The chromatin remodeler Brg1 activates enhancer repertoires to establish B cell identity and modulate cell growth

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Early B cell development is orchestrated by the combined activities of the transcriptional regulators E2A, EBF1, Foxo1 and Ikaros. However, how the genome-wide binding patterns of these regulators are modulated during B lineage development remains to be determined. Here we found that in lymphoid progenitor cells, the chromatin remodeler Brg1 specified the B cell fate. In committed pro-B cells, Brg1 regulated contraction of the locus encoding the immunoglobulin heavy chain (Igh) and controlled expression of the gene encoding the transcription factor c-Myc (Myc) to modulate the expression of genes encoding products that regulate ribosome biogenesis. In committed pro-B cells, Brg1 suppressed a pre-B lineage–specific pattern of gene expression. Finally, we found that Brg1 acted mechanistically to establish B cell fate and modulate cell growth by facilitating access of lineage-specific transcription factors to enhancer repertoires.

The developmental progress of B cells can also be characterized by the status of the rearrangement of immunoglobulin-encoding genes. The locus encoding the immunoglobulin heavy chain (Igh) undergoes recombination of the diversity (D) region and joining (J) region (D11-JH recombination) starting at the BLP stage, whereas rearrangement of the variable (V), D and J regions (VH-DJH rearrangement) occurs at the pro-B cell stage. Recombination of the locus encoding the immunoglobulin light chain is initiated and completed at the small pre-B cell stage. Recombination of Igh coincides with commitment to the B lineage and is tightly regulated to ensure that DJ1-JH recombination precedes VH-DJH rearrangement and that the entire VH repertoire is represented. The mouse VH repertoire is composed of over 100 VH segments divided across different families that are segregated into proximal and distal VH segments11. During developmental progression, distal VH segments are brought into closer spatial proximity to IJH segments through locus contraction12. Contraction of the Igh locus is controlled by multiple transcription factors, including E2A, YY1 and Pax5 (refs. 13–15).

Lineage-specific transcriptional regulators such as E2A, EBF1 and Foxo1 act mainly by binding to distally located enhancer elements that are characterized by DNase I hypersensitivity, active histone marks and non-coding transcription16. On the one hand, enhancers displaying the histone marks of histone H3 monomethylated at Lys4 (H3K4me1), histone H3 dimethylated at Lys4 (H3K4me2) and histone H3 acetylated at Lys27 (H3K27ac) are considered active and are bound by the histone acetyltransferase p300 (ref. 17). On the other hand, enhancers without deposition of H3K27ac are thought to be in a poised state17.

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Enhancers activate transcription by looping to their cognate promoter regions. Promoter-enhancer interactions are facilitated by the mediator or cohesin complexes. Super-enhancers, which represent mediator or cohesin complexes. Promoter-enhancer interactions are facilitated by the mediator or cohesin complexes. Super-enhancers, which represent mediator or cohesin complexes. Enhancer elements need to be regulated genes and are characterized by a high density of binding of promoters and transcription factors. Enhancers activate transcription by looping to their cognate promoter regions. Promoter-enhancer interactions are facilitated by the mediator or cohesin complexes. The use of the BAF complex underlies its specific polymorphic composition. The BAF complex consists of at least 14 subunits encoded by 28 genes. The BAF complex is essential for accessibility to transcription factor–regulators closely associated with a B lineage–specific transcriptional stages to orchestrate B cell development. Specifically, we found that Brg1 controlled pro-B cell growth and prevented premature pre-B cell differentiation by permitting EBF1, Ikaros and Pax5 access to a distally located Myc super-enhancer. Together these observations show how a lineage-specific chromatin remodeler specifies cell fate, regulates cell growth and enforces developmental checkpoints.

**RESULTS**

**Brg1 specifies the B cell fate**

Published studies have indicated an important role for Brg1 in early B cell development. However, it has remained unclear how Brg1 expression acts to orchestrate B cell fate. As a first approach to addressing this question, we depleted the CLP compartment of Brg1 expression through the use of mice with loxP-flanked alleles encoding Brg1 (Smarca4^fl/fl) deleted by Cre recombinase expressed from the gene encoding the interleukin 7 (IL-7) receptor α-chain (IL7r^Cre/+). The use of IL7r^Cre/+ leads to efficient recombination of loxP-flanked alleles in the lymphoid compartment with an onset at the CLP stage in the BM. To alleviate potential defects due to heterozygous nature of IL7r^Cre+, we directly compared Smarc4^fl/Il7r^Cre/+ mice with their Smarc4^fl/Il7r^Cre/++ littermates (control mice). Smarc4^fl/Il7r^Cre/+ mice did not display gross abnormalities, and their BM cellularity was normal (Fig. 1a). B cell cellularity, however, was more than tenfold lower in Smarc4^fl/Il7r^Cre/+ mice than in the Smarc4^fl/Il7r^Cre/++ control mice (Fig. 1b). In concordance with published results, the cellularity of the pro-B cell, pre-B cell, immature B cell and mature B cell compartments was greatly reduced in BM

![Figure 1](image-url)
derived from Smarca4fl/flIl7rCre+/mice relative to the cellularity of those compartments in the Smarca4fl/flIl7rCre/+ control mice (Fig. 1c and Supplementary Fig. 1). In contrast, the number of ALPs and BLPs was not altered in Smarca4fl/flIl7rCre/+ mice relative to the number of these cells in their Smarca4fl/flIl7rCre/+ littermates (Fig. 1d).

To determine whether B cells detected in Smarca4fl/flIl7rCre/+ mice had undergone efficient depletion of Brg1 expression, we crossed Smarca4fl/flIl7rCre/+ mice with mice containing sequence encoding yellow fluorescent protein (YFP) inserted into the ubiquitously expressed Rosa26 locus. In these Rosa26YFP/+ mice, YFP expression is blocked by a loxP-flanked ‘stop’ site, which is removed in cells that express Cre recombinase. On the one hand, large proportion of Ly6D+ CLPs (>90%) from Smarca4fl/flIl7rCre/+Rosa26YFP/+ mice displayed YFP expression, indicative of efficient deletion in the BLP compartment (Fig. 1e). On the other hand, a substantial proportion of pro-B cells and pre-B cells from Smarca4fl/flIl7rCre/+Rosa26YFP/+ mice lacked YFP expression (Fig. 1e), which suggested that Brg1 expression was not completely abrogated in the pro-B cells and pre-B cells that developed in Smarca4fl/flIl7rCre/+ mice.

To determine whether Brg1 expression in hematopoietic progenitor cells is required before and/or at the CLP cell stage, we depleted cells of Brg1 expression through the use of mice with transgenic expression of tamoxifen-inducible Cre linked to the estrogen receptor (ER-Cre). For this, we injected CD45.2+ Smarca4fl/flER-Cre and Smarca4fl/flER-Cre BM cells together with CD45.1+ wild-type BM cells into CD45.1+ recipient mice. At 10 weeks after transplantation, we injected tamoxifen into the recipient mice and assessed the presence of CLPs in these mice. Treatment of the mice with tamoxifen resulted in the generation of considerably fewer CLPs from Smarca4fl/flER-Cre (Smarca4fl/fl) BM cells than from Smarca4fl/flER-Cre (Smarca4fl/fl) BM cells (Supplementary Fig. 2a). The abundance of BLPs was further reduced in the absence of Brg1, and developing Smarca4fl/fl B cells were almost completely undetectable (Supplementary Fig. 2a,b). Together these observations indicated that Brg1 was required for establishment of the B cell fate.

**Genome-wide occupancy by Brg1 in pro-B cells**

As a first approach to determining how Brg1 regulates pro-B cell development, we examined the expression of Smarca4 transcripts during hematopoiesis. For this purpose, we isolated RNA from LSK (Lin−Sca-1−c-Kit+ cells), LMPPs (lymphoid-primed multipotent progenitors), ALPs, BLPs, pro-B cells, pre-B cells, immature B cells and mature B cells from wild-type mice and analyzed Smarca4 expression. We found that Smarca4 transcripts were absent or their abundance was low in the majority of hematopoietic progenitors but their abundance was elevated in BLPs relative to that in the rest of the hematopoietic progenitor cells (Fig. 2a). In cells committed to the B cell lineage, Smarca4 expression was highest in pro-B cells but was decreased in pre-B cells (Fig. 2a).

To assess the spectrum of Brg1-bound sites across the pro-B cell genome, we performed chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) with an antibody to Brg1. We found that Brg1 occupancy was abundant (13,000 bound sites), with the majority of sites present cut-off for tag count as determined by HOMER software. (Fig. 2a). Quantification of Brg1 and p300 tags in BLPs was not completely abrogated in the pro-B cells and pre-B cells that lacked YFP expression (Fig. 2b), indicative of efficient deletion in the BLP compartment (Fig. 2a).

**Figure 2** Genome-wide occupancy by Brg1 across the pro-B cell genome. (a) Expression of Smarca4 and Smarca2 transcripts during hematopoiesis and early B cell development (horizontal axis); results are presented relative to those of the control gene Actb. (b) Distribution of 13,169 Brg1-binding sites across genomic regions in Rag1−/− pro-B cells. TSS, transcription start site; TTS, transcription termination site; UTR, untranslated region. (c) Heat map of ChIP-Seq data showing distribution of the deposition of H3K4me1, H3K4me2 and H3K4me3 in Rag1−/− pro-B cells, assessed in a 6-kilobase window across Brg1-bound sites, gated on genome-wide Brg1-bound sites. (d) Quantification of Brg1 and p300 tags in Rag1−/− pro-B cells, assessed in a 200–base pair window of combined Brg1- and p300-binding sites. Binding sites present cut-off for tag count as determined by HOMER software. (e) Occupancy by Brg1, p300 and E2A across Pax5 (chromosome 4, positions 44,703,000–44,730,000 in the mm9 NCBI assembly of the mouse genome) and Cd19 (chromosome 7, positions 133,551,000–133,564,000 in mm9) in Rag1−/− pro-B cells. (f) Association of cis-regulatory sequences with Brg1 occupancy; letter size indicates nucleotide frequency. Numbers at left indicate significance of motif occurrence (binomial test). (g) Brg1 ‘reads’ for a 100–base pair window centered on 5,000 Ikaros-, E2A-, EBF1-, Pax5-, PU.1- and CTCF-binding sites. Data are representative of two experiments (a; error bars, s.e.m. of n = 2 independent cell sorts), or one experiment (b–g).
Figure 3  Brg1 is required for the establishment of an accessible B lineage–specific enhancer repertoire. (a) ATAC-Seq tag coverage in ALPs and BLPs from Smarcd<sup>fl/fl</sup>/I<sup>°</sup>/I<sup>°</sup> and Smarca<sup>fl/fl</sup>/I<sup>°</sup>/I<sup>°</sup> mice, plotted as a function of genomic distance from E2A- (left), Ikaros- (middle), and CTCF-bound sites (right). Each symbol represents an individual mouse; small horizontal lines indicate the mean. (b) ATAC ‘reads’ and deposition of H3K4me2 or occupancy by E2A, Ikaros or EBF1 (left margin) across intergenic genomic regions flanking a 3′ Foxo1 enhancer (left; chromosome 3, positions 52,243,000–52,253,000) or a Cd79a regulatory region (right; chromosome 7, positions 25,677,000–25,687,000) in B cells at various stages of development (left margin); pre-pro-B cells are represented by E2A-deficient multipotent progenitors, and pro-B cells were derived from Rag1<sup>−/−</sup> mice. (c) Abundance of E2A protein in BM Lin<sup>+</sup> cells (CD11b<sup>+</sup>Ter119<sup>+</sup>CD3<sup>+</sup>NK1.1<sup>+</sup>GR1<sup>+</sup>), ALPs, BLPs and BM B cells (CD19<sup>+</sup>) from E2A-GFP mice, presented as mean fluorescence intensity (MFI). Each symbol represents an individual mouse; small horizontal lines indicate the mean. (d) RT-PCR analysis of Tcf3, Ebf1 and Ikzf1 in ALPs and BLPs sorted as in a, presented relative to Actb expression. NS, not significant (*P > 0.05); *P < 0.05 and **P < 0.01 (two-tailed unpaired Student’s t-test). Data are from two independent experiments (a,b) or are representative of one experiment (c,d); average and s.d. of biological triplicates in d).

To evaluate the degree of overlap between Brg1 occupancy and an active enhancer repertoire, including super-enhancers, we compared Brg1-bound sites with those occupied by p300. Notably, a large fraction of Brg1–bound sites overlapped with those occupied by p300 (Fig. 2d), which indicated that Brg1 associated mainly with an active enhancer repertoire. As expected, we observed Brg1 occupancy across enhancer elements that exhibited binding of E2A and p300 and were associated with the expression of a subset of B lineage–specific genes, including Pax5 and Cd79a (Fig. 2e). At a genome-wide level, sites occupied by Brg1 showed considerable enrichment for consensus binding motifs for the transcription factors E2A, EBF1, IRF, Ikaros, Pax5, and Runx (Fig. 2f). Consistent with that analysis, sites occupied by Brg1 were closely associated with those occupied by Ikaros and, to a lesser degree, with E2A-, EBF-, Pax5- and Pu.1-bound sites (Fig. 2g). In contrast, Brg1-bound sites were not frequently associated with sites occupied by the transcriptional repressor CTCF (Fig. 2g). We observed binding of Brg1 at super-enhancers, including the Foxo1 and I<sub>α</sub>p<sub>5δd</sub> super-enhancers (Supplementary Fig. 3a). The proportion of Brg1 bound to super-enhancers was comparable to that of transcription factors known to associate with super-enhancers, such as E2A, Pax5 and Med1 (a member of the mediator complex) (Supplementary Fig. 3b). To assess the strength with which Brg1 bound to super-enhancers, we normalized the number of tag counts to the size of the enhancers, since the median size of super-enhancers is approximately tenfold greater than that of typical enhancers<sup>19</sup>. We found that unlike results obtained for Med1, the strength of Brg1’s binding was not greater at super-enhancers than at typical enhancers (Supplementary Fig. 3c).

Published studies have established that Ikaros interacts with Brg1 (refs. 27,28). Such findings raised the question of whether Ikaros recruits Brg1 to the pro-B cell enhancer repertoire. Hence, we investigated the binding of Ikaros in progenitors of B cells using E2A-deficient pre-pro-B cells<sup>29</sup>. We found that Ikaros bound to a spectrum of sites in pre-pro-B cells distinct from that observed in pro-B cells, even though the expression of Ikaros protein was similar in both cell types<sup>30</sup> (Supplementary Fig. 4a). Notably, the ‘<i>de novo</i>’ enhancer repertoire associated with pre-B cells (‘new’ compared with that in pre-pro-B cells) displayed overlapping
patterns of occupancy by Ikaros and occupancy by Brg1 (Supplementary Fig. 4a). Furthermore, pro-B cell–specific Brg1-bound sites were associated with regions that underwent depletion of nucleosomes during the transition from the pre-pro-B cell stage to the pro-B cell stage (Supplementary Fig. 4b). Collectively, these results showed that Brg1 binding was closely associated with a de novo enhancer repertoire that was established during the transition from pre-pro-B cell to pro-B cell.

**Brg1 facilitates access to enhancer repertoires**

As Brg1 is a chromatin remodeler and is required for specification of the B cell fate, we considered the possibility that Brg1 might serve a crucial role in the generation of a de novo enhancer repertoire by facilitating the depletion of nucleosomes across an ensemble of B lineage–specific enhancers. The expression of genes associated with the establishment of B cell identity is activated in BLPs, which suggests that a pro-B cell–specific enhancer repertoire is established in the BLP compartment. As a first approach to exploring this possibility, we used ATAC (‘assay for transposase-accessible chromatin’) sequencing (ATAC-Seq) to capture open chromatin sites31. ATAC-Seq generates results comparable to those obtained by mapping of DNase I–hypersensitivity sites and involves incubation of nuclei with a transposase. The transposase ‘preferentially’ integrates into active
regulatory regions and simultaneously fragments and tags the genome with sequencing adapters. This technique can be used for small numbers of cells, including progenitor populations. To identify accessible enhancer repertoires in progenitors of B cells, we sorted ALPs and BLPs from Smarca4fl/fl Cre/+ and Smarca4fl/fl Cre/+ mice. We incubated the sorted cells with transposase and sequenced the results at a global scale. Next, we plotted ATAC-Seq tag counts as a function of genomic separation from E2A-, Ikaros-, and EBF1- or CTCF-bound sites in ALPs and BLPs (Fig. 3a). These data indicated that at a developmental stage at which progenitor cells become specified to the B lineage, the enhancer repertoire associated with a B lineage–specific program of gene expression became depleted of nucleosomes. In contrast, Brg1-deficient BLPS displayed either no increase in the number ATAC reads for enhancer repertoires associated with occupancy by E2A, Ikaros or EBF1 relative to that in ALPs or a reduced increase compared with that of Brg1-deficient BLPS (Fig. 3a). Genomic regions associated with CTCF occupancy showed comparable ATAC tag counts in both Brg1-sufficient and Brg1-deficient ALPs and BLPS (Fig. 3a). Since E2A and EBF1 directly regulate Foxo1 expression in the BLP compartment, we investigated whether putative enhancers associated with the Foxo1 locus underwent depletion of nucleosomes. We found that, indeed, Brg1 expression was essential for the eviction of nucleosomes across a distally located Foxo1 enhancer associated with occupancy by E2A and EBF1 (Fig. 3b, left). Likewise, Brg1 expression was required for the depletion of nucleosomes across a regulatory element located within the Cd79a locus (which encodes the immunoglobulin α-chain) (Fig. 3b, right).

The increase in accessibility observed in BLPS relative to accessibility in ALPs might have simply reflected increased abundance of the B lineage–specific transcription factors. To exclude this possibility, we examined E2A protein expression in ALPs and BLPS from mice expressing green fluorescent protein (GFP)-tagged E2A (E2A-GFP mice). We found that abundance of E2A protein did not change during the ALP-to-BLP transition (Fig. 3c). Likewise, the abundance of transcripts encoding E2A (Tcf3) and Ikaros (Ikzf1) was equivalent in ALPs and BLPS (Fig. 3d). Thus, the enhancement in open chromatin seen at E2A-binding sites in Brg1-sufficient BLPS relative to its presence in ALPs could not readily be explained in terms of elevated protein expression. Instead, we suggest that Brg1 facilitated the depletion of nucleosomes across an enhancer repertoire closely associated with a B lineage–specific program of gene expression.

**Brg1 is essential for promoting Igh locus contraction**

Since Brg1 abundance is particularly substantial in pro-B cells and Brg1 binds together with factors that are associated with contraction of the Igh locus, we assessed occupancy of the Igh locus by Brg1. As reported, Brg1 bound to JH segments (Fig. 4a). However, occupancy by Brg1 was not limited to the JH elements. Instead, binding of Brg1...
was dispersed throughout the Igh locus and overlapped with the deposition of H3K4me2 and regions depleted of nucleosomes, as revealed by ATAC-Seq (Fig. 4a). Upon inspecting Hi-C data (meta-analysis of chromosome-conformation capture followed by deep sequencing) generated from pro-B cells, we observed an intricate pattern of significant short-range genomic interactions that spanned the entire cluster of \( V_H \) segments (Fig. 4a), consistent with an organization of bundles of loops\(^{33,34} \). Notably, we identified a giant loop connecting the extreme ends of the Igh locus and thus identified the boundaries that define the Igh topological domain (Fig. 4a). We also identified a spectrum of long-range genomic interactions involving distal \( V_H \) region clusters and Igh segments across the 3’ end of the locus and found that a subset of these putative anchors showed enrichment for Brg1 occupancy (Fig. 4a,b). Genomic interactions involving Brg1 showed enrichment mainly for binding of lineage-specific transcription factors, including Pax5, EBF1, YY1, Ikaros and E2A, as well as Med1, relative to the general occupancy of the transcription factor on the Igh locus (Fig. 4c).

To determine whether Brg1 modulated the accessibility of transcription factor–binding sites across the Igh locus, we assessed ATAC-Seq ‘reads’ in Brg1-sufficient and Brg1-deficient pro-B cells. We isolated pro-B cells from B220\(^+\) cell populations obtained from Smarca4\(^{-/-}\)ER-Cre and Smarca4\(^{fl/fl}\)ER-Cre BM and expanded these populations in the presence of IL-7 and stem-cell factor. We found that the vast majority of pro-B cells underwent depletion of Brg1 protein upon culture of the cells for 3 d in the presence of tamoxifen (data not shown). As expected, loss of Brg1 expression was closely associated with decreased chromatin accessibility for a large fraction of sites (50%) bound by lineage-specific transcription factors, but not those bound by CTCF (Fig. 4d,c). The abundance of RNA transcripts encoding lineage-specific transcription factors was not affected by depletion of Brg1 expression (Supplementary Fig. 5a). Consistent with reduced binding of Pax5 and YY1, we observed a reduction in antisense transcription associated with distal regulatory regions called ‘PAIR elements’\(^{35,36} \) (Supplementary Fig. 5c).

Our findings indicated that Brg1 occupancy across the Igh locus was closely associated with transcription factor–binding sites. The transcription factors that bind to these sites have been linked to contraction of the Igh locus\(^{13-15} \). These observations raised the possibility that Brg1 acts to evict nucleosomes positioned across regulatory elements that mediate long-range genomic interactions. To investigate this possibility, we measured the spatial distances separating the distal \( V_H \) region cluster from a genomic region located 3’ of the Igh locus. Specifically, we obtained pro-B cells from mice deficient in recombination-activating gene 1 (Rag1\(^{-/-}\) mice) to look at germline configuration of the Igh locus and depleted the cells of Brg1 expression through the transduction of short hairpin RNA (shRNA) directed against Smarca4. We then examined the topology of the Igh locus in the transduced pro-B cells by three-dimensional fluorescence in situ hybridization. Notably, we found that the Igh locus displayed significant long-range decontraction of the locus in the pro-B cell population upon depletion of Brg1 expression (Fig. 4f and Supplementary Fig. 5b). Since reduced contraction should lead to ‘preferred’ use of the proximal \( V_H \) segments, we assessed the use of proximal and distal \( V_H \) segments in pre-B cells sorted from Smarca4\(^{fl/fl}\)IgM\(^{-/-}\)IgD\(^{-/-}\) or Smarca4\(^{fl/fl}\)IgM\(^{-/-}\)IgD\(^{-/-}\) mice. In agreement with the reduction in contraction of the Igh locus in Rag1\(^{-/-}\) pro-B cells depleted of Brg1, we found significantly more use of proximal \( V_H \) segments than distal \( J_H \) segments in Smarca4\(^{fl/fl}\)IgM\(^{-/-}\)IgD\(^{-/-}\) pre-B cells than in Smarca4\(^{fl/fl}\)IgM\(^{-/-}\)IgD\(^{-/-}\) pre-B cells (Fig. 4g). Together these observations indicated that Brg1 was essential for promoting contraction of the Igh locus.

**Brg1 regulates Myc expression to promote pro-B cell growth**

To determine whether in committed pro-B cells Brg1 acts to maintain active enhancer repertoire, we isolated RNA from Brg1-sufficient and Brg1-deficient pro-B cells and analyzed the RNA by high-throughput sequencing technologies for cDNA (RNA-Seq). We found that approximately 1,800 genes displayed significant differences in transcript abundance in pro-B cells depleted of Brg1 expression relative to their abundance in Brg1-sufficient pro-B cells (Fig. 5a). We found that at least 1,000 genes underwent a change in expression of at least 2-fold or more upon depletion of Brg1 (Supplementary Table 1). Notably, gene-ontology analysis showed that the absence of Brg1 affected the expression of a spectrum of genes encoding products associated with ribosome biogenesis (Fig. 5b). The abundance of transcripts encoding products associated with the metabolism of rRNA and the synthesis of non-coding RNA was also lower in Brg1-deficient...
Figure 7 Brg1 expression acts to maintain the pro-B cell compartment. (a) Difference in transcript abundance in Smarcad1/−/− pro-B cells versus Smarcad1+/− pro-B cells plotted against that in CLPs versus pro-B cells (left), and in Smarcad1/−/− pro-B cells versus Smarcad1+/− pro-B cells plotted against that in pre-B cells versus pro-B cells (right), as determined by microarray by the Immunological Genome Project Consortium; colors indicate transcripts significantly less abundant (blue) or more abundant (red) in Smarcad1/−/− pro-B cells versus Smarcad1+/− pro-B cells (difference of over twofold for each). Spearman correlation = −0.05 (left) or 0.37 (right). (b) Flow cytometry of B cells from Smarcad1+/+Il7rCre/+ and Smarcad1+/+Il7rCre/+ mice, identifying pro-B cells (IgM−IgD−c-Kit+CD25−) and pre-B cells (IgM−IgD−c-Kit+CD25+) (left) and the ratio of pre-B cells to pro-B cells in those mice (right). Numbers adjacent to outlined areas (left) indicate percent c-Kit+CD25− cells (top left) or c-Kit+CD25+ cells (bottom right) among PI−CD19+IgM−IgD− singlets. * P < 0.05 (two-tailed paired Student’s t-test). (c) Expression of transcripts from genes associated with c-Myc occupancy in pro-B cells and pre-B cells. * P = 2 × 10−2 (two-tailed paired Student’s t-test). Data are representative of two experiments (a (Smarca4+/+ and Smarcad1+/− pro-B cells)), three experiments (a (CLPs, pro-B cells and pre-B cells), c) or three independent experiments (b).

Pro-B cells than in Brg1-sufficient pro-B cells (Fig. 5b). Finally, we found that the abundance of Myc transcripts was significantly lower in Brg1-deficient pro-B cells than in Brg1-sufficient pro-B cells (Fig. 5a). Real-time PCR analysis confirmed the downregulation of Myc expression in Brg1-deficient pro-B cells and also in Rag1−/− pro-B cells transduced with shRNA directed against Smarcad1 (Fig. 5c).

The decrease in the abundance of Myc transcripts upon depletion of Brg1 expression raised the question of how Brg1 regulates Myc expression. As a first approach to addressing this question, we inspected the Myc locus for Brg1 occupancy. Brg1 occupancy was particularly prominent in a distally located regulatory element that has been shown to modulate Myc expression in leukemic cells37 (Fig. 5d,e). We found that in pro-B cells, the distally located super-enhancer showed deposition of H3K4me2 and was associated with occupancy by Brg1, EBF1, Ikaros and Pax5 (Fig. 5d,e). Furthermore, Hi-C analysis showed that in pro-B cells, the super-enhancer contacted the Myc promoter with relatively high frequency (Fig. 5d). To assess whether Brg1 expression was needed to permit B lineage–specific transcription factors access to the Myc super-enhancer, we examined Ikaros occupancy by ChIP in wild-type and Brg1-deficient pro-B cells. Indeed, occupancy by Ikaros at the Myc super-enhancer was much lower in the absence of Brg1 than in the presence of Brg1 (Fig. 5f). To directly assess whether Brg1 expression was required for the eviction of nucleosomes across the Myc super-enhancer, we analyzed chromatin accessibility in Brg1-sufficient and Brg1-deficient pro-B cells by ATAC-Seq. As expected, we found that Brg1 expression modulated the accessibility of chromatin to the Myc super-enhancer (Fig. 5e).

c-Myc is known to control cell growth by regulating ribosome biogenesis38. To determine whether c-Myc directly regulated the expression of genes encoding products that regulate ribosome biogenesis, we examined c-Myc occupancy in pro-B cells by ChIP-Seq. Notably, in pro-B cells, we observed binding of c-Myc in promoter regions of genes encoding products associated with ribosome biogenesis, including Tbr1 and Gar1 (Fig. 6a). These findings raised the possibility that in pro-B cells, Brg1 activated Myc expression, which in turn, acted to induce the expression of an ensemble of genes encoding products involved in ribosome biogenesis. We found that the expression of genes associated with the binding of c-Myc in pro-B cells was significantly lower in Brg1-deficient than in Brg1-sufficient pro-B cells (Fig. 6b). Globally, approximately 12% of the genes with lower transcript abundance in Brg1-deficient pro-B cells than in Brg1-sufficient pro-B cells were direct targets of c-Myc (Supplementary Table 1).

To assess directly whether Brg1 acts to regulate pro-B cell growth, we monitored the proliferation of pro-B cells in vivo in Smarcad1+/+Il7rCre/+ and Smarcad1+/+Il7rCre/+ mice through incorporation of the thymidine analog BrdU. Specifically, at 4 h after injection of BrdU, we isolated BM from Smarcad1+/+Il7rCre/+ and Smarcad1+/+Il7rCre/+ mice and analyzed incorporation of BrdU in the pro-B cell compartment. As we expected, BrdU incorporation indicated a significantly lower degree of proliferation in the pro-B cell population isolated from Smarcad1+/+Il7rCre/+ mice than in that from Smarcad1+/+Il7rCre/+ mice (Fig. 6c). Together these observations indicated that in pro-B cells, Brg1 permitted a subset of B lineage–specific transcription factors access to a super-enhancer to directly activate expression of the gene encoding c-Myc, which in turn induced the expression of genes encoding products associated with ribosome biogenesis, to control the growth of pro-B cells.

Brg1 enforces a pro-B cell–specific transcription signature

To examine in greater detail the spectrum of genes affected by the loss of Brg1 expression, we compared the differences in transcript abundance in Brg1−/− pro-B cells versus Brg1−/− pro-B cells to the differences during the CLP–to–pro-B cell transition. The transcription signatures of pro-B cells depleted of Brg1 expression relative to those of wild-type pro-B cells did not correlate with the changes in expression that occurred during the CLP–to–pro-B cell transition (Fig. 7a, left). However, the transcription profiles of Brg1−/− pro-B cells versus those of wild-type pro-B cells correlated with changes in transcription signatures that accompanied the pro-B cell–to–pre-B cell transition (Fig. 7a, right). For example, the abundance of Cd79b transcripts was greater, whereas the abundance of Dntt transcripts was lower, in the absence of Brg1 than in the presence of Brg1 (data not shown). These data suggested that Brg1 acted in pro-B cells to suppress a pre-B lineage–specific pattern of gene expression. To confirm these findings in vivo, we examined Smarcad1+/+Il7rCre/+ and Smarcad1+/+Il7rCre/+ mice for changes in the ratio of pro-B cells to pre-B cells. As predicted, we found that the ratio of small pre-B cells
to pro-B cells was significantly increased in mice that had undergone depletion of Brg1 expression (Fig. 7b). Notably, the large pre-B cell compartment was the most severely affected among the B cell compartments from the BM (Supplementary Fig. 6a), consistent with a requirement for Brg1 in the promotion of cell growth. In further support of that notion, we found that sorted pro-B cells derived from Smarca4fl/flER-Cre mice led to a ‘preferential’ loss of large cells and an increase in the fraction of small pre-B cells upon culture of the cells with tamoxifen, compared with their abundance among sorted and treated Smarca4fl/flER-Cre pro-B cells (Supplementary Fig. 6b,c). To determine how these findings mechanistically related to changes in transcription signatures during the pro-B cell–to–pre-B cell transition, we assigned c-Myc-binding sites to the nearest transcription start site and compared c-Myc occupancy with gene-expression patterns in pro-B cells and pre-B cells. Notably, we found that the expression of an ensemble of genes associated with c-Myc-bound sites in pro-B cells was closely associated with a pro-B cell developmental stage–specific program of gene expression and was downregulated at the pre-B developmental stage (Fig. 7c). Together these data indicated that Brg1 acted to prevent the premature differentiation of pro-B cells into pre-B cells by regulating c-Myc expression.

**DISCUSSION**

It is now established that during developmental progression, transcriptional regulators act together to establish de novo enhancer repertoires as cells differentiate along distinct trajectories. The development of early progenitors of B cells is among the best characterized in terms of the transcription factors that modulate the developmental progression. However, it is less clear how lineage-specific transcription factors activate de novo or poised enhancer repertoires to initiate lineage-specific programs of gene expression. Published studies have demonstrated that chromatin remodelers, including the BAF complex, act to promote nucleosome depletion to permit transcription factors access to their cognate binding sites. Here we mapped the spectrum of Brg1-binding sites across the pro-B cell genome. We found that Brg1 occupancy did not overlap with that of one distinct transcription factor but instead overlapped with that of combinations of transcription factors, including E2A, EBF1, Ikaros and members of the IRF family. How, then, is Brg1 recruited to a B lineage–specific enhancer repertoire with different and unique combinations of transcription factor–binding sites? We found that in pro-B cells, occupancy by Brg1 and p300 overlapped across the majority of the pro-B cell enhancer repertoire. p300 is recruited to active enhancer elements by directly interacting with a multitude of transcription factors. Thus, we propose that in the CLP compartment, E2A, Foxo1, Ikaros and EBF1 bind to a repertoire of primed and partially opened enhancers. This binding results in the recruitment of p300, a histone acetylase, to an ensemble of B lineage–specific enhancers. The sequestration of p300 to poised enhancers leads to the deposition of H3K27ac, which recruits Brg1, which in turn evicts and/or slides nucleosomes away from the repertoire of poised enhancers.

The role of Brg1 in B cell development is not restricted to specification of the B cell fate. Our data indicated multiple roles for Brg1 in cells committed to the B lineage, including contraction of the Igh locus. During early B cell development, the Igh locus undergoes large-scale alterations that merge the proximal and distal VH regions into a single domain as progenitor cells differentiate into pro-B cells. How does a chromatin remodeler such as Brg1 promote the merging of chromatin domains? We found that Brg1 bound to multiple sites scattered across the entire Igh locus. Published studies have demonstrated that contraction of the Igh locus is regulated by multiple transcription factors, including E2A, Pax5, Ikaros and YY1 (refs. 13–15). Here we demonstrated that Brg1 expression was essential for the eviction of nucleosomes across regulatory elements of the Igh locus containing clusters of binding sites for Pax5, YY1, E2A and EBF1. We found that a subset of these sites was associated with long-range genomic interactions that connected the distal VH region cluster with more proximally located anchors possibly to promote Igh locus contraction.

Our data also indicated that Brg1 acted in committed pro-B cells to maintain Myc expression and promote cell growth. Brg1 was needed to activate Myc expression by associating with a distally located super-enhancer. The distal super-enhancer of Myc has been identified in acute myelogenous leukemia, in which Brg1 is essential for maintaining Myc expression and promoting the development of leukemia. We found that Brg1 was critical for maintaining accessibility of this super-enhancer and allowing binding of transcription factors. Downregulation of Myc expression caused further downregulation of the expression of genes encoding products associated with the control of lymphocyte growth. Specifically, a wide spectrum of genes encoding products associated with ribosome biogenesis, including factors associated with nucleoli and members of the exosome complex, were downregulated in the absence of Brg1 and exhibited c-Myc binding. Consistent with those findings, we found that the large pre-B cell population was ‘preferentially’ reduced in Brg1-deficient mice compared with that in Brg1-sufficient mice. Thus, we suggest that Brg1 expression promotes the population expansion of pro-B cells and large pre-B cells by elevating the abundance of c-Myc. The c-Myc in increased abundance, in turn, associates with a repertoire of promoters of genes encoding products associated with cell growth and protein synthesis. During the transition from the pro-B cell stage to the small resting pre-B cell stage, Brg1 levels decrease, as do c-Myc levels. Interestingly, we found that depletion of Brg1 expression in the pro-B cell compartment led to the aberrant activation of genes upregulated at the pre-B cell developmental stage. Since we found that occupancy by c-Myc was associated with genes with higher expression at the pro-B cell stage than at the pre-B cell stage, we suggest that premature exit from the cell cycle through downregulation of c-Myc expression induces a pre-B cell developmental stage–specific program of gene expression, consistent with published observations connecting exit from the cell cycle with developmental progression.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** GEO: ATAC-Seq data, Brg1 and Ikaros ChIP-Seq data, and RNA-Seq data, GSE66978.

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

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AUTHOR CONTRIBUTIONS
C.B. performed and analyzed the majority of the experiments; C.S.M. performed fluorescence in situ hybridization; A.N.C. analyzed RNA-Seq data; R.M. sorted B cells at various developmental stages; H.-R.R. provided IÎ²/Î²Cre+ mice; C.B. and C.M. wrote the manuscript; and C.M. supervised the study.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Smarcd4fl/fl, IrFcre1, Rosa26<εFP>Cre, ER-CRE and E2A-GFP mice were bred and housed in specific pathogen–free conditions in accordance with the Institutional Animal Care and Use Committee of University of California, San Diego.

Flow cytometry. BM cells were harvested from femur, tibia and crista iliac. Fc receptors in cells were blocked (anti-CD16/CD32 (2.4G2: eBioScience) and anti-Fc (eBioscience)) with the Institutional Animal Care and Use Committee of University of California, San Diego.

PERC. Cells were analyzed with an LSRII and were sorted on a FACSAria II (BD Biosciences).

RNA-Seq. Total RNA was isolated with an RNAeasy Mini Kit (Qiagen). RNA was treated with Turbo DNase (Life Technologies). mRNA was purified from total RNA with a Dynabeads mRNA purification kit (Life Technologies). cDNA was generated with a First-Strand Synthesis Kit (Life Technologies) and a combination of random hexamers and oligo(dT) in presence of actinomycin D. Second-strand synthesis was performed with dUTP instead of dTTP. The double-stranded cDNA was sonicated to a length of 200–400 base pairs with an S220Focused-ultrasonicator (Covaris). Sonicated cDNA was ligated to adapters (NEBNext primer kit). The resulting DNA was treated with uracil-N-glycosylase before amplification by PCR with the indexing primers (NEBNext primer kit). Following PCR, fragments were selected by size. To perform RNA-Seq analysis, we sequenced at a depth of 10× to 15×106 reads per sample with an Illumina HiSeq2500. Raw sequencing files underwent quality control with the FastQC tool (Babraham Bioinformatics). Alignment and trimming of reads was performed with the Omicsoft Sequence Aligner algorithm against mm10 as a reference (mm10 NCBI assembly of the mouse genome) with Array Studio software (Omicsoft). RNA transcripts were quantified by RSEM methods (RNA-Seq by expectation-maximization) as implemented in Array Studio (Omicsoft). Principal-component analysis was then performed to check for possible batch effects and outliers. Abundance values (counts) were normalized, and values for samples were compared with the DESeq package of the R project for statistical computing.

ATAC-Seq. ATAC-Seq was performed as described31. Cells were lysed, then nuclei underwent ‘tagmentation’ (transposase-based fragmentation) for 30 min at 37 °C with the Nextera DNA Sample Preparation Kit. The resultant ‘tagmented’ DNA was cleaned up with DNA Clean and Concentrator columns (ZymoResearch). Library fragments were amplified with 1x NEB Next PCR Master Mix and custom Nextera PCR primers 1–6. The number of cycles was determined by quantitative PCR as described31. Libraries were purified on DNA Clean and Concentrator columns. Libraries were sequenced on a HiSeq 2500 system as single reads. Reads were aligned to mm9 with Bowtie software (Johns Hopkins University) with the parameter –m 1. Data were analyzed with the HOMER suite of tools (hypergeometric optimization of motif enrichment). Tag directories were generated with the parameter –tbp 1, which removed most of the ‘reads’ arising from mitochondrial DNA.

ChIP and ChIP-Seq. Cells were fixed for 15 min in PBS containing 1.5 mM ethylene glycol-bis(succinimidyldiacetate). Cells were washed twice with PBS and pellets were frozen in liquid nitrogen. Nonspecific binding was blocked in 30 µl protein G Dynabeads (Life Technologies) by incubation with 0.5% BSA (wt/vol) in PBS. Magnetic beads were bound with 6 μg of the appropriate antibody. Antibodies used were as follows: anti-Brg1 (07-478; Millipore) and anti-Ikaros (Ik-C; a gift from S. Smale). Crosslinked cells were lysed in lysis buffer 1 (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 and 0.25% TritonX-100) and were washed with lysis buffer 2 (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA and 0.5 mM EGTA). Cells were resuspended and then were sonicated (20 W) in lysis buffer 3 (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate and 0.5% N-lauroylsarcosine) for ten cycles of 10 s each on ice with intervals of 50 s on ice between cycles. Lysates were cleared by centrifugation, and Triton X-100 was added at a final concentration of 1%. Lysates were then incubated overnight at 4 °C with the previously prepared magnetic beads. Beads were washed once with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100 and 1 mM EDTA), once with RIPA buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100 and 1 mM EDTA), once with LiCl wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate and 1 mM EDTA) and finally twice with TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA). Bound complexes were eluted with 100 mM NaCl, 0.5% SDS, 20 mM Tris-HCl, pH 8.0, and 200 µg/ml proteinase K for 1 h, and DNA was purified with a QIAquick PCR Purification Kit (Qiagen) and eluted in 20 µl water. DNA was quantified with a NanoDrop ND-1000. ChIP-Seq libraries were prepared using Nextera XT library preparation kit (Illumina) and sequenced on the Illumina Hiseq 2500.
were eluted from the beads by incubation for 30 min at 65 °C with shaking in elution buffer (10 mM Tris-HCl, pH 8.0, 0.5% SDS, 300 mM NaCl and 5 mM EDTA). Crosslinks were reversed by incubation overnight at 65 °C. RNA and protein were digested in the supernatants with RNase A and proteinase K, and DNA was purified with ChIP DNA Clean and Concentrator Columns (Zymo Research).

Libraries were prepared with the NEBNext primer set and were selected by size by 8% PAGE. Libraries were run on Illumina HiSeq 2500. Reads were aligned to mm9 with Bowtie software with the parameter –m 1. Data were analyzed with HOMER software.

For PCR after ChIP with anti-Ikaros, the primers were as follows: site 1, 5′-CCTGGTTCCGAGAAGTCACT-3′ and 5′-GCACTCTCTGGAAAGGTCT-3′; site 2, 5′-TCTCCTAGCCCTAAGACCA-3′ and 5′-GGAGTTGCCCTTGAGATCC-3′.

**Hi-C analysis.** Reads were aligned to mm9 with Bowtie software with the parameter –m 1. Data were analyzed with HOMER software. Interactions were prepared for presentation with the Circos software package. The results for all pairwise comparisons of the various features at the interactions end-points were visualized with the cytoscape software platform.

**Imaging.** Fluorescent in situ hybridization was performed as described\(^1\). The bacterial artificial chromosome probes used were RP23-201H14 and RP24-189H12 from the BACPAC Resource Center at Children’s Hospital Oakland Research Institute. Three-dimensional fluorescent images were acquired on a deconvolution microscope (Deltavision). Optical sections (z-stacks) 0.2 μm apart were obtained throughout the cell volume in the appropriate channels. Distances between the probes were obtained with the softWoRx program by calculation of the distances between the centers of mass of the probes. Probe RP23-201H14 was labeled with Alexa Fluor 568–5-dUTP and probe RP24-189H12 was labeled with Alexa Fluor 488–5-dUTP.

**PCR analysis of Igh recombination.** Genomic DNA was purified from sorted pre-B cells on DNeasy Mini Columns (Qiagen). PCR was performed on 25, 6.75 or 1.2 ng of genomic DNA with forward primers 5′-GAASAMCCTGTWCCTGCAAATGASC-3′ (V\(_{H}\)7183) and 5′-CARCACAGCCTWCATGCARCTCARC-3′ (V\(_{H}\)558) and reverse primer 5′-CTCACAAGAGTCCGATAGACCTTGAGATCC-3′ (I\(_{H}\)3). PCR products were separated by electrophoresis through a 1.5% agarose gel, then were blotted onto nylon membrane and hybridized with radioactive probe. Signals were detected with a Phosphorimager (GE Healthcare) and were analyzed with ImageJ software.

**GEO accession codes for publicly available data sets.** PU.1, GSE21512; c-Myc, p300 and H3K4me2, GSE40173; E2A, H3K4me1 and H3K4me3, GSE21978; EBF, GSE53595; Pax5, GSE38046; YY1, GSE43008; Med1, GSE44288; and Hi-C, GSE35519 and GSE40173.

**Statistical methods.** Data were analyzed with a two-tailed Student t-test for two-group comparisons. P values of less than 0.05 were considered statistically significant. No blinding was used for the animal studies, and no animal was excluded from the analysis. For fluorescence in situ hybridization, the investigator measuring the distance between the probes was unaware of the sample identity. The distance between the probes was measured only in cells in which the probes could be detected for both alleles.