-negative region data are summarized in the penultimate lowest panel of the figure. Binding of Pax5 and E47 at the Vκ24-140 and dv-36 regions is summarized in the lowest panel. Presence of YY1, EZH2, SMC4, SMC2, BRRN1, Pax5, or E47 at each site is indicated with a '+'. Question marks indicate inconclusive DNA binding.

marrow cultures with RNAi oligonucleotides against condensin subunit SMC4 or with scrambled RNAi oligonucleotides, and observed a 90% knock-down of SMC4 (Figure 7A). Reduction in IL-7 from 5 to 0.1 ng/ml in these cultures enables observation of Igκ rearrangement. We tested for rearrangement of Vκ38-93 and Vκ12-44 (which fail to rearrange in a YY1ΔREPO background) to Jκ2 and Jκ5 by PCR with V- and J-specific primers. DNA from control splenic B cells yielded the expected PCR products indicative of Vκ-Jκ rearrangement (Figure 7B, lanes 1 and 2). As expected, little rearrangement was observed in bone marrow cultures grown in 5 ng/ml IL-7 (Figure 7B, lanes 3 and 4). However, withdrawal of IL-7 to 0.1 ng/ml revealed Vκ-Jκ rearrangement in the control scrambled RNAi samples (Figure 7B, lanes 5 and 6). This rearrangement was greatly impaired at Vκ38-93 in the SMC4 knock-down samples (Figure 7B, lanes 7 and 8). On the contrary, rearrangement of the Vκ12-44 gene was unaffected by SMC4 knock-down (Figure 7B, lanes 5–8). Similarly, YY1 knock-down resulted in reduced rearrangement of the Vκ38-93, Vκ21-4, and Vκ2-139 genes but had little impact on Vκ8-27 gene rearrangement. In summary, our condensin and YY1 knock-down studies indicate that YY1 and condensin complex proteins function in Vκ-Jκ rearrangement.

Potential role of other transcription factors in controlling Ig κrearrangements

Two regions of the Vκ locus continue to rearrange in the YY1ΔREPO background. These regions include the areas around Vκ genes 24-140 and dv-36. We hypothesized that other transcription factors implicated in Ig rearrangements such as Pax5 and E47 (Schlissel *et al*, 1991; Choi *et al*, 1996; Bain *et al*, 1999; Romanow *et al*, 2000; Ebert *et al*, 2011) might bind to these regions to mediate structures needed for Vκ-Jκ rearrangement. We assayed for binding of these transcription factors to the Vκ24-140 and dv-36 regions by ChIP and found strong binding by both factors to these regions but no binding to a DNA segment from the NFκB2 gene (Figure 8A). Binding of Pax5 and E47 to these regions is summarized in Figure 6, lowest panel.

We propose that YY1 mediates formation of multiple loops within the Igκ locus forming structures conducive for rearrangement of many Vκ genes. When YY1 PcG function is ablated, as with the YY1ΔREPO mutant, these loops do not form and instead only loops may held other transcription factors, such as Pax5 and E47, remain to generate a more limited Vκ repertoire (see model in Figure 8B).

Discussion

We found that the YY1 REPO domain, which mediates YY1 PcG function, is critical for B-cell development. Our rearrangement assays indicated that the heavy chain rearrangement pattern was largely normal, but that the Igκ gene rearrangement pattern was severely altered. The highly skewed Vκ repertoire in YY1ΔREPO mice could be caused by either a direct effect on Igκ locus structure needed for normal rearrangement, or could be the result of a developmental block prior to Igκ rearrangement and rare cells that bypass this block yield an atypical repertoire. Though these scenarios are

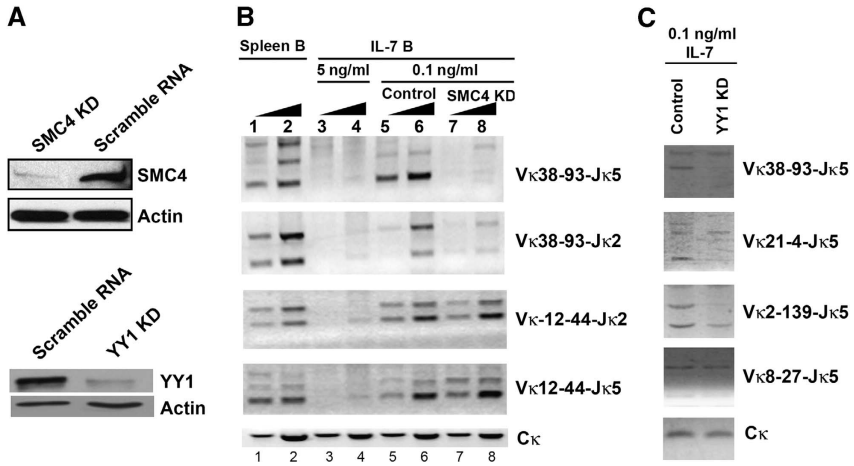

Figure 7 Condensin subunit or YY1 knock-down inhibits Igκ rearrangement of some Igκ V genes. (**A**) SMC4 and YY1 knock-down by siRNA in IL-7 cultured B cells. Cell lysates from SMC4 or YY1 knock-down, or scrambled RNA, were subjected to western blot for SMC4 or YY1. β-Actin was used as a loading control. (**B**) Knock-down of SMC4 impairs recombination of Vκ38-93 to Jκ2 and Jκ5 gene segments. IL-7 cultured B cells electroporated with either SMC4 siRNA or scrambled siRNA were treated with either 5 or 0.1 ng/ml IL-7 right after the electroporation and incubated 72 h. Three-fold serially diluted DNA samples were used for PCRs to detect rearrangement of Vκ38-93 or Vκ12-44 regions to downstream Jκ2 or Jκ5 regions. PCR of the Cκ region was used as an internal control. (**C**) Knock-down of YY1 results in decreased recombination of Vκ38–93, Vκ21-4, and Vκ2-139, respectively. IL-7 cultured B cells were electroporated with YY1 siRNA or scrambled siRNA and the IL-7 concentration was lowered from 5 to 0.1 ng/ml right after the electroporation. After 72 h, DNA samples were used for PCRs for rearrangement of Vκ38-93, Vκ21-4, Vκ2-139, or Vκ8-27. PCR of the Cκ region was used as an internal control.

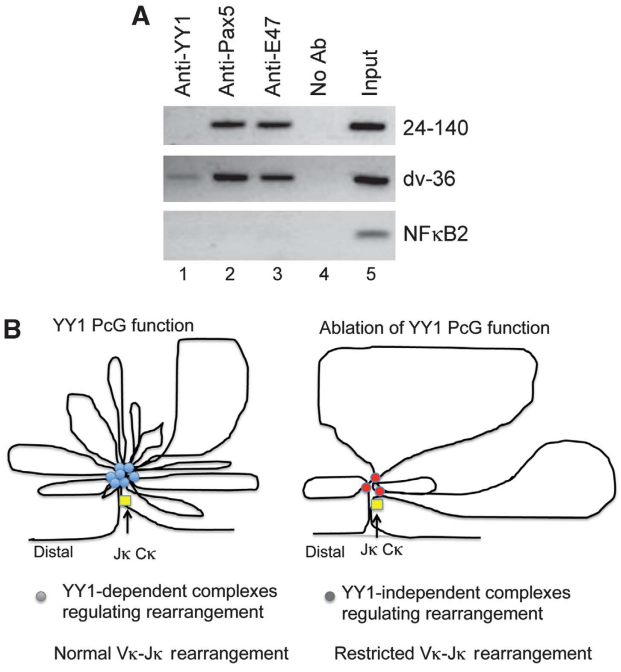

Figure 8 Model for YY1 PcG function and Igκ locus structure. (**A**) Pax5 and E47 bind to the Vκ24-140 and dv-36 regions. Chromatin from IL-7 cultured bone marrow cells was subjected to ChIP with the antibodies shown above the lanes and the primers shown on the right. (**B**) Model of Igκ locus loop structure in the presence and absence of YY1 PcG function. Source data for this figure is available on the online supplementary information page.

not mutually exclusive, we favour the hypothesis that YY1 function has a direct effect on Igκ locus structure for the following reasons. First, the Vκ repertoire in individual YY1ΔREPO mice showed considerable constancy with the same Vκ genes showing rearrangement and the same Vκ genes lacking rearrangement. This selective effect of the REPO domain deletion impacting rearrangement of many but not all Vκ genes is most consistent with a direct effect on some aspect of a subset of Vκ genes (such as locus structure) rather than a general effect on Vκ-Jκ rearrangement or a developmental block prior to Igκ rearrangement. Second, 60% of Vκ genes that did rearrange in YY1ΔREPO mice used two or more Jκ segments similar to wild-type mice and YY1 reconstituted mice (69 and 62%, respectively). Rare cells escaping a developmental block would more likely show a clonal phenotype with rearrangement to a single Jκ gene. Third, we found that the YY1 REPO domain, which we previously showed recruits PcG proteins to DNA (Wilkinson *et al*, 2006), physically interacts with condensin and cohesin proteins involved in large-scale chromosomal structures. Cohesin proteins have been implicated in Ig locus structure and rearrangement as well as T-cell receptor rearrangement (Degner *et al*, 2009, 2011; Seitan *et al*, 2011), but connections between Ig rearrangement and the condensin complex have not been previously observed. The YY1-condensin/cohesin interaction is consistent with a direct effect on rearrangement. Fourth, YY1, PcG, and condensin proteins co-localized at multiple sites across the Igκ locus. Such binding sites suggest a direct role in Igκ locus contraction and rearrangement. Finally, knock-down of YY1 and condensin proteins reduced rearrangement of Vκ genes. Again, this effect on rearrangement is most consistent with a direct effect on Igκ locus structure. However, we cannot exclude non-rearrangement related effects of the YY1 REPO domain on differentiation or cell proliferation.

The Vκ rearrangement pattern we observed in wild-type and YY1 reconstituted mice is similar, but not identical to the repertoire observed in C57BL/6 mice by Nemazee and coworkers (Aoki-Ota *et al*, 2012). While the Vκ usage pattern in our mice showed 77% of the same Vκ genes used, there were differences in the most frequently rearranged genes. Only five out of seven of the most frequently used Vκ genes identified by Aoki-Ota *et al* were shared by our mice, and these accounted for only 13.5–15.1% of all rearrangements

compared to over 40% by Aoki-Ota *et al*. The reasons for these differences are not clear, but may relate to methodological differences in V gene amplification or housing and environmental differences of our mice. At any rate, these differences do not impact our conclusions regarding the skewed repertoire in YY1ΔREPO mice compared to YY1 mice.

The implication of cohesin proteins in Ig rearrangement is interesting (Degner *et al*, 2009, 2011), particularly since conditional deletion of cohesin subunit Rad21 in thymocytes impacts T-cell receptor rearrangement and thymocyte differentiation (Seitan *et al*, 2011). Condensin and cohesin complex proteins regulate chromosome contraction and cohesion during mitosis and meiosis (Haering, 2009; Hudson *et al*, 2009). These complexes can mediate long-distance chromosomal interactions (D'Ambrosio *et al*, 2008; Haeusler *et al*, 2008), and kleisin-β, a member of the condensin II complex is important for T-cell development as is cohesin subunit Rad21 (Gosling *et al*, 2007; Seitan *et al*, 2011). Cohesin subunit Rad 21 (a kleisin family protein) is recruited to CTCF binding sites throughout the immunoglobulin loci during B lymphocyte development (Degner *et al*, 2009). As condensin I is involved in the process of physically compacting DNA in the presence of hydrolysable ATP (Strick *et al*, 2004), condensin complex proteins may also participate in bringing V genes in the Ig locus into close proximity with D and J gene segments. However, a role for condensin complex proteins in Ig locus contraction or rearrangement has never been established. PcG proteins are also able to mediate long-distance interactions between their binding sites in Polycomb response elements (PREs) and promoter DNA sequences (Lanzuolo *et al*, 2007). Since YY1 recruits PcG proteins to DNA via the REPO domain (Srinivasan and Atchison, 2004; Wilkinson *et al*, 2006), the recruitment of EZH2 to multiple sites across the Igκ locus could also assist in long-distance interactions leading to contraction and rearrangement.

It should be noted that RNA expression profiles of PcG protein EZH2, and condensin subunit proteins SMC4, SMC2, CAP-G, CAP-H (BRRN1), and CAP-D2 peak during B-cell development at the pre-B cell stage (www.immgen.org). Expression levels are also high in pro-B cells, but peak in pre-B cells, then drop in immature B-cell stages. This expression pattern is coincident with the timing of Ig rearrangement and is consistent with a role in Ig locus contraction and rearrangement. However, this timing is also coincident with high levels of proliferation in pre-B cells suggesting a possible effect of the YY1ΔREPO mutant on the pre-B proliferative burst during development. All factors peak again in germinal centre B cells (www.immgen.org), suggesting possible roles in proliferation, class switch recombination, or somatic hypermutation.

Based upon (a) the crucial nature of the YY1 REPO domain for B-cell development, (b) the ability of this domain to recruit PcG proteins to DNA, (c) the physical interaction of the REPO domain with condensin proteins, (d) the co-localization of YY1, EZH2, and condensin proteins across the Igκ locus, (e) the effect of condensin subunit knock-down on Vκ-Jκ rearrangement, and (f) the high level of EZH2 and condensin proteins in pro-B cells and peak levels of expression in pre-B cells, we propose the following mechanism. We propose that YY1 binds to clusters of sites spanning the Igκ locus. Concomitant with YY1 DNA binding to the Igκ locus, increased EZH2 and condensin subunit expression results in these proteins binding to the same DNA regions, presumably due to interactions with YY1. The nucleated PcG and condensin proteins then mediate long-distance interactions between the clusters of YY1 binding sites resulting in either contraction or maintenance of the Igκ locus in a looped or rosette structure. This relatively compacted structure thus assists in somatic rearrangement of a relatively large fraction of Vκ genes. Immediately upon maturation to the immature B-cell stage, EZH2 and condensin protein expression drops dramatically (www.immgen.org), thus facilitating de-contraction of the Ig loci and perhaps assisting in regulation of the allelic exclusion process. In a YY1ΔREPO background, specific loops would be lost and only loops independent of YY1 function, perhaps maintained by Pax5 or E47 (see Figure 8B) would remain and by default would represent the only Vκ genes available for rearrangement.

The mechanism we propose here is likely distinct from the mechanisms that operate to generate skewed Igκ repertoires observed in two recent studies. Xiang *et al* (2011)) deleted the Sis enhancer lying between the Vκ and Jκ regions and found increased proximal Vκ gene usage and reduced distal Vκ rearrangement. Deleting this enhancer did not impact Igκ locus contraction, but instead appeared to inhibit proximal Vκ gene rearrangement and to increase distal Vκ rearrangement, perhaps by controlling CTCF and Ikaros binding in the Vκ-Jκ intervening sequence. Similarly in $CTCF^{-/-}$ mice, De Almeida *et al* (2011) observed no change in IgH rearrangement but saw skewed Igκ rearrangement. Loss of CTCF resulted in increased proximal and decreased distal Vκ gene rearrangement. CTCF was found to limit interactions of the Igκ enhancers with proximal Vκ genes, and CTCF deletion increased these interactions leading to increased proximal Vκ rearrangement (De Almeida *et al*, 2011). Deletion of CTCF sites in the IgH locus near the DFL16.1 gene also increased V_H proximal rearrangement and decreased V_H distal rearrangement (Guo *et al*, 2011b; Feeney and Verma-Gaur, 2012). The Sis and CTCF mechanisms thus control enhancer interactions with proximal gene sequences and thus appear to be distinct from the YY1 controlled mechanism we have identified here as YY1ΔREPO does not result in changes in proximal versus distal Vκ rearrangement.

The selective effect of the YY1ΔREPO mutant on Igκ rearrangement rather than IgH rearrangement is also interesting. Some mechanism must effectively target the YY1 effect we observe here to the Igκ locus. Future studies will be required to test key features of our proposed model and to determine how YY1 nucleates PcG and condensin complex proteins to the Igκ locus. As YY1 is a ubiquitously expressed protein, recruitment of EZH2 and condensin proteins may require a stage-specific YY1 post-translational modification or expression of other regulatory proteins.

Materials and methods

Animals

We used *mb1-CRE yy1*$^{f/f}$ mice in which the *yy1* promoter region and exon 1 are flanked by loxP sites. In these mice, the endogenous *yy1* gene is conditionally knocked out at the early pro-B cell stage by the action of mb1-driven Cre recombinase (Liu *et al*, 2007). All experiments involving animals were approved by the IACUC of the University of Pennsylvania and conform to the appropriate regulatory standards.

Bone marrow transduction and transplantation

Bone marrow cells were harvested from 6- to 8-week-old *yy1*$^{f/f}$ *mb1-CRE* mice 4 days after intravenous injection of 250 mg/kg 5-fluorouracil (5-FU). Cells were cultured overnight in DMEM with 10% FBS, L-glutamine, IL-3 (6 ng/ml), IL-6 (5 ng/ml), and SCF (100 ng/ml), then washed and resuspended in retroviral supernatant containing polybrene (4 μg/ml) and the same cytokine cocktail for spin infection at 1290 *g* for 90 min. A second round of spin infection was performed 24 h following the first one. At least 5×10^5 cells were injected intravenously into lethally irradiated (9 Gy) recipient C57BL/6 mice. Antibiotic containing drinking water was provided for recipient mice for 2 weeks post transplantation.

PCR detection of Ig rearrangement

Genomic DNA from sorted GFP$^+$ pre-B or splenic B cells from MigR1, YY1, or YY1ΔREPO reconstituted mice were amplified by PCRs with primers described previously (Fuxa *et al*, 2004). PCR products were visualized by Southern blot with an oligo probe (5′-AGCCTTCAGGACCAAGATTCTCTGCAAACG-3′) detecting the region upstream of J_H3. For Igκ rearrangement, DNA samples were amplified by real-time PCR and by traditional PCRs. Real-time PCR primers (provided by William Garrard; University of Texas) are shown in Supplementary Table II. GAPDH was used as an internal control for normalization. DNA samples were also used for traditional PCRs for Igκ recombination (94°C for 10 min followed by 35 cycles at 94°C for 1 min, 61°C for 1 min, 72°C for 1 min and end with 72°C for 5 min) with primers described previously (Li *et al*, 2001).

Transcriptome sequencing

First-strand cDNA synthesis was performed using a gene-specific primer targeting the Cκ region (GSP1: 5′-CACAGGTATAGCTGTT ATG-3′) following manufacturers' instructions (Superscript III RT kit, Invitrogen). The 3′ end of the cDNA was tailed with dCTP using TdT. The tailed cDNA was amplified using Abridged Anchor Primer (AAP) (Invitrogen) and GSP2 (5′-TGCTGCTCATGCTGTAGGTG-3′) in a PCR with denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min using Taq polymerase (Invitrogen). Nested PCR with 1:25 dilution of the PCR amplified product used Universal Amplification Primer (UAP) (Invitrogen) and GSP3 (5′-GATGTTAACTGCTCACTGGATG-3′). Products were gel purified, barcoded, and transcriptome sequenced using GS FLX and GS Junior Systems (Roche).

Computational approaches

The mouse Igκ genomic sequence (chr6: +67 800 000–71 200 00) was obtained from the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway) version hg18 (NCBI36). Consensus software with default parameter settings was used to define YY1 and Pax5 consensus binding sites (Hertz *et al*, 1990). These binding sites were aligned and used to construct the computational model. The PSPM (Wang and Hannenhalli, 2006) was used to scan the whole mouse Igκ locus by a sliding-window approach with 1 bp steps.

ChIP assays

ChIP was carried out according to the Upstate Biotechnology protocol using 10×10^6 IL-7 cultured B cells or 3T3 fibroblasts. Anti-YY1 (H414, Santa Cruz Biotechnology), -EZH2 (Upstate), -SMC4 (ab17958, Abcam), -SMC2 (ab10399, Abcam) -BRRN1 (ab62815, Abcam), Pax5 (sc1974x, Santa Cruz Biotechnologies), and E47 (114811A, Pharmingen) antibodies were used followed by protein G or protein A conjugated bead precipitation. Pre-immune controls used normal rabbit IgG. DNA samples were analysed by conventional PCR or real-time PCR using a Roche Lightcycler 1.5. ChIP primer sequences are shown in Supplementary Table III. Relative enrichments for each region are presented as the percentage of input. The mouse actin-B promoter region was used as a negative control for YY1 binding and the RPL30 promoter region as a positive control.

Knock-down of SMC4 and YY1

Five million bone marrow IL-7 culture cells were electroporated with 1 nmol of *Silencer*® Negative Control #1 siRNA (AM4636, Ambion), SMC4 siRNA (Sigma), or YY1 siRNA (SASI_Mm01_00125709, Sigma) at 290 V for 10 msec and allowed to grow in IMDM media with 15 ng/ml IL-7 for 24 h. Subsequently, the IL-7 concentration was lowered to 0.1 ng/ml for 48 h and cells were harvested for protein and DNA preparation. Primers used for Vκ rearrangement assays are shown in Supplementary Table II.

Retroviral constructs and plasmids

Flag-tagged YY1 or flag-tagged YY1ΔREPO cDNAs were cloned into the *Hpa*I site of GFP-expressing MSCV-IRES-GFP vector (MigR1) by blunt end ligation. High titer retroviral supernatants were prepared following transient transfection of HEK293 cells. Retroviral envelopes with ecotropic host ranges (pHIT123) were used. Plasmids pcDNA3.1 GAL and pcDNA3.1GALREPO were described previously (Wilkinson *et al*, 2006).

Flow cytometric analysis and cell sorting

Bone marrow or spleen cells were stained with antibodies for FACS analysis. PE anti-CD43, APCcy7 anti-CD19, PECy7 anti-IgM, and Biotin anti-CD5 were purchased from BD Biosciences-Pharmingen. APC anti-AA4-1, AF700 anti-B220, PE Cy5.5 anti-IgM, Pacific-Blue anti-CD21, PECy7 anti-CD23, and DAPI were obtained from eBioscience. Acquisitions were performed on LSRII (BD) cell sorters. Data were analysed with BD FlowJo software. Freshly prepared spleen cell suspensions were stained with APCcy7 anti-CD19, AF700 anti-B220, and PE anti-CD3. Bone marrow cell suspensions were stained for PE anti-CD43, APC anti-AA4-1, AF700 anti-B220, APCcy7 anti-CD19, and PECy7 anti-IgM. GFP$^+$CD19$^+$B220$^+$ and CD3$^-$ splenic B cells and GFP$^+$B220$^+$CD43$^-$ IgM$^-$AA4.1$^+$ bone marrow pre-B cells were sorted by the University of Pennsylvania Flow Cytometry and Cell Sorting facility.

Cell culture

HEK 293 cells and 3T3 cells were cultured in DMEM with 10% FBS. Bone marrow cells from C57BL/6 mice were isolated, plated onto OP9 feeder cells and were cultured in IMDM with 10% FBS, 5 ng/ml IL-7, 50 μM 2-mercaptoethanol, 1 × L-glutamine, 1 × non-essential amino acids, and 1 × penicillin/streptomycin. Ten days after culturing, 90% of bone marrow cells were B220$^+$IgM$^-$CD43$^+$ by flow cytometric analysis.

Endogenous co-immunoprecipitation

IL-7 cultured B-cell lysates (1 mg) were immunoprecipitated with 3 μg YY1 antibody (H414, Santa Cruz) or the same amount of rabbit IgG control followed by incubation with protein A beads overnight. Lysates from primary pro-B cells from rag −/− mice were immunoprecipitated with SMC4 antibody (Abcam 17958) or normal rabbit IgG. Samples were fractionated on SDS–PAGE gels and were immunoblotted for SMC4 (ab17958, Abcam) BRRN1 (ab62815, Abcam), EZH2 (sc-172638, Santa Cruz Biotechnology), SuZ12 (ab12073, Abcam), or YY1 (H414, Santa Cruz Biotechnology).

Mass spec analysis

HEK293 cells were transfected with pcDNA-GALREPO or pcDNA-GAL vector plasmids by the lipofectamine 2000 method (Invitrogen). Forty-eight hours post transfection, nuclear extracts were made from the transfected cells by the Nuclear Complex co-IP kit according to manufacturer's protocol (Active Motif). Nuclear extracts were immunoprecipitated with GAL antibody beads (Santa Cruz). The bound proteins were fractionated by SDS–PAGE, the gel was silver stained, and bands specific for GALREPO were excised. Protein identities were determined by MALDI-TOF mass spec analyses performed at the Proteomics Core Facility of the University of Pennsylvania.

Western blots

GFP$^+$ lymphocytes were sorted from YY1ΔREPO reconstituted mice. Total cell extracts were prepared in 2 × SDS sample buffer (Biorad). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, western blotting, and peroxidase-based chemiluminescence detection were performed according to standard laboratory protocols. YY1 antibody (H414, Santa Cruz) was used to detect the endogenous and Flag-tagged YY1. Bands were quantified by using ImageJ software.

Quantitative real-time PCR

GFP$^+$pre-B cells were sorted from MigR1, YY1, or YY1ΔREPO reconstituted mice. RNAs from the sorted cells were made by

the RNeasy plus mini kit (QIAGEN) and subjected to the reverse transcriptase-PCR (RT–PCR) procedure. Murine primers for YY1 were sense CCCACGGTCCCAGAGTCCA and antisense TGTGCGCA AATTGAAGTCCAGT. Hprt was used as an internal control for normalization. Hprt primers were sense CTCCTCAGACCGCTTTT TCC and antisense TAACCTCCTTCATCATCGCTAATC. All amplifications crossed intron-exon boundaries to exclude genomic DNA amplification.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: XP performed bone marrow reconstitution; XP and AB performed co-IP experiments; XP YH, MC, and WJQ performed FACS analyses; XP, MP, AB, and SH performed Ig rearrangement assays and RNAi knock-down studies; MP performed transcriptome studies; JW performed bioinformatics analyses; XP, FW, MP, SH, and KZ performed ChIP studies; HL and YS provided YY1 conditional knock-out mice and intellectual input; DA, MC, and MLA designed and evaluated experiments, XP and MLA wrote the manuscript with edits from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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