Histone reader BRWD1 targets and restricts recombination to the Igk locus

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B lymphopoiesis requires that immunoglobulin genes be accessible to RAG1-RAG2 recombinase. However, the RAG proteins bind widely to open chromatin, which suggests that additional mechanisms must restrict RAG-mediated DNA cleavage. Here we show that developmental downregulation of interleukin 7 (IL-7)-receptor signaling in small pre-B cells induced expression of the bromodomain-family member BRWD1, which was recruited to a specific epigenetic landscape at Igk dictated by pre-B cell receptor (pre-BCR)-dependent Erk activation. BRWD1 enhanced RAG recruitment, increased gene accessibility and positioned nucleosomes 5′ to each Igk recombination signal sequence. BRWD1 thus targets recombination to Igk and places recombination within the context of signaling cascades that control B cell development. Our findings represent a paradigm in which, at any particular antigen-receptor locus, specialized mechanisms enforce lineage- and stage-specific recombination.

The defining event of B lymphopoiesis is immunoglobulin gene recombination1. Studies in mice have shown that rearrangement begins with the Igk locus and recombination of diversity (D) to joining (J) gene segments in pre-pro B cells, followed by recombination of variable (V) gene segments to DJ segments in late pro-B cells2. After in-frame recombination, the expressed immunoglobulin μ-chain assembles with the surrogate light chain (λ) to form a pre-B cell receptor (pre-BCR). Expression of the pre-BCR–induced E2A can then bind the Eκ enhancer (Eκi) and recruits polycomb repressive complex 2 (PRC2), which decorates regional chromatin, including Jκ and Cκ, with trimethyl groups at lysine 27 of histone H3 (H3K27me3) (ref. 9). Expression of the pre-BCR is associated with subsequent escape from IL-7R-dependent STAT5 activation2 leading to cell cycle exit10 and de-repression of Igk6. Pre-BCR–induced E2A can then bind the Eκi and 3′ κ-enhancer (3′Eκ′), recruit histone acetyltransferases and augment Igk transcription.5,11

Some studies indicate that transcription itself is required for recombination6,12, whereas others have noted discordance between transcription and recombination13,14. It might be that the epigenetic state associated with transcriptional activation is a more universal requirement of antigen-receptor gene recombination, as H3K4me3, a mark of open chromatin, directly recruits RAG2 (refs. 15–17). This observation directly links the epigenetic landscape to recombination.

The idea of a role for H3K4me3 in recombination suggests specific restrictions on how accessibility is regulated at immunoglobulin genes targeted for recombination. Nucleosomes would have to be present in targeted loci to recruit RAG2. However, nucleosomes at recombination signal sequences (RSSs, which include nonamer and heptamer motifs) inhibit RAG-mediated cleavage18–20, and in vitro, nucleosomes are preferentially positioned over RSS sites1,18. These data suggest that nucleosomes bearing H3K4me3 would need to be positioned adjacent to RSSs by mechanisms not solely reliant upon the underlying DNA sequence21,22.

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Furthermore, it is not clear whether the known mechanisms of gene accessibility and recombination are sufficient to restrict recombination to specific immunoglobulin gene loci at particular developmental transitions. In small pre-B cells, both RAG1 and RAG2 are recruited to thousands of sites bearing H3K4me3 (refs. 1,23). Furthermore, cryptic RSSs, which can be cleaved by RAG24,25, are predicted to occur at millions of sites across the genome26. Yet in small pre-B cells, recombination is normally restricted to the Igk loci. These observations suggest that there must be additional, unknown factors that target and restrict recombination to Igk in small pre-B cells.

Here we demonstrate that the dual bromodomain-family member BRWD1 targets Igk for recombination. BRWD1 is rapidly induced after escape from IL-7R signaling and is then recruited to Igk by a specific epigenetic code imparted by pre-BCR-dependent signals. Binding of BRWD1 at Igk both opens regional chromatin and positions nucleosomes relative to DNA GAGA motifs to enable RAG recruitment and Igk recombination.

RESULTS

STAT5 directly represses Brwd1

In pro-B cells, STAT5-mediated repression is usually associated with stable silencing of target genes through subsequent stages of B lymphopoiesis28. In our study, of the 47 genes repressed by STAT5 in pro-B cell5, only two genes, Igk and Brwd1 (Fig. 1a), were immediately and strongly induced upon transition to the small pre-B cell stage. BRWD1 was a direct target of STAT5, which bound the Brwd1 promoter region, and STAT5 binding was associated with coincident and flanking H3K27me3 repressive marks (Fig. 1b). Brwd1 demonstrates an expression pattern similar to that of Igk throughout B cell development, and like Igk, it is expressed primarily in the B cell lineage. BRWD1 is a histone lysine-acetylation reader27 and a member of the dual bromodomain and WD40 repeat protein families that associate with the SWI/SNF chromatin-remodeling complex28. These features predict nuclear localization. Indeed, confocal microscopy of flow cytometry–sorted wild-type primary B cell progenitors indicated that BRWD1 expression was induced after the pro-B cell stage and that most BRWD1 resided in the nucleus (Fig. 1c).

BRWD1 is required for B lymphopoiesis

To examine whether BRWD1 is important in B lymphopoiesis, we obtained Brwd1–mutant mice29 harboring an ethylnitrosourea-induced single point mutation at the exon 10–intron 10 junction of Brwd1 (Brwd1mut). These mice, originally derived on a C57BL/6 background, were then extensively backcrossed to C3HeB/FeJ mice to isolate the mutation to a 1.8-Mb region on chromosome 16 (ref. 29). cDNAs from bone marrow (BM) and splenic B220+ B cells10 from wild-type and Brwd1mut mice were sequenced to confirm that the identified mutation induced exon skipping and a frame shift that generated premature stop codons (Supplementary Table 1 and Supplementary Fig. 1a–c). Immunoblotting of Brwd1mut splenic B cells with an antibody specific for the N-terminal domain of BRWD1 did not detect either wild-type BRWD1 bands or any species with a smaller molecular weight (Supplementary Fig. 1d).

Next, we harvested BM and spleens from Brwd1mut and wild-type littermate control mice and analyzed B lymphopoiesis by flow cytometry (Fig. 2). There were diminished frequencies of later-stage B cell progenitors in Brwd1mut mice (Fig. 2a). Total numbers of B220+ BM cells were decreased in Brwd1mut mice as compared with wild-type mice, mainly because of a reduction in the IgM+ B cell population (Fig. 2b). The defect began in small pre-B (Lin−B220+CD43−IgM−FSClo) cells and progressively worsened in later BM developmental stages (Fig. 2c).

No significant B cell developmental defects were detected in heterozygous Brwd1mut+/+ mice (Supplementary Fig. 2a). The developmental defect in Brwd1mut mice persisted into the periphery; these mice had small spleens (data not shown) and decreased numbers of total splenocytes and splenic B220+CD19+ B cells (Fig. 2d). We detected defects in the frequencies and numbers of splenic transitional (B220+CD19+CD93−), immature (B220+CD19+IgM−IgD−), mature (B220+CD19+IgM+IgD−) and follicular (B220+CD19+CD21−CD23+) B cells (Fig. 2e–h). In contrast, B cells (B220+CD19+CD21−CD23−) in the marginal zone were relatively preserved (Fig. 2g,h). Subsequent analysis of early common progenitors, other hematopoietic lineages and thymocytes showed no substantial defects (Supplementary Fig. 2b–l).

To assess the competitive fitness of Brwd1mut cells in vivo, we reconstituted sublethally irradiated Rag2−/−Il2rg−/− mice with Lin−Scalc1−c-Kit− (LSK) progenitor cells from either wild-type CD45.1 and wild-type CD45.2 mice (Fig. 3a) or wild-type CD45.1 and Brwd1mut CD45.2 mice (Fig. 3b), with equal amounts of cells from each type of mouse in each mixture. After 5 weeks, we harvested BM and analyzed it by flow cytometry (Fig. 3a,b). LSK cells from wild-type CD45.1 and CD45.2 mice were equally competent to reconstitute B lymphopoiesis (Fig. 3c). LSKs from Brwd1mut mice also were able to reconstitute the pro-B and large pre-B cell compartments (Fig. 3d). However, wild-type LSKs contributed fourfold to fivefold more small pre-B cells than did Brwd1mut cells, and this bias persisted into the immature and...
mature B cell compartments (Fig. 3d). Numbers of splenic Brwd1mut B cells were also severely diminished (Supplementary Fig. 3a). In the T cell lineage, there was some skewing toward CD4+ cells that may have reflected low expression of BRWD1 in double-positive thymocytes (Supplementary Fig. 3b).

The observed defects in B lymphopoiesis in Brwd1mut mice were not associated with apparent increased frequencies of apoptotic B cell progenitors (Supplementary Fig. 3c). We noted that slightly more Brwd1mut large and small pre-B cells progressed through the cell cycle (Supplementary Fig. 3d). Expression of Ccdn2 mRNA was slightly diminished and that of Ccdn3 mRNA was slightly increased in Brwd1mut pre-B cells compared with pre-B cells from wild-type littermate controls (Supplementary Fig. 3e). These results suggest that the observed defects in B lymphopoiesis were not due to increased apoptosis or diminished proliferation.

Igk recombination requires BRWD1

Igk recombination occurs in small pre-B cells, so we sorted those cells from wild-type and Brwd1mut mice. kGT was decreased approximately twofold in Brwd1mut mice as compared with wild-type controls (Fig. 4a), and overall Vκ-Jκ recombination was diminished approximately fivefold compared with that in wild-type mice (Fig. 4b). Quantitative PCR (qPCR) for Vκ-Jκ recombination revealed a similar defect in recombination8 (Fig. 4d). The distribution of Iκκ use in rearranged Igk from wild-type and Brwd1mut small pre-B cells was similar (Supplementary Fig. 3f). Vκ1, Vκ4, Vκ6 and Vκ8 were the most common Vκ segments detected in rearranged Igk (Supplementary Fig. 3g). Finally, the frequency of cells expressing immunoglobulin λ-chain among immature B cells isolated from BM and spleen was also modestly reduced in Brwd1mut mice as compared with wild-type mice (Supplementary Fig. 3h).

To confirm that BRWD1 is required for Igk recombination, we used pre-B cells from Irf4−/−Irf8−/− mice, which rapidly proliferate in vitro with IL-7 and undergo Igk recombination upon IL-7 withdrawal9. IL-7 withdrawal robustly induced the expression of Brwd1 (Fig. 4e). We then used retrovirus to express a short hairpin RNA (shRNA) targeting Brwd1 (or a control shRNA) in cultured pre-B cells from Irf4−/−Irf8−/− mice. In the presence of IL-7, both Brwd1 expression and kGT were low, and this was not affected by the Brwd1-specific shRNA (Fig. 4e,f). However, after withdrawal of IL-7, we noted reductions in the induction of Brwd1 by approximately 75% and in kGT by 55% in Irf4−/−Irf8−/− pre-B cells expressing the Brwd1-specific shRNA (Fig. 4e,f). Igk recombination after IL-7 withdrawal was attenuated by fivefold in Irf4−/−Irf8−/− cells expressing the Brwd1-specific shRNA (Fig. 4g–i). Therefore, both in vivo and in vitro studies indicated a critical role for BRWD1 in Igk recombination.

BRWD1 is recruited to histone H3K9AcS10pK14Ac marks at Igk

In vitro studies of the BRWD1 bromodomains predict recruitment to H3K9Ac, phosphorylated H3S10 (H3S10p) and H3K14Ac23. Therefore, we isolated pro-B and small pre-B cells from wild-type BM by flow sorting and subjected nuclear preparations to chromatin immunoprecipitation (ChIP) with antibodies to H3K9Ac, H3S10p and H3K14Ac or BRWD1 followed by qPCR for the indicated regions of the Igk locus including 3′Eκ (Fig. 5a–c). In pro-B cells, the Igk locus was essentially devoid of detectable H3K9Ac or H3S10pK14Ac epigenetic marks. However, in the small pre-B cell pool there was robust induction of H3K9Ac and H3S10pK14Ac through Jκκ, Eκκ and Cκ (Fig. 5a,b).
BRWD1 was preferentially recruited to Jκ through Cκ regions marked with both H3K9Ac and H3S10pK14Ac (Fig. 5c). Jκ segments were negligible in ChIP assays with BRWD1-specific antibodies from Brwd1mut small pre-B cells (Supplementary Fig. 4a).

It is possible that BRWD1 decorates Igk with H3K9Ac and H3S10pK14Ac. Therefore, we subjected nuclear lysates from wild-type and Brwd1mut small pre-B cells to ChIP-qPCR with antibodies to H3K9Ac or H3S10pK14Ac as described above. The absence of BRWD1 in small pre-B cells did not substantially change the magnitude of either H3K9Ac or H3S10pK14Ac at the Igk locus (Supplementary Fig. 4b,c). These data suggest that BRWD1 is recruited to a pre-existing, specific epigenetic landscape.

Erk induces H3S10 phosphorylation at Igk

The pre-BCR activates Erk10, which can directly phosphorylate H3S10 (refs. 31,32). Therefore, we examined whether blocking
the Erk pathway in cultured Ifng−/−Irβ8−/− cells diminished H3S10 phosphorylation. With IL-7 there was modest H3S10 phosphorylation at Iκκ, Eκκ and Cκ (Fig. 5d) that increased after IL-7 withdrawal. This induction was greatly attenuated in Ifng−/−Irβ8−/− cells expressing either dominant-negative Ras (DN-Ras) or DN-MEK (Fig. 5d). We next examined whether H3S10 phosphorylation could be a consequence of E2A induction. We cultured Ifng−/−Irβ8−/− cells expressing an estrogen receptor (ER)-Iκκ fusion in the presence or absence of 4-hydroxytamoxifen10. However, E2A inhibition did not appreciably diminish Igk H3S10 phosphorylation (Fig. 5e).

We next examined whether E2A was specifically required for Igk H3K9Ac and H3K14Ac. Ifng−/−Irβ8−/− cells expressing ER-Iκκ were cultured as above and assayed by ChIP with antibodies to H3K9Ac and H3K14Ac. Withdrawal of IL-7 induced robust expression of H3K9Ac and H3K14Ac at Iκκ through Cκ (Fig. 5f,g) that was significantly attenuated by Iκκ. Finally, we determined whether Erk activation was needed for BRWD1 recruitment. Expression of DN-Ras or DN-MEK in Ifng−/−Irβ8−/− cells diminished BRWD1 recruitment to Igk after IL-7 withdrawal (Fig. 5h). Overall, these observations suggest that downstream of the pre-BCR, Erk signaling sets the epigenetic landscape at Igk to recruit BRWD1.

**BRWD1 regulates Igk locus accessibility**

Reanalysis of pro-B cell and splenic B cell (CD19+) DNase-Seq data33,34 showed that the Jκ−Cκ regions of the Igk locus were inaccessible in pro-B cells but accessible in splenic B cells (Fig. 5i). We next assessed whether BRWD1 has a role in opening the Igk locus in small pre-B cells. We subjected nuclear lysates from flow-isolated wild-type and Brwd1mut pro-B cells, small pre-B cells and splenic B cells to ChIP with H4K16Ac-specific antibodies35. In wild-type pro-B cells, there was little H4K16Ac at the Igk locus. However, after the transition to the small pre-B cell stage, H4K16Ac expression became robust at Igk (Fig. 5j). In contrast, in Brwd1mut small pre-B cells, this mark was almost absent. These data suggest that BRWD1 binds to Igk and facilitates chromatin decompaction.

**BRWD1 is recruited to H3K9Ac and H3S10pK14Ac genome-wide**

We next assessed the correlations among H3K9Ac, H3S10pK14Ac and BRWD1 recruitment across the entire genome. We subjected nuclear
Figure 6 Recruitment of BRWD1 to H3K9Ac and H3S10pK14Ac genome-wide. (a) Overlap of peaks (P < 10\(^{-7}\)) obtained by ChIP-seq for BRWD1, H3K9Ac and H3S10pK14Ac from purified wild-type small pre-B cells. Data are representative of two independent experiments. (b) ChIP-seq analysis of the binding of BRWD1, H3K9Ac and H3S10pK14Ac at the Igk locus in purified wild-type small pre-B cells presented as the smoothed density (where “density” indicates sequence ‘read’). Data are representative of two independent experiments. Igk locus shows the locations of V\(_κ\), J\(_κ\) and C\(_κ\) gene segments (mm9 chromosome 6: 70,653,572–70,676,748). (c) Alignment of BRWD1, H3K9Ac and H3S10pK14Ac enrichment in ChIP-seq peaks. The y-axis represents the normalized immunoprecipitation (IP)-signal distribution for ChIP peaks centered at 0 bp. (d) Percentage of peaks containing at least one extended GAGA motif (GA\(_{11}\)). Data are average ± s.d. from 100 bootstrapping runs for each peak group. (e) Total number of extended GAGA motif (GA\(_{11}\)) peaks was coincident with BRWD1 peaks (Fig. 6a and Supplementary Fig. 5a). More than 64% of these overlapping peaks were also coincident with H3K9Ac. The concordance of BRWD1, H3K9Ac and H3S10pK14Ac peaks at the Igk locus was particularly good, with peaks clustered at J\(_κ\) and E\(_κ\) (Fig. 6b). Comparison of genome-wide normalized immunoprecipitation signal distributions for BRWD1, H3K9Ac and H3S10pK14Ac confirmed extensive peak overlap (Fig. 6c). When we considered the ChIP-Seq data sets both separately and in combination, we found that 37%–62% of peaks were in gene regulatory regions (intragenic or promoter) and 38%–63% were intergenic (Supplementary Fig. 5b).

There was no evidence of BRWD1 binding to Igf or IgI (which encodes the \(λ\)-light chain) in small pre-B cells (data not shown). Furthermore, J\(_{H}\) and J\(_{L}\) were not marked with H3K9Ac or H3S10pK14Ac in those cells. IgI is normally rearranged in a small fraction of immature B cells\(^5\). Therefore, we examined whether BRWD1 binds IgI in immature B cells from mice that cannot rearrange Igk (Igk\(^{del}\))\(^{36}\) and found no detectable binding of BRWD1 to J\(_{κ}\) in the cells (Supplementary Fig. 5c). These data indicate that BRWD1 is a specific mediator of Igk recombination in small pre-B cells.

BRWD1 binds at GAGA motifs genome-wide

We next used \textit{de novo} prediction of motifs to assess DNA sequences occurring at single and coincident ChIP-Seq peaks. In individual peaks, the most significant DNA motifs observed were similar to the binding motifs of ISRE (BRWD1 and H3S10pK14Ac) and Sox12 (H3K9Ac), both of which contain repetitive GA (CT) elements (Supplementary Fig. 5d). At peaks coincident in two or more ChIP-Seq data sets, long repetitive sequences of GAGA were overrepresented (BRWD1-H3S10pK14Ac, \(P < 10^{-599}\), and BRWD1-H3K9Ac-H3S10pK14Ac, \(P < 10^{-264}\)) (Supplementary Fig. 5e,f). Further analysis showed that extended GAGA motifs (GA\(_{11}\)) were most enriched in BRWD1 and H3S10pK14Ac peaks, with a prevalence of up to 79% (Fig. 6d). In some data sets, the total number of extended GAGA motifs exceeded the total number of peaks (Fig. 6e), which indicated that some peaks contained multiple GAGA motifs. These data demonstrate a remarkable enrichment of GAGA motifs at sites of H3S10pK14Ac and BRWD1 recruitment.

BRWD1 regulates accessibility and nucleosome positioning

In Drosophila, GAGA motifs recruit Trithorax-like (TRL), which regulates gene accessibility.\(^{37,38}\) Therefore, to determine whether BRWD1 regulates gene accessibility genome-wide, we assessed nuclear lysates from flow-sorted wild-type and Brwd1\(^{mut}\) small pre-B cells by transposase-accessible chromatin with sequencing (ATAC-Seq)\(^{39}\). Comparison at the Igk locus in Brwd1\(^{mut}\) small pre-B cells showed diminished accessibility at the J\(_κ\) region with relatively little change in overall accessibility at the E\(_κ\) and C\(_κ\) regions (Fig. 7a and Supplementary Table 2). The J\(_κ\) region was approximately 2.7 times more accessible in wild-type small pre-B cells relative to the whole-genome average accessibility. In contrast, the J\(_κ\) region in Brwd1\(^{mut}\)
Figure 7 BRWD1 regulates chromatin accessibility and nucleosome positioning in vivo. (a) Accessibility (open chromatin) at Jκ, Eκ and Cκ in wild-type and Brwd1mut small pre-B cells. The y axis represents tags per million reads. Data are representative of two independent experiments (10^5 cells per sample). (b) Quantitative measurement of accessibility in the Jκ region and whole genome of wild-type and Brwd1mut small pre-B cells. The Jκ region was defined as 70,672,000–70,675,000 of chromosome 6 (mm9). *P < 0.0001 versus whole-genome average accessibility (unpaired t-test). (c) Accessibility around individual Jκ segments at single-nucleotide resolution in wild-type and Brwd1mut small pre-B cells. The RSSs, nonamer (G/AGTTTTTGT) and heptamer (CAGTGTG) motifs, and coding sequences of each Jκ region are provided. Data are representative of two independent experiments. (d) Nucleosome positioning at Jκ, Eκ and Cκ in wild-type and Brwd1mut small pre-B cells. The nucleosome signal represents the difference in normalized density between the simulated signal and background data obtained from the same data; “signal” is from read pairs with large insert sizes, and “background” is from read pairs with short insert sizes. Data are representative of two independent experiments. (e–h) DNA footprint analysis of a 1-kb region for wild-type and Brwd1mut small pre-B cells at BRWD1 peaks (e), BRWD1-H3K9Ac-H3S10pK14Ac peaks (f), GAGA motifs (n = 136) (g) and poly(A) motifs (n = 737) (h) centered on 0. For the nucleosome differential (y axis), values greater than 0 indicate the presence of a nucleosome, and values less than 0 represent nucleosome-free peaks or motifs.

In wild-type small pre-B cells, nucleosomes were positioned between Jκ segment exons, leaving the RSSs and Jκ segments nucleosome free (Fig. 7d and Supplementary Fig. 7a). In marked contrast, in Brwd1mut small pre-B cells, nucleosomes were positioned over each RSS and Jκ segment. Furthermore, whereas in wild-type small pre-B cells EκE was primarily free of nucleosomes, in Brwd1mut cells we noted accumulation of nucleosomes over the E2A binding site E box2 (Fig. 7d). At 3'Eκ there was also an accumulation of nucleosomes in Brwd1mut small pre-B cells (Supplementary Fig. 7b), which suggested that BRWD1 might regulate nucleosome organization through long-range loops.

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Extensive recombination at Iκg could distort apparent accessibility. However, comparison of Iκg in Rag1−/− pro-B cells, wild-type small pre-B cells and wild-type splenic B cells showed that Vκ segment accessibility was similar in Rag1−/− pro-B cells and wild-type small pre-B cells (Supplementary Fig. 6c–e). In contrast, apparent accessibility throughout Iκg was greatly diminished in splenic B cells. Therefore, in small pre-B cells, Iκg was poised for recombination, but substantial recombination had not yet occurred.

In Brwd1mut small pre-B cells there was greatly diminished accessibility at each RSS and at the exons encoding Jκ1, Jκ2 and Jκ4 compared with that in wild-type small pre-B cells (Fig. 7f). For Jκ5, the loss of accessibility observed in Brwd1mut small pre-B cells was most prominent at the RSS nonamer motif. In contrast, changes in accessibility at EκE were subtle, with a slight overall shift in the distribution of accessibility (Fig. 7a).

It has been postulated that nucleosomes must flank recombining Iκ segments as RAG2 is recruited to H3K4me3 (ref. 15). As predicted, in wild-type small pre-B cells, nucleosomes were positioned between Jκ segment exons, leaving the RSSs and Jκ segments nucleosome free (Fig. 7d and Supplementary Fig. 7a). In marked contrast, in Brwd1mut small pre-B cells, nucleosomes were positioned over each RSS and Jκ segment. Furthermore, whereas in wild-type small pre-B cells EκE was primarily free of nucleosomes, in Brwd1mut cells we noted accumulation of nucleosomes over the E2A binding site E box2 (Fig. 7d). At 3'Eκ there was also an accumulation of nucleosomes in Brwd1mut small pre-B cells (Supplementary Fig. 7b), which suggested that BRWD1 might regulate nucleosome organization through long-range loops.

Genome-wide, BRWD1 binding was associated with nucleosome depletion and enhanced DNA accessibility (Fig. 7e). Similarly, BRWD1-H3K9Ac-H3S10pK14Ac peaks were locally associated with nucleosome-free DNA (Fig. 7f). These associations were also observed in BRWD1-H3S10pK14Ac and H3S10pK14Ac peaks, but not in BRWD1-H3K9Ac or H3K9Ac peaks (Supplementary Fig. 7c).
The largest effect of BRWD1 on nucleosome placement was observed at extended GAGAG motifs that were enriched in BRWD1, H3K9Ac and H3S10pK14Ac (Supplementary Fig. 7d). These extended GAGAG motifs were normally relatively free of nucleosomes in wild-type small pre-B cells, whereas in Brwd1mut cells nucleosomes tended to occupy motifs in these peaks. Indeed, all ten peaks at which chromatin remodeling occurred had at least one GAGAG motif, whereas none of the six other peaks did.

**Figure 8** BRWD1 is required for recruitment of RAG1 and RAG2 to Igk. (a) Overlap of RAG1, RAG2 and H3K4me3 ChIP-Seq peaks (P < 10⁻⁷). (b) Coincidence of BRWD1, RAG1, RAG2 and H3K4me3 at the Jκ-Cκ region of the Igk locus. (c) Overlap of the indicated peaks (“Open” means accessible by ATAC-Seq) with RAG1, RAG2 and H3K4me3, with the distribution of peaks within regions of DNA given. The total number of peaks in each group is shown at right. (d) ChiP-qPCR for RAG1 and RAG2 at Jκ. Hbb-b2 (encoding β-globin) and Actg1 (encoding α-actin) genes in wild-type and Brwd1mut small pre-B cells (n = 3). *P < 0.001 versus wild-type (unpaired t-test). (e) ChiP-qPCR with H3K4me3-specific antibodies or control IgG from flow-sorted wild-type pre-B and small pre-B cells to detect various regions of Igk (n = 3). Data presented as average ± s.d.

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RAG1 and RAG2 was reduced by approximately fourfold in Brwd1mut small pre-B cells compared with that in wild-type cells (Fig. 8d). In contrast, RAG2 recruitment to Actg1 (ref. 15) was similar in the two cell lines. Expression of Rag1 and Rag2 was only slightly diminished in Brwd1mut small pre-B cells compared with wild-type cells (Supplementary Fig. 7f). Furthermore, the expression of other genes implicated in pre-B cell proliferation and Igk recombination, such as Tcf3 (encoding E2A; also known as Tcf2a), Pax5, Ikaros (encoding IKAROS), Ifng, Ifnb, Smarca4 (encoding BRG1) and Myc, was not significantly altered in Brwd1mut small pre-B cells compared with that in wild-type cells (Supplementary Fig. 7g). Rag2 binds H3K4me3, and therefore decreased RAG recruitment might reflect diminished nucleosome occupancy or methylation. However, there was only a modest decrease (30%) in amounts of H3K4me3 across GAGA motifs 42, providing a possible mechanism for the observed reduction.

In contrast, RAG2 recruitment to Tcf3 (encoding E2A), Ikaros, Pax5, and Smarca4 was not appreciably altered in Brwd1mut pre-B cells compared with wild-type cells (Supplementary Fig. 7g). Tcf3 (encoding E2A), Ikaros, Pax5, and Smarca4 are all lineage-restricted and stage-restricted mechanisms that remodel chromatin at Jk, and requires BRWD1 for efficient RAG protein binding.

DISCUSSION

Numerous findings have indicated that accessibility of antigen-receptor genes to recombination and recruitment of RAG proteins are required for recombination4,13. However, recent evidence has also made it clear that the usual mechanisms of accessibility are not sufficient to explain the cell-lineage-specific and stage-specific recombination of antigen-receptor loci1,2,23. However, under physiological conditions, only Igk is targeted for recombination in these cell lines. Here we show that a complex, lineage-specific and stage-restricted mechanism remodels the chromatin landscape at Jk to enable assembly of the RAG protein complex at RSS sites poised for recombination.

Central to productive assembly of the recombination machinery at Jk, and to opening the Igk locus to recombination, is BRWD1. Like Igk, Brwd1 transcription is repressed by STAT5 yet rapidly induced after a loss of IL-7R signaling. Subsequent targeting of BRWD1 is mediated by a very specific and relatively genome-wide restricted epi-genetic code that is dependent upon pre-BCR signaling. Downstream of the pre-BCR, activation of Erk induces phosphorylation of H3S10, a possible direct substrate of Erk32. Erk can be directly recruited to GAGA motifs42, providing a possible mechanism for the observed increase in amounts of H3S10p at GAGA-enriched DNA sequences. Erk also induces free nuclear E2A that binds and recruits histone acetyltransferases to E2A, where they acetylate regional histones at H3K9 and H3K14 (refs. 9,11). This specialized epigenetic code largely restricts BRWD1 to the putative recombination center at Jk. In contrast, the RAG proteins, which are recruited to general features of open chromatin, are broadly recruited to the region from which Igk is initiated. The coordinated control of BRWD1 expression and recruitment by IL-7R and pre-BCR, respectively, ensures that Igk recombination is restricted to small pre-B cells and follows Igh recombination2.

Remarkably, BRWD1 was required for the positioning of nucleosomes relative to GAGA motifs genome-wide. Although long GAGA motifs were highly enriched at BRWD1 sites, five nucleotide GAGA motifs were found 5′ to each functional Igk segment. In Drosophila, five nucleotide GAGAG motifs can recruit TRL41, which enhances gene accessibility to transcription factors37. It has been postulated that TRL travels along GAGAG motifs to slide or eject nucleosomes38, functions consistent with what we observed with BRWD1. Only one known mammalian molecule shares sequence homology with TRL: ThPOK (also known as cKrox)43. However, ThPOK has not been shown to position nucleosomes in a GAGA-dependent manner. Therefore, to the best of our knowledge, BRWD1 is the first mammalian protein associated with TRL-like functions.

Previous in vitro studies1,16,17,44 predicted that the specific chromatin-remodeling events associated with BRWD1 enable RAG-mediated recombination. Overall accessibility at Jk was increased by BRWD1 in our study, yet the most marked and consistent increase was at the Jk RSS nonamers, which have been proposed as the initial sites of Rag1 recruitment1,44,45. BRWD1 was also required for the precise positioning of nucleosomes flanking the RSSs and Igk exons. This is predicted to both enable H3K4me3-mediated RAG2 recruitment and help position the RAG complex at the RSSs.

Our data suggest that there are important differences between the accessible states associated with enhanced transcription and those that enable immunoglobulin gene recombination. Transcription is usually associated with nucleosome positioning at transcription start sites and a relative depletion of nucleosomes at exons46. However, BRWD1 binding resulted in precise placement of nucleosomes flanking RSSs and Igk exons, changes that would not necessarily be reflected in transcriptional changes. Indeed, Kgt was decreased only twofold in Brwd1mut pre-B cells compared with wild-type cells, despite the large difference in nucleosome positioning throughout the Igk region and greatly diminished Igk recombination. This apparent discrepancy between Kgt and Igk recombination suggests that although transcription might be permissive for recombination47, recombination efficiency is not proportional to the magnitude of transcription.

BRWD1 lacks identifiable catalytic domains, and it is unlikely that it directly mediates nucleosome positioning or other BRWD1-associated functions such as acetylation of H4K16. Rather, we propose that BRWD1 serves as a platform that binds to specific epigenetic landscapes, where it assembles and coordinates the activities of other chromatin regulators. BRWD1 can recruit the ATP-dependent chromatin-remodeling protein SMARCA4 (also known as BRG1) (ref. 28), the catalytic subunit of the mammalian SWI/SNF complex. In vivo, SMARCA4 binds immunoglobulin gene segments when they are accessible to RAG1 or RAG2 and is required for recombination48,49. Other binding partners of BRWD1 remain to be identified.

It seems that other molecules or processes can partially compensate for BRWD1 deficiency. B cell lymphopoiesis was not ablated in Brwd1mut mice, and in those B cells able to transit into the periphery there was some restoration of H4K16Ac at Jk. There are two BRWD1 paralogues in the mouse genome: pleckstrin homology interacting protein (PHIP) and BRWD3. Like BRWD1, both PHIP and BRWD3 contain WD40 repeats and tandem BROMO domains. Both have >60% amino acid sequence homology with BRWD1 through large N-terminal domains29, and both are expressed in developing B lymphocytes. However, neither PHIP expression nor BRWD3 expression is induced in pre-B cells, and Igk chromatin structure was remarkably aberrant in Brwd1mut mice. These observations suggest that BRWD1 and BRWD1-associated molecules are the major determinants of Igk chromatin structure in small pre-B cells.

Our results show that opening the Igk locus to recombination requires coordinated regulation of stage-specific signaling processes and the induction of BRWD1, whose expression is highly restricted. These findings raise the possibility that those molecules regulating recombination at other antigen-receptor loci, including Igh, Igk, Tcrb and Tcra, are also specialized, developmentally restricted and tightly regulated by signals critical for key developmental transitions. We postulate that such layers of combinatorial specificity ensure that antigen-receptor gene recombination is sequential and occurs only in the correct developmental contexts.
METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. ChIP-Seq and ATAC-Seq data sets have been deposited in the GEO database with accession number GSE63302.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
M.M. designed, carried out and analyzed most of the experiments including ChIP-Seq and ATAC-Seq, oversaw the entire project and wrote the first draft of the manuscript. K.M.H. assisted M.M. in flow cytometry in some of the experiments, as well as in confocal microscopy, immunoblotting, shRNA experiments and adoptive-transfer studies. A.T. assisted in flow cytometry in some of the experiments. M.M.-C. performed ChIP-Seq and ATAC-Seq analysis with M.M. N.B. worked with M.M.-C. G.T. generated RAG1, RAG2 and H3K4me3 ChIP-Seq data. J.H.T. assisted M.M. in development of the ATAC-Seq methodology. J.B. assisted M.M. in HS10p ChIP-J.J.E. generated and provided advice about BrdU/DNA mice. D.G.S. collaborated on RAG and H3K4me3 ChIP-Seq data. M.R.C. oversaw the entire project and prepared the final draft of the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Online Methods.

Mice. Wild-type (C57BL/6 and C57BL/6 backcrossed to C3H/HeJ), Ifng−/−/Ifnrb−/− (C57BL/6), Brdwlmut (C57BL/6-C3H/HeJ), Ragl−/− (C57BL/6) and Ifgkl (BALB/c) mice were housed in the animal facilities of the University of Chicago. Ragl−/−, Rag2−/−, Ragl−/−B18 Igk−/− mice in with Rag1(D708A) transgenic, and Ragl−/−B18 Igk−/− mice were housed in the animal facility of Yale University. Mice were used at 6–12 weeks of age, and experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Chicago.

Isolation, culture and flow cytometry of BM B cell progenitors. BM was collected from wild-type mice, and cells were resuspended in staining buffer (3% vol/vol FBS in PBS). Erythrocytes were lysed, and cells were stained with antibodies specific for CD11c (HL3), NK1.1 (PK136), TCRβ(50 or biotin). Pro-B cells (Lin−CD19+B220+CD43+IgM−), large pre-B cells antibodies specific for CD11c (HL3), NK1.1 (PK136), TCRβ collected from wild-type mice, and cells were resuspended in staining buffer in the animal facility of Yale University. Mice were used at 6–12 weeks of age, at a concentration of 10 ng/ml (high IL-7) or 0.1 ng/ml (low IL-7) (ref. 10).

Cell cycle analysis. B cell progenitors at different developmental stages were flow sorted and then incubated in a solution containing propidium iodide. The analysis was done on a FACScan (Becton Dickinson) as described16. The proportion of cells in G1, S and G2-M phases of the cell cycle was analyzed with FlowJo and Cell Quest software (Becton Dickinson).

Apoptosis assays. Apoptosis was evaluated via flow cytometry with fluorescence-conjugated annexin V (BD Pharmingen). When cell-surface staining was completed, the cells were washed and incubated in annexin V binding buffer with annexin V at a 1:20 dilution for 20 min at 25 °C. The cells were then washed, resuspended in annexin V binding buffer, and immediately analyzed by flow cytometry. We carried out annexin V staining in conjunction with staining with the vital dye 7-amino-actinomycin D (7-AAD) to differentiate early apoptosis (annexin V−7-AAD−) from late apoptosis or necrosis (annexin V−7-AAD+).

PCR analysis of Igk rearrangements. Semi-quantitative PCR with genomic DNA was done as described10 (primers are shown in Supplementary Table 1). For PCR analysis of Igk rearrangements, we used small pre-B cells from wild-type and Brdwlmut mice or Ifng−/−/Ifnrb−/− pre-B cell populations (cultured for 48 h in high or low concentrations of IL-7). Degenerate Vκ and Igk intron primers10 and fivefold template dilutions were used for PCR. A region in Eκ was controlled to allow the generation of genomic DNA (primers Eκ-F and Eκ-R). DNA from wild-type splenic IgM+ B cells was used as a positive control. The intensity of the band for each reassembly product was divided by that for the corresponding Igk intron fragment, and the resulting value was then normalized to values obtained from IgM+ B cells, given a value of 1. Quantitative analysis of Vκ−Iκ1 reassembly was done by qPCR (primers degVκX and κ1F-κ1R; Supplementary Table 1), with Eκ primer product used as a control.

Analysis of Igk usage. Total RNA was extracted with TRIzol (Invitrogen) for flow-sorted small pre-B cells from wild-type and Brdwlmut mice. cDNA was synthesized as described above. Vκ−Iκ1 rearrangements were amplified by PCR with high-fidelity Taq enzyme (Roche) and specific primers (Supplementary Table 1). PCR amplicons were then cloned with the TOPO-TA cloning kit (Invitrogen) and sequenced (UC core facility). Unique sequences were analyzed for Igk usage.

Chromatin immunoprecipitation. We used a ChiP assay kit according to the manufacturer’s instructions (Millipore 17-295) (ref. 9). Samples were immunoprecipitated with antibodies specific for H3K9ac (Millipore, 06-942, lot 2279810), H3S10pK14Ac (Millipore, 07-180, lot 2208929), Brdwl (1-5, Santa Cruz Biotechnology, sc-83517, lot J1508), H3S10p (Millipore, 06-570, lot 220541), H4K16Ac (Millipore, 07-329, lot 2073125), H3K4me3 (Millipore, 07-473, lot 2430389), rabbit IgG (101-000-00; Jackson ImmunoResearch Labs), RAG1 or RAG2 (ref. 15). Purified DNA was then analyzed with quantitative real-time PCR primers (Supplementary Table 1).

ChIP-Seq. Chromatin from flow-sorted small pre-B cells (4×10^6 to 7×10^6) was used for each ChiP experiment with antibodies to H3K9ac, H3S10p, and BRD1 as described above. DNA libraries were prepared from the sheared chromatin (200–600 bp). Libraries were sequenced on an Illumina HiSeq2000. The sequences were aligned to the mm9 reference genome (National Center for Biotechnology Information build mm9_NCBI_build_37.1) with Bowtie alignment software, and only reads with unique matches were retained.

ChIP-Seq peak calling and motif analysis. Peaks for ChIP-Seq samples were called with MACS2 at a p value threshold of 10^-7. We generated peak groups by considering overlapping peak regions of at least 10 bp. HOMER software (hyergeometric optimization of motif enrichment) for de novo motif discovery and next-generation sequencing analysis was used for new prediction of motifs in the peaks. Additionally, we carried out de novo motif searches independently on each peak group with meme, asking for the top ten motifs. GA repeat motifs were obtained from manual filtering of the motifs found by meme.
For further motif analysis and DNA footprinting, we recalled peaks at a P value threshold of $10^{-7}$. We then searched for the GA repeat motifs in each of these peak groups using MAST\textsuperscript{50}, counting both the total number of hits for the motif and the fraction of sequences with at least one hit for the motif.

Assay for transposase-accessible chromatin with sequencing (ATAC-Seq). ATAC-Seq was done as described\textsuperscript{39}. Flow-sorted small pre-B cells (1 \times 10^6) from wild-type and Brwd1\textsuperscript{mut} mice were used for each ATAC-Seq assay. To prepare nuclei, we centrifuged cells at 500g for 5 min, washed them with ice-cold PBS and centrifuged them again at 500g for 5 min. Cells were lysed with cold lysis buffer (10 mM Tris- HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl\textsubscript{2} and 0.1% IGEPAL CA-630). Immediately after lysis, nuclei were spun at 500g for 10 min at 4°C. Supernatant was carefully pipetted away from the pellet after centrifugation. Immediately after the nuclei prep, the pellet was resuspended in the transposase reaction mix (25 µl 2× Tagment buffer, 2.5 µl Tagment DNA enzyme (Illumina, FC-121-1030) and 22.5 µl nuclease-free water). The transposition reaction was carried out at 37°C for 30 min. After transposition, the sample was purified with a Qiagen MinElute kit. After purification, we amplified library fragments using Nextera PCR Primers (IlluminaNextera Index kit) and NEBnext PCR master mix (New England BioLabs, 0541) for a total of 10–12 cycles followed by purification with a Qiagen PCR cleanup kit.

The amplified, adapter-ligated libraries were size-selected with Life Technologies’ E-Gel SizeSelect gel system in the range of 150–650 bp. We quantified the size-selected libraries with an Agilent Bioanalyzer and via qPCR in triplicate using the KAPA Library Quantification Kit on the Life Technologies’ E-Gel SizeSelect gel system in the range of 150–650 bp. We quantified library fragments using Nextera PCR Primers (IlluminaNextera Index kit) and NEBnext PCR master mix (New England BioLabs, 0541) for a total of 10–12 cycles followed by purification with a Qiagen PCR cleanup kit.

Quality control and DNA alignment. All raw sequence data were quality-trimmed to a minimum Phred score of 20 with Trimomatic\textsuperscript{51}. Alignment to reference genome mm9 was done with BWA\textsuperscript{52}. For ATAC-Seq data, read pairs where one pair passed quality trimming but the other did not were aligned separately and merged with the paired-end alignments. PCR duplicates were removed using Picard MarkDuplicates, and alignments with an edit distance from the reference of more than 2, or those that were mapped to the reference multiple times, were removed.

ATAC-Seq analysis. Read-alignment positions were adjusted according to their strand: +4 bp for + strand alignments, and −5 bp for – strand alignments. We called open chromatin regions using Zinba\textsuperscript{53} with a window size of 300 bp, an offset of 75 bp, and a posterior probability threshold of 0.8.

For nucleosome positioning, we filtered properly paired alignments by their fragment size. Fragments less than 100 bp in size were considered nucleosome free and were replaced with a single BED region and used as a background. Those with sizes between 180 and 247 bp were considered mononucleosomes and were replaced with a single BED region; those with sizes between 315 and 473 bp were considered dinucleosomes and were replaced with two BED regions, each spanning half the overall fragment length; and those with sizes between 558 and 615 bp were considered trinucleosomes and were replaced with three BED regions, each spanning one-third of the overall fragment length. The mono-, di- and trinucleosome regions were concatenated and used as the nucleosome signal. The resulting BED regions were analyzed with DANPOS\textsuperscript{54} with the parameters –p 1 –a 1 –d 20 –clonalcut 0 to identify regions enriched or depleted for nucleosomes.

We obtained DNA footprinting data by combining bigWig enrichment tracks for ChIP-Seq and ATAC-Seq data over specified BED regions (combinations of peak calls or motif hits). ChIP-Seq enrichment data were generated by MACS2 as described above. Open chromatin enrichment data from ATAC-Seq were generated from the read-adjusted alignments with custom scripts and normalized to reads per million alignments, and nucleosome-positioning enrichment data were obtained from DANPOS\textsuperscript{54}. DNA footprinting scores were averaged over 10-bp bins from enrichment tracks with custom scripts.

For correlations between signals, we used the UCSC genome browser’s bigWigToWig tool to extract profiles for the Igk loci from each of the enrichment tracks. Different enrichment profiles were compared using both Pearson and Spearman correlation coefficients; the latter was included to prevent regions of very high enrichment from dominating the correlation.

Statistical analysis. Data were analyzed via unpaired t-test and analysis of variance followed by the test of least-significant difference for comparisons within and between groups. All categories in each analyzed experimental panel were compared, and P values less than 0.05 were considered significant. All P values less than 0.001 were rounded to facilitate comparisons of results.

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