Distinct roles for E12 and E47 in B cell specification and the sequential rearrangement of immunoglobulin light chain loci

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The E2A gene products, E12 and E47, are critical regulators of B cell development. However, it remains elusive whether E12 and E47 have overlapping and/or distinct functions during B lymphopoiesis. We have generated mice deficient for either E12 or E47 and examined their roles in B cell maturation. We show that E47 is essential for developmental progression at the prepro-B cell stage, whereas E12 is dispensable for early B cell development, commitment, and maintenance. In contrast, both E12 and E47 play critical roles in pre-B and immature B cells to promote immunoglobulin λ (Igλ) germline transcription as well as Igλ VJ gene rearrangement. Furthermore, we show that E12 as well as E47 is required to promote receptor editing upon exposure to self-antigen. We demonstrate that increasing levels of E12 and E47 act to induce Igλ germline transcription, promote trimethylated lysine 4 on histone 3 (H3) as well as H3 acetylation across the Jα region, and activate Igλ VJ gene rearrangement. We propose that in the pre-B and immature B cell compartments, gradients of E12 and E47 activities are established to mechanistically regulate the sequential rearrangement of the Ig light chain genes.
regulators that contain an HLH dimerization domain, as well as a basic DNA binding region that is located immediately N terminal to the HLH domain. E proteins form homodimers or heterodimers with other E proteins or other members of the HLH family (Murre, 2005). These protein complexes have the ability to act either as transcriptional activators or repressors. In vertebrates, four E proteins have been identified. These include the E2A proteins, E12 and E47, which only differ in their basic DNA binding region, as well as HEB and E2-2, both of which give rise to distinct isoforms generated through alternative initiation of transcription (Cornelissen et al., 1991; Wang et al., 2006). E proteins also have the capacity to interact with antagonistic HLH proteins, named Id proteins. Id proteins contain an HLH domain but lack a basic region, and upon interacting with E proteins, inactivate their DNA binding activity (Benezra et al., 1990). E and Id proteins are widely expressed throughout the entire hematopoietic system. They play critical roles at virtually every step of hematopoiesis to promote developmental progression, expansion, and survival of developing lymphocytes (Lazorchak et al., 2005; Murre, 2005).

The E2A proteins are active at the HSC cell stage, where they are required for the maintenance of the stem cell pool (Yang et al., 2008; Semerad et al., 2009). They remain active during the development of HSCs into LMPPs, common lymphoid progenitors (CLPs), and prepro–B cells (Zhuang et al., 2004; Borghesi et al., 2005; Dias et al., 2008; Yang et al., 2008; Semerad et al., 2009). In the absence of E2A, the LMPP and CLP compartments are severely reduced and the pro–B cell compartment is completely absent (Dias et al., 2008; Semerad et al., 2009). In both the LMPP and CLP compartment, the E2A proteins have been postulated to act in concert with other transcriptional regulators, such as PU.1 and Ikaros, to promote further developmental progression (Singh et al., 2007; Nutt and Kee, 2007).

B cell development in E2A-ablated mice is arrested at the prepro–B cell stage before the onset of IgH DJ gene rearrangement (Bain et al., 1994; Zhuang et al., 1994). A similar block in B cell development is also observed in EBF-deficient mice, and recent data have demonstrated that the E2A proteins act upstream of EBF to promote B-lineage maturation (Lin and Grosschedl, 1995; Ikawa et al., 2004; Roessler et al., 2007). Specifically, forced EBF expression in E2A-deficient bone marrow cells relieves the block at the prepro–B cell stage (Seet et al., 2004). These data suggest that in the absence of E2A, B lymphopoiesis is blocked before the initiation of B cell development, owing to the inability to activate Ebf1 transcription in prepro–B cells. The E2A proteins are also required to promote a B-lineage program of gene expression in pro–B cells, because conditional deletion of Tcf/le2a revealed that the E2A proteins act to maintain the expression of Ebf1 and Pax5 in pro–B cells. The block at the prepro–B cell stage in E2A-deficient bone marrow is also relieved by the enforced expression of Pax5, likely because of the ability of Pax5 to regulate Ebf1 expression (Lazorchak et al., 2006b; Kwon et al., 2008). In addition, multiple pro–B cell–specific genes contain binding sites for E2A, EBF, and Pax5 in putative promoter and enhancer regions, raising the possibility that the combined activities of E2A, EBF, and Pax5 initiate the B cell fate (Nutt and Kee, 2007).

Upon expression of a pre–BCR, E47 protein levels transiently decline, whereas Id3 levels are elevated (Schebesta et al., 2002; Quong et al., 2004). E12 protein levels have not been studied in detail. At the small pre–B cell stage, E47 levels increase again to induce Igk VJ gene rearrangement (Quong et al., 2004). E47 expression remains high at the immature B cell stage when interaction of the BCR with self-antigen promotes receptor editing (Quong et al., 2004). During positive selection within the transitional B cell compartments, E47 levels decrease and then decline further at the IgM ‘‘IgD’’ stage (Quong et al., 2004; Kwon et al., 2008). We have previously proposed a model in which high abundance of E47 enforces the immature B cell checkpoint (Quong et al., 2004). In the presence of self-reactivity, E47 levels remain high to promote continuous Igk locus accessibility and receptor editing. The expression of an innocuous BCR would lead to a decline of E47 levels, suppressing further Ig light chain recombination and promoting developmental progression toward the mature B cell stage. The E2A proteins initiate Igk VJ gene rearrangement by directly binding to the E-box sites located within the Igk intronic (iEκ) and 3’ enhancers (Murre et al., 1989). Mutational analysis of the two E2A binding sites in iEκ affects Igk rearrangement as severely as deletion of the entire enhancer, providing unambiguous evidence for a critical function of E-box sites in Igk enhancer function (Greenbaum and Zhuang, 2002a; Inlay et al., 2004). Enforced expression of E47 or E12 readily promotes Igk locus accessibility to the recombination machinery in nonlymphoid cells, and E2A-deficient pre–B cells are defective in their ability to undergo Igk VJ gene rearrangement (Romanow et al., 2000; Goebel et al., 2001; Lazorchak et al., 2006a). Finally, secondary rearrangements and receptor editing are severely impaired in E2A-heterozygous mice (Quong et al., 2004).

Because the E2A proteins fulfill fundamental roles in so many aspects of the lymphoid branch of the hematopoietic system, it is important to determine in detail how they exert their unique effects. In particular, although the role of E2A proteins in lymphocyte development is now well established, it is less clear whether E12 and E47 have overlapping and redundant roles or whether they perform distinct functions. Previous studies have described the phenotypes in mice that lack E47 expression (Bain et al., 1997). B cell development in E47-ablated mice was blocked at a similar stage as in E2A-deficient mice, although low levels of Ebf1 and Pax5 transcripts were detectable (Bain et al., 1997). However, because of the applied gene targeting strategy, these mice showed a substantially lower abundance of E12, and it remained unclear whether the defects that were detected were caused by a decrease in E12 and E47 rather than E47 alone (Bain et al., 1997).

To determine the individual roles of E12 and E47 in B cell development, we have generated E12- and E47-deficient
mice and analyzed them for abnormalities during B lymphopoiesis. We found that deletion of E47 leads to a block in early B cell development, whereas E12 is dispensable for B cell specification. During later stages of development, however, there is a critical dependence on E12 as well as E47 expression in the pre–B and immature B cell compartment. Specifically, using both in vitro and in vivo approaches, we show that E12 and E47 play a crucial role in promoting Igα VJ gene rearrangement and receptor editing. Specifically, we demonstrate that the E2A proteins modulate trimethylated lysine 4 on histone 3 (H3K4me3) across the Igα J regions. These data link E2A, epigenetic marking, and the recruitment of the RAG proteins in a common pathway. Finally, we propose a model in which a gradient of E12 and E47 acts to sequentially regulate Igk and Igα VJ gene rearrangement.

RESULTS
Essential role for E47 but not E12 in early B cell development
To examine the individual roles of E12 and E47 in B cell development, mice were generated that were either deficient in E12 or E47 (Fig. 1 A). To generate E12−/− and E47−/− mice, exons encoding for either E12 or E47 were flanked with loxP sites by homologous recombination in embryonic stem (ES) cells (Fig. 1 A). Genomic DNA was isolated from targeted ES cells and analyzed by Southern blotting for correct targeting (Fig. 1 B). Mutant mice were generated from cells carrying the floxed E12 or E47 alleles and crossed with transgenic mice that express the Ella−/− transgene to delete the loxP-flanked DNA regions in the germline (Lakso et al., 1996). Mice carrying specific deletions in either E12 or E47 were identified using PCR on genomic DNA (Fig. 1 C).

To determine whether E12 and/or E47 is required to promote early B cell development, bone marrow derived from E12−/− and E47−/− mice was examined for cellularity as well as for the presence of B cell markers. In bone marrow and spleen derived from E47−/− mice, B cells were virtually absent (Fig. 2, A and B). In contrast, the number of B cells in E12−/− mice was not significantly altered as compared with wild-type mice (Fig. 2, A and B).

Previous studies using E2A−/− mice have revealed that B cell development in these mice is blocked at the prepro–B cell stage, because the majority of the cells are arrested in Hardy fraction A (Bain et al., 1997). To more precisely determine the arrest in B cell development in E47−/− mice, bone marrow was isolated and analyzed by flow cytometry for the expression of B220, CD43, heat stable antigen (HSA), and BP-1 (Fig. 2 C; Hardy and Hayakawa, 2001). B cell development in E47−/− mice was partially blocked in fraction A and a substantial fraction of cells could be detected in fraction B, whereas virtually no B cells could be detected in fraction C (Fig. 2, C and D). As expected, the B220+c-kit+ compartment was also severely reduced in E47−/− bone marrow (Fig. S1). Interestingly, E12−/− mice showed a slightly higher percentage of fraction C cells among the B220−CD43+ population than wild-type controls (Fig. 2, C and D).

To determine whether the excision of the E47 exon affects E12 mRNA abundance, LMPPs and CLPs were isolated from wild-type and E47−/− bone marrow, and mRNA was isolated and examined for the expression of E12 by quantitative PCR (Fig. S2). Preliminary data show that in both the LMPP and CLP compartment, E12 levels in the E47-deficient cells were similar to that of their wild-type counterparts (Fig. S2). These data indicate that the block in early B cell development in E47−/− mice is specifically caused by the depletion of E47 expression. Collectively, these data show that E12 does not play a critical role in early B-lineage specification. In contrast, E47 is indispensable at the earliest stages of B cell commitment.

E12 is required to promote the development of B cells expressing Igα
As described in the previous section, B cell development in E12−/− mice seems to be slightly blocked at the transition from Hardy fraction C to the small pre–B cell stage, raising the possibility that E12 is required during the small pre–B cell stage. To examine this possibility, bone marrow derived from E12−/− mice was analyzed for the abundance of pro–B cells (B220+IgM+IgD−), pre–B cells (B220+IgM+CD23−), immature B cells (B220int IgM+), and recirculating B cells (B220hi IgM+) using flow cytometry (Fig. 3 A). As predicted, the size of the pre–B cell compartment was slightly reduced in E12−/− bone marrow (Fig. 3 A). On the other hand, the pro–B, immature B, and mature B cell compartments were not altered in E12−/− bone marrow as compared with wild-type controls (Fig. 3 A). Similarly, the cellularity of immature B cells (B220+IgM+IgD−), transitional B cells (B220+IgM+IgD+), mature B cells (B220+IgM+IgD+), follicular B cells (B220+CD23+CD21+), and marginal zone B cells (B220+CD23−CD21hi) in the spleen was similar in E12−/− as compared with wild-type mice (Fig. 3 B).

Previous studies demonstrated a critical role for the E2A proteins in receptor editing (Quong et al., 2004). Furthermore, a substantial reduction in the fraction of Igα-expressing cells was observed in E2A−/− mice. To determine whether E12 plays a critical role in the development and/or survival of Igα-expressing cells, E12−/− bone marrow was analyzed for the presence of Igα+ cells by flow cytometry. Strikingly, the fraction of Igα-expressing B cells was substantially reduced in E12−/− mice as compared with those of wild-type littermates (Fig. 3 C and D). Similarly, total numbers of Igα+ cells in the bone marrow of E12−/− mice were significantly reduced as compared with those of wild-type mice (Fig. 3 D). Comparable reductions were observed among newly formed B220int and recirculating B220hi bone marrow B cells as well as in splenic B cells (Fig. 3, C and D). In contrast, the fraction of Igα-expressing cells was slightly elevated in the bone marrow as well as in the spleen of E12−/− mice, resulting in a much higher Igk/Igα ratio in E12−/− mice (Fig. 3 E). In summary, these data show that E12 plays a distinct and essential role in the developmental progression and/or survival of B cells that express Igα.
Defective receptor editing in E12<sup>−/−</sup> mice

The aforementioned data show that the Igk/Igλ ratio was severely perturbed in E12<sup>−/−</sup> B cells, raising the possibility that E12 plays a critical role in promoting Ig<sub>λ</sub> VJ gene rearrangement upon exposure to self-antigen. To determine whether E12 plays a role in promoting receptor editing, bone marrow cells were isolated from wild-type and E12<sup>−/−</sup> mice and transplanted into recipients that constitutively express a κ-macroself transgene (Ait-Azzouzene et al., 2005). 6 wk after transplantation, bone marrow as well as spleen cells were harvested and examined for the expression of Igλ, Igk, and B220 (Fig. S3). As shown previously, the bone marrow of recipients expressing the κ-macroself transgene that received wild-type cells showed an increased frequency of Igλ<sup>+</sup> B cells as compared with recipients that lacked κ-macroself expression (Fig. S3 B). Similarly our preliminary results show that mice that received E12<sup>−/−</sup> bone marrow cells also have increased percentages of Igλ<sup>+</sup> cells if recipients expressed the κ-macroself transgene as compared with nontransgenic control recipients (Fig. S3 B). This increase in Igλ-expressing cells was similar or even greater in the absence as compared with the presence of E12. However, the fraction of Igλ-expressing cells in κ-macroself recipients reconstituted with E12<sup>−/−</sup> cells was significantly lower as compared with those reconstituted with bone marrow cells from wild-type littermate controls (Fig. S3, A and B).

As expected, tolerance was maintained because no Igk-expressing cells were detectable in the spleen of recipients expressing the κ-macroself antigen transplanted with either wild-type or E12<sup>−/−</sup> bone marrow (Fig. S3, C, bottom). The fraction of Igλ-expressing cells in the spleens of recipients that express the κ-macroself transgene was significantly lower in E12<sup>−/−</sup> bone marrow as compared with wild-type chimeras (Fig. S3, C and D, top). Furthermore, the overall decrease in the total number of splenic B cells was much more substantial in E12<sup>−/−</sup> chimeras as compared with controls (Fig. S3 D, bottom). Collectively, these data indicate that

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**Figure 1. Generation of E12<sup>−/−</sup> and E47-deficient mice.** (A) Structure of the targeted Tcfe2a locus. The alternatively spliced E12 and E47 exons, respectively, were flanked with loxP sites (indicated by arrows). In addition, a tetracysteine tag (black bar) was inserted into exon 20, 3' of the coding region, to produce tagged E47 and E12 proteins. Furthermore, a neomycin (neo) resistance gene was introduced to screen for targeted ES cells. The EcoRI (E) fragments as well as the probe used for screening of the ES cells are indicated. After crossing of Tcfe2a<sup>fl_E12</sup>- or Tcfe2a<sup>fl_E47</sup>-bearing mice with a germline Cre-deleter strain (Ella-Cre), the corresponding Tcfe2a<sup>−/−</sup> or Tcfe2a<sup>−/−</sup> strains were obtained. (B) Southern blot analysis of EcoRI-digested DNA from correctly targeted ES cells of the indicated genotype. The position of the probe is shown in A. (C) PCR analysis of genomic DNA from mice of the indicated genotype. Primer positions are depicted in A.
upon exposure to self-antigen, the induction of receptor editing does not fully require E12 activity; however, E12 critically modulates the magnitude of the Igλ response.

**E47 and E12 abundance is limiting during the development of Igλ-expressing B cells**

The observations described in the previous section demonstrate that E12 plays a critical role in the developmental progression of Igλ-expressing cells upon exposure to self-antigen. Because B cell development in E47−/− mice was blocked at the Hardy fraction A/B cell stage, we were not able to assess how the complete absence of E47 affects the developmental progression of B cells beyond the pro–B cell stage. To address this issue and to compare the individual roles of E12 and E47 at the pre–B and immature B cell stage, we examined E12+/− and E47+/− mice for the abundance of

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**Figure 2. Essential role for E47 but not E12 in early B cell development.**

(A and B) Representative FACS analysis of bone marrow (A) and spleens (B) from wild-type, E47−/−, and E12−/− mice. Numbers indicate percentages of B cells within the live cell fraction. Mean percentages of B cells (B220+CD19+) in the live cell gate as well as absolute B cell numbers in bone marrow (A) and spleens (B) are depicted. At least four independent experiments using one to three mice per group were performed. Horizontal bars represent the mean. ***, P < 0.001. (C) Flow cytometry of bone marrow B cells from wild-type, E47−/−, and E12−/− mice. Numbers indicate percentages of B cells within the live cell fraction (left) or the B220+CD43+ pro–B cell fraction (right). At least four independent experiments using one to three mice per group were performed. (D) Mean percentages of fraction A (HSA−BP-1−), fraction B (HSA+BP-1−), and fraction C (HSA+BP-1+) cells within the pro–B cell (B220+CD43+) compartment of wild-type, E47−/−, and E12−/− mice. At least four independent experiments using one to three mice per group were performed. ns, not significant.
Igλ-expressing cells. Bone marrow cells derived from wild-type, E12<sup>+/−</sup>, E47<sup>+/−</sup>, and E12<sup>−/−</sup> mice were isolated and examined for the expression of Igλ. Both E12<sup>+/−</sup> and E47<sup>+/−</sup> mice showed a substantial reduction in the fraction of Igλ<sup>+</sup> B cells (Fig. 4, A and B). Similarly, the fraction of Igλ-expressing cells in the spleen derived from E12<sup>+/−</sup> and E47<sup>+/−</sup> mice was significantly reduced as compared to wild-type mice (Fig. 4 C).

Figure 3. E12 is required to promote the development of B cells expressing Igλ. Flow cytometric analysis of B cell populations in wild-type and E12<sup>−/−</sup> mice. (A) Mean percentages of depicted cell types within the live cell gate and total numbers of pro-B cells (B220<sup>+</sup>IgM<sup>−</sup>c-kit<sup>+</sup>), pre-B cells (B220<sup>−</sup>IgM<sup>−</sup>CD45<sup>+</sup>), immature B cells (B220<sup>−</sup>IgM<sup>−</sup>IgD<sup>+</sup>CD43<sup>+</sup>CD25<sup>+</sup>), and recirculating B cells (B220<sup>−</sup>IgM<sup>−</sup>IgD<sup>−</sup>CD25<sup>−</sup>) in the bone marrow. Six independent experiments using one mouse per group were performed. (B) Mean percentages of depicted cell types within the live cell gate and total numbers of immature B cells (B220<sup>−</sup>IgM<sup>−</sup>IgD<sup>−</sup>) in the spleen. Six independent experiments using one mouse per group were performed. (C) Representative FACS analysis of Igλ<sup>−</sup> B cells in the bone marrow and spleen. Numbers indicate percentages of the depicted gates within the live cell fraction. Nine independent experiments using one mouse per group were performed. (D) Mean percentages of newly formed Igλ<sup>−</sup>B cells (B220<sup>−</sup>IgM<sup>−</sup>IgD<sup>−</sup>) and recirculating Igλ<sup>−</sup>B cells (B220<sup>−</sup>IgM<sup>−</sup>IgD<sup>−</sup>) in the bone marrow as well as Igλ<sup>−</sup>B cells (B220<sup>−</sup>IgM<sup>−</sup>IgD<sup>−</sup>) in the spleen as gated in C. Nine independent experiments using one mouse per group were performed. (E) Percentages of Igκ<sup>+</sup>B cells within the B220<sup>−</sup> fraction as well as the ratio of Igκ<sup>+</sup> to Igλ<sup>+</sup>B cells in the bone marrow (left) and spleen (right). Six independent experiments using one mouse per group were performed. All bar graphs show means ± SEM. **, P ≤ 0.01; ***, P < 0.001 (values are not significantly different from wild-type controls if there is no p-value depicted).
Although the E12 and E47 exons were specifically excised in the null mutant mice, it remained possible that E12 and E47 cross-regulate each other. To explore this possibility, we isolated mRNA from sorted pre–B cells derived from wild-type, E12+/−, E47+/−, or E12−/− mice and determined the mRNA levels of E12 and E47 by quantitative PCR (Fig. 4 D). Pre–B cells derived from E47+/− showed slightly elevated levels of E12 mRNA (Fig. 4 D, left). In contrast, E47 levels in E12−/− pre–B cells were not significantly altered (Fig. 4 D, right). E12−/− pre–B cells, on the other hand, showed a modest reduction in E47 levels (Fig. 4 D, right).

These data indicate that E47 levels are modestly but significantly reduced in E12−/− pre–B cells. On the other hand, E47 abundance is not significantly affected in E12+/− pre–B cells. Furthermore, these data indicate that depletion of E47 slightly but significantly elevates the expression of E12. Thus the defect in Igα-expressing cells in E47+/− and E12−/− mice cannot be explained by abnormalities in the abundance of either E12 or E47. Collectively, these data demonstrate that both E12 and E47 play a critical role in the developmental progression of Igα-expressing B cells.

**E12 and E47 facilitate Igα locus accessibility**

Given that Igα-expressing cells were less abundant in E12+/−, E47+/−, and E12−/− mice, we considered the possibility that E12 proteins act to regulate Igα VJ gene rearrangement. To address this issue, DNA was isolated from sorted pre–B cells derived from wild-type, E12+/−, E47+/−, and E12−/− mice and analyzed by semiquantitative PCR for the presence of IgαDJ and Igα VJ joining is not perturbed in pre–B cells isolated from E47+/−, E12+/−, and E12−/− mice (Fig. S4). Recombining sequence (RS) rearrangements were only modestly affected in E12+/−, E47+/−, and E12−/− mice (Fig. 5 B, left and middle). In contrast, Igα V1-J1 rearrangement was significantly reduced in pre–B cells isolated from both E12−/− and E47+/− mice (Fig. 5 B, right). As predicted, E12−/− pre–B cells showed a significant decrease in the levels of Igα VJ gene rearrangements as compared with wild-type mice and, importantly, also with E47+/− pre–B cells (Fig. 5 B, right). These data indicate that both E12 and E47 act to promote Igα VJ gene rearrangement.

To determine whether the decrease in Igα VJ gene rearrangements in E12−/− and E47+/− bone marrow cells was caused by lower levels of RAG1 and/or RAG2 expression, mRNA was isolated from sorted pre–B cells and examined by quantitative PCR for the expression of RAG1/2. Both RAG1 and RAG2 mRNA levels were not significantly altered in E12+/− and E47+/− as compared with wild-type mice (Fig. S5). To determine whether a reduction in E12 and/or E47 affected Igα germline transcription, mRNA from sorted pre–B cells was analyzed for the expression of Igα I germline transcripts. Consistent with the decrease in Igα VJ gene rearrangements in E12−/− and E47+/− pre–B cells, Igα I germline transcript levels were significantly reduced as compared with wild-type (Fig. 5 C). As expected, a further significant reduction in Igα I germline transcripts was observed in E12−/− pre–B cells (Fig. 5 C, right). To determine whether this defect in Igα VJ gene rearrangements in E12−/− pre–B cells, Igα I germline transcript levels were significantly reduced as compared with wild-type pre–B cells. This is consistent with the observation that Igα VJ gene rearrangements are altered in E12−/− pre–B cells (Fig. 5 C).

Next, we compared Igα V1-J1 rearrangements in FACS-sorted pre–B cells (B220+CD43+CD19−) from mice of the indicated genotypes. At least three independent experiments using one mouse per group were performed. (D) Quantitative PCR analysis of mouse per group were performed. (D) Quantitative PCR analysis of E12 and E47 abundance is limiting during the development of Igα-expressing B cells. (A–C) Mean percentages and total numbers of newly formed Igα+ B cells (B220+Igα+IgM+; A) and recirculating Igα+ B cells (B220+Igα+IgM−; B) in the bone marrow as well as Igα+ B cells (B220+Igα+IgM+; C) in the spleen as gated in Fig. 3 C from mice of the indicated genotypes. At least three independent experiments using one mouse per group were performed. (D) Quantitative PCR analysis of E12 and E47 transcripts in FACS-sorted pre–B cells (B220+IgM−CD19−) from the bone marrow of wild-type, E12+/−, E47−/+ and E12−/− mice. Levels were normalized to hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1) transcripts and relative levels to wild-type controls are shown (2−ΔΔCt). Bar graphs show means ± SEM of three independent experiments. *, P ≤ 0.05; **, P ≤ 0.01; ***, P < 0.001 (values are not significantly different from wild-type controls if there is no p-value depicted). nd, not detectable.
reduction in Igκ germline transcription was observed in E12−/− pre-B cells (Fig. 5 C). Collectively, these data suggest that E12 and E47 activate Igκ VJ gene rearrangement by modulating Igκ locus accessibility to the recombination machinery.

Consistent with a role for E12 and E47 in modulating Igκ locus accessibility are previous observations indicating the presence of E12 and E47 binding sites in the Igκ enhancer regions (Rudin and Storb, 1992). To determine whether E12 and E47 directly interact with E-box sites present in the Igκ enhancer, B cells were isolated from wild-type and E12−/− bone marrow, cross-linked with formaldehyde, and immunoprecipitated with an anti-E2A antibody. Subsequently, DNA was isolated from the immunoprecipitates and examined for the presence of regions containing Eκ1-3

Figure 5. E12 and E47 facilitate Igκ locus accessibility. (A) Schematic diagram of the mouse Igκ and light chain (Igκ and Igλ) loci. V, D, J, and C segments as well as the RS in Igκ are depicted as gray boxes and the enhancers are depicted as white boxes. Analyzed gene rearrangements are shown by arrows on top of the loci, whereas germline transcripts are shown by arrows under the gene locus. (B) Quantification of Southern blots from PCR analysis of Igκ gene rearrangements in FACS-sorted pre-B cells (B220+IgM−CD25+ Igκ) from mice of the indicated genotypes. PCR products from reactions of fourfold serial dilutions were electrophoresed on agarose gels and abundance was quantitated by Southern blotting using specific probes. Shown are means ± SEM relative to wild-type signal. *, P ≤ 0.05; ***, P < 0.001 (values are not significantly different from wild-type controls if there is no p-value depicted). Three independent experiments using one mouse per group were performed. (C) Quantitative PCR analysis of Igκ germline transcripts (Igκ T) in FACS-sorted pre-B cells (B220+IgM−CD25+) from the bone marrow of wild-type, E12−/−, E47−/−, and E12−/− mice. Levels were normalized to Hprt1 transcripts and relative levels to wild-type controls are shown (2−ΔΔCt). Shown are means ± SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***, P < 0.001 (values are not significantly different from wild-type controls if there is no p-value depicted). Three independent experiments using one mouse per group were performed. (D) ChIP assays using anti-E2A antibodies on MACS-purified total B220+ bone marrow cells from wild-type and E12−/− mice. Relative enrichment of the bound DNA over input was determined by quantitative PCR using specific primers for the Igκ enhancers Eκ1-3 and Eκ2-4. Shown are means ± SEM. Two independent experiments using one mouse per group were performed. IRS1, intronic recombination sequence 1.
and Eα2+4 using the appropriate primers. Both E12 and E47 interacted with the Igα enhancer regions, Eα1+3 and Eα2+4, because the enrichment observed in wild-type bone marrow cells was severely reduced in E12−/− cells but not abolished (Fig. 5 D). Thus, we conclude that the residual binding to the Igα enhancer regions is caused by E47 (Fig. 5 D). Collectively, these data demonstrate that both E12 and E47 interact with E-box sites present in the Igα enhancers, activate Igα germline transcription, modulate Igα locus accessibility, and promote Igα V-J gene rearrangement.

**Attenuation of IL-7 signaling elevates E12 and E47 abundance**

Previous observations have indicated that E47 abundance is low in cycling large pre-B cells but is elevated in small resting pre-B cells (Quong et al., 2004). Because Ig light chain gene rearrangement is initiated by pre-BCR signaling as well as attenuation of IL-7 signaling, we examined the temporal expression of E12 and E47 in bone marrow cells that express an innocuous BCR upon withdrawal of IL-7 signaling. In brief, B cells expressing the hen egg lysozyme transgene (HEL-Tg) were isolated from the bone marrow and grown for a period of 4 d in the presence of IL-7. Upon withdrawal of IL-7 from the B cell cultures, the abundance of Igα germline transcripts as well as E12 and E47 transcript levels were determined at various time points using quantitative PCR. Upon attenuation of IL-7–mediated signaling, E12 and E47 transcript levels increased gradually (Fig. 6 A). Interestingly, the relative increase in E12 expression was modestly but consistently larger than that of E47 expression, with the ratio of E12 to E47 expression increasing over time (Fig. 6 A). Concomitantly with this increase in E12 and E47 mRNA levels, we observed an increase in Igα germline transcription consistent with a model in which E12 directly regulates Igα germline transcription (Fig. 6 B). To determine whether E12 is required for the induction of Igα germline transcription, E12−/−, HEL-Tg and E12−/+ HEL-Tg bone marrow cells were cultured in the presence of IL-7 for 4 d, followed by withdrawal of IL-7. RNA was isolated at the indicated time points and examined for the presence of Igα germline transcription. Consistent with these observations, Igα germline transcript levels were significantly reduced in E12−/+ immature B cells (Fig. 6 B).

**E2A proteins modulate epigenetic marks across the Igα J regions**

Intriguing studies have recently indicated a correlation between epigenetic marking and antigen receptor assembly. Specifically, a noncanonical plant homeodomain located within the noncore region of RAG2 was shown to interact with H3K4me3 (Liu et al., 2007; Matthews et al., 2007; Ramón-Maiques et al., 2007). Furthermore, across the JA region, H3K4me3 and H3 acetylation (H3ac) were shown to be specifically enriched during the pre-B and immature B cell stages (Xu and Feeney, 2009). These observations raise the possibility that the E2A proteins regulate Igα V-J gene rearrangement by promoting the recruitment of RAG2 to the recombination signal sequences by, directly or indirectly, modulating trimethylation of H3K4. To address this issue, B220+ bone marrow cells from wild-type and E12−/+ mice were cultured in the presence of IL-7 and stem cell factor for 8 d to obtain a homogeneous culture of B220+CD19+CD43+ pro–B cells. After IL-7 withdrawal for 18 h, we examined the JA region for H3K4 trimethylation as well as H3ac by chromatin immunoprecipitation (ChIP; Fig. 7, A and B). Interestingly, both...
H3K4me3 and H3ac across the Jα region were significantly reduced in the absence of E12 (Fig. 7, A and B).

Collectively, these data indicate that E12 expression is elevated in immature B cells to initiate Igα germline transcription and to promote Igα locus accessibility. Based on these, as well as previous observations, we propose that in immature B cells initially, E12 and E47 abundance is low, preventing accessibility of the Igα locus to the recombination machinery. However, increasing levels of E12 and E47 would eventually lead to relative high occupancy of E-box elements present in the Igα enhancer, inducing Igα germline transcription and permitting the activation of Igα VJ gene rearrangement (Fig. 8). Furthermore, we suggest that the E2A proteins act to promote epigenetic marking of histone residues located at the Jα regions to permit the recruitment of RAG2 to the recombination machinery.

DISCUSSION

The Tcfe2a locus encodes for two highly related proteins that differ in their DNA binding region through alternative splicing of one exon (Murre et al., 1989; Sun and Baltimore, 1991). From previous studies, it is apparent that the transcription factor E2A is not only indispensable for commitment to the B cell lineage but fulfills critical functions throughout B lymphopoiesis that include maintenance, DNA recombination, receptor editing, marginal versus follicular zone development, and class switch recombination (Greenbaum and Zhuang, 2002b; Murre, 2005). We have previously generated E47-deficient mice that also showed substantially reduced abundance of E12 caused by the insertion of the neomycin gene. Thus, an important unresolved question is whether E12 and E47 fulfill distinct and/or overlapping roles during B lymphopoiesis. In this study, we have generated two mouse strains that selectively delete either the E12 or the E47 exon through the usage of the loxP system, enabling us to study their specific roles during B cell development.

Previous studies have shown that B cell development in E2A-deficient mice is completely blocked at the prepro–B cell stage (Bain et al., 1994; Zhuang et al., 1994). Overexpression of either E12 or E47 has the ability of triggering commitment to the B cell lineage, raising the possibility that both play a role in B cell specification and/or commitment (Bain et al., 1997). Our study shows that E47 is strictly required for progression beyond the prepro–B cell stage and that endogenous levels of E12 cannot compensate for its loss. In striking contrast, E12-deficient mice show almost normal numbers of mature B cells in the bone marrow and spleen. However, the pre–B cell compartment is slightly reduced in E12-deficient bone marrow. We note that the decrease in the pre–B cell compartment of E12-deficient bone marrow might be caused by the twofold decrease in E47 mRNA levels (Fig. 4 D). Thus, in the presence of endogenous levels of E47 protein, E12 is dispensable for the specification of the B cell fate. In turn, this suggests that E47 but not E12 activates a B lineage–specific program of gene expression.

In addition to regulating lineage decisions through the activation of gene expression of B cell–specific genes, the E2A proteins also play critical roles in regulating Ig gene...
rearrangements. Both E47 as well as E12 were originally identified as factors that interact with the κE2 site of the iEx. Deletion of the two E-boxes in iEx affects Igκ recombination to the same degree as deletion of the complete enhancer (Murre et al., 1989; Inlay et al., 2004). Forced E12 or E47 expression, in conjunction with RAG1 and RAG2, promotes Igκ VJ gene rearrangement in nonlymphoid cells (Romanow et al., 2000). Furthermore, Igκ VJ gene rearrangements in E2A-deficient pre-B cells are completely abolished (Lazorchak et al., 2006b; Kwon et al., 2008).

Collectively, these studies establish the E2A proteins as key regulators of Igκ VJ gene rearrangement. We show in this study, however, that Igκ VJ gene rearrangement is not affected in E12-deficient mice. Because B cell development in E47-deficient mice is completely blocked at the prepro-B cell stage and because we were not able to generate E47+/− mice that would permit the stage-specific deletion at the pre-B or immature B cell stage, we could not assess directly whether E47 is the critical component in promoting Igκ VJ DNA recombination. However, because E2A-deficient B cells are completely defective in Igκ VJ recombination and E12 activity is not required, we infer that E47 is the main factor required to promote Igκ VJ joining.

In contrast, we show in this study that both E47 and E12 are required for proper Igλ gene rearrangement. As described, E12-deficient B cells show a reduction in E47 mRNA levels that is similar to that observed in B cells from E47-heterozygous mice. Because E47-heterozygous B cells also have a significant defect in Igλ VJ gene rearrangement, E12-deficient B cells cannot be used to evaluate the role of E12 in Igλ VJ gene rearrangement. Therefore, E12-heterozygous B cells that show normal levels of E47 were used to unambiguously demonstrate a critical role for E12 in Igλ VJ gene rearrangement. We emphasize, however, that in addition to E12, E47 also plays a critical role in modulating Igλ VJ gene rearrangement.

There are several mechanisms that may underpin the decrease in Igλ-expressing cells in E12- or E47-deficient pre-B cells. First, it is conceivable that E12 and E47 act to promote the survival and/or life span of immature B cells, because previous studies have demonstrated a critical role for the E proteins in B cell survival (Kee et al., 2002; Lazorchak et al., 2006b). In this case, however, survival of in vitro–cultured E12+/− B cells expressing the HEL-Tg mouse is not affected, yet they do not efficiently up-regulate Igλ germine transcripts. Thus, although we cannot exclude the possibility that the E2A proteins act, in part, to promote cell survival at the immature B cell stage in vivo, we consider it unlikely that this is their sole function. A second possibility is that RS deletion is regulated by E12 and E47. RS is a DNA element that carries a canonical recombination signal sequence. It is located ∼25 kbp downstream of the Igκ constant region and rearranges by DNA recombination to Vk elements and to elements present in the Jκ-Cκ intronic region. The absence of RS deletion substantially interferes with the development of Igλ-producing cells (Vela et al., 2008). Thus, it is conceivable that Igλ production in E12-ablated bone marrow cells is perturbed because of a reduction in RS deletion. However, in this study we show that RS deletion is only modestly affected in E12-deficient pre-B and immature B cells carrying unmanipulated BCRs. A third possibility is that E12 and E47 are required to activate the expression of RAG1/2. However, E12- and E47-heterozygous pre-B cells did not show decreased levels of either RAG1 or RAG2 mRNA. A fourth possibility, and one that we favor, is that E12 and E47 binding to the Igλ enhancers directly allows the recombinase access to the recombination signal sequences.

It is now well established that the Ig light chain loci rearrange sequentially. Specifically, Igκ VJ rearrangements are initiated before the onset of Igλ VJ gene rearrangement (Nadel et al., 1990; Engel et al., 1999). It has remained unclear how the sequential rearrangement of the Ig light chain genes is achieved during the developmental progression of B-lineage cells. We suggest that E12 and E47 are critical players that regulate the sequential activation of Igκ versus Igλ loci. Previous observations have demonstrated that E47 abundance in large cycling pre-B cells is low but elevated during the transition toward the small pre-B cell stage (Quong et al., 2004). We also have previously proposed that at the pre-B cell stage, concurrent with the cessation of proliferation, E2A levels are elevated to initiate Igκ VJ gene rearrangement (Quong et al., 2004). In this study, we show that attenuation of IL-7 signaling elevates both E12 and E47 expression. We speculate that once pre-B cells migrate away from IL-7-expressing stroma, E47 as well as E12 levels increase to establish a gradient of E2A activity. This gradient of E2A activity promotes the sequential activation of Igκ versus Igλ VJ gene rearrangement. In such a model, relatively low levels of E47 would be sufficient to bind the E-box sites present in the Igκ enhancer and to promote Igκ locus accessibility. Initial levels of E47 are sufficient to achieve high occupancy binding to E-box sites present in the Igκ but not the Igλ locus. The differences in the degree of occupancy by E12 and E47 in the Igκ and Igλ enhancers might be caused by interactions with other regulators. IRF-4 is an attractive candidate because it has been shown to bind cooperatively with the E proteins to sites present in the Igκ enhancers (Johnson et al., 2008). Thus, initially, E47 levels might be limiting and act in concert with IRF-4 to drive Igκ VJ gene rearrangement but not to promote Igλ accessibility and DNA recombination. Later, during the pre-B to immature B cell transition, the absence of an Igκ or Igλ chain or the expression of a self-reactive BCR would increase E12 and E47 abundance to levels that allow sufficient occupancy at sites present in the Igκ enhancers and, thus, drive Igλ VJ recombination. On the other hand, because high levels of E47 reduce cell size and ultimately promote cell death, we suggest that the increase in E2A levels restricts the time that is available for the B cells to generate a useful receptor (Fig. 8; Engel et al., 2001; Engel and Murre, 2004). If, however, an innocuous BCR is generated, E12 and E47 abundance declines, resulting in a decrease in Igκ...
and IgA occupancy and the promotion of positive selection (Quong et al., 2004). An intriguing question is how the E2A proteins modulate the assembly of such a diverse set of antigen receptor loci, and the data described in this study may help to illuminate the mechanism by which the E2A proteins contribute to antigen receptor recombination. Recently, it was shown that RAG2 associates with H3K4me3, establishing a direct link between epigenetic marking and antigen receptor assembly (Liu et al., 2007; Matthews et al., 2007; Ramón-Maiques et al., 2007). In this study, we demonstrate that E12 elevates the abundance of H3K4me3 across the IgA J regions in B cells poised to undergo IgA VJ gene rearrangement. Clearly, E12 is not the sole factor responsible for H3K4me3 because residual H3K4me3 remains across the Jα region in E12-deficient B cells, and consequently, other factors must contribute as well. There are several possible mechanisms of how E12 might modulate the degree of H3K4me3. First, it is possible that E12 contributes directly to the recruitment of methyltransferase activity acting on H3K4. Alternatively, E12 may function indirectly by activating IgA germline transcription, enabling methyltransferases and acetylases to travel with the RNA pol II complex, which then may lead to the trimethylation of H3K4. Collectively, these data have implications for the role of the E proteins in modulating the assembly of the large majority of antigen receptor loci (Murre, 2005). We suggest that the E proteins, directly or indirectly and in conjunction with other factors, act to promote epigenetic marking of antigen receptor loci across J regions to permit the recruitment of the RAG2 component of the recombinase.

In conclusion, this study demonstrates the multiple and unique functions displayed by the E2A isoforms E12 and E47. E47 is essential during B cell specification, commitment, and maintenance and it is necessary but not sufficient during the sequential rearrangement of the Igα and Igλ loci. E12 and E47 are both required to promote Igα VJ rearrangement by modulating, at least in part, the trimethylation of H3K4 across the Jα region. Finally, we propose that in the pre-B and immature B cell compartments, a gradient of E12 and E47 activity is established to mechanistically regulate the sequential rearrangement of the Ig light chains.

MATERIALS AND METHODS

Generation of specific E12- and E47-deficient mice. The linearized targeting vector, containing either the E12 or the E47 exon flanked by two loxP sites as well as a C-terminal tetracycline tag (Martin et al., 2005), was electroporated into 129Sv/Ev ES cells, and correctly targeted clones were identified by Southern blot analyses using the enzymes and outside probe depicted in the figures. After injection into blastocysts and generation of chimeric mice the correctly germline-targeted mouse was backcrossed to Ella-1 mice to generate E12- and E47-deficient mice (Lakso et al., 1996). Mice were genotyped using primers in Table S2. After injection into blastocysts and generation of chimeric mice the correct germline-targeted mouse was backcrossed to the Ella-1 mice to generate E12- and E47-deficient mice (Lakso et al., 1996). Mice were genotyped using primers in Table S2. All experiments using mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego (UCSD).

Flow cytometry and cell sorting. FACS analyses were performed with 8–12-wk-old mice. Single cells were stained with various fluorochrome-conjugated antibodies purchased from eBioscience or BD. Data were collected with a flow cytometer (LSRII; BD) and were analyzed with FlowJo software (Tree Star, Inc.). Specific cell populations were either sorted on a FACSAria (BD) or isolated by magnetic cell sorting with anti-B220 or anti-biotin microbeads (Miltenyi Biotec) according to the manufacturer’s protocol. The following antibodies were used for flow cytometry: anti-B220 (RA3-6B2); CD19 (1D3); CD43 (R2/60); BP-1 (6C3); CD24/HSAM1(M1/69); CD117/c-kit (2B8); CD25 (PC61); IgM (II/41); IgD (11–26); CD21 (8D9); CD23 (B34); IgM light chain (187.1); Igα1, A2 and A3 light chain (R26–46); Flt3 (AF10.1); CD127/IL-7Rα (ATR34); Sc1 (D7); Ter119 (TER–119); Gr1 (RB6–8C5); CD11b/Mac1 (M1/70); and CD3e (145–2C11) and CD11c (N418). For CLP/LMPP sorting, the lineage cocktail contained antibodies against B220, Ter119, Gr1, CD11b/Mac1, CD3ε, and CD11c. Unspecific antibody binding was prevented by preincubation with CD16/CD32 Fc blocking solution (eBioscience).

Quantitative RT-PCR. Total RNA was isolated using the DNeasy kit (QIAGEN), and cDNA was prepared with oligo-dT primers using SuperScriptII reverse transcription (Invitrogen). Quantitative PCR was performed using Brilliant SYBR Green Master Mix (Agilent Technologies) on the Mx3000P cycler (Agilent Technologies) using the gene-specific primers listed in Table S2 and a 60°C annealing temperature.

Bone marrow chimera. Recipients were either κ-macrophage transgenic mice or littermate controls that carried the CD45.1 allele and have been previously described (Ait-Azzouzene et al., 2005). Recipients received 1,000 rads of γ irradiation from a Cs source the day before transfer. Bone marrow donors were all of the CD45.2 allotype. Bone marrow cell suspensions were depleted of T cells by treatment with biotinylated anti-Thy1.2 antibodies and anti-biotin microbeads (Miltenyi Biotec). 10 million cells were transferred i.v. per recipient. After 6 wk, recipients were killed and their lymphoid tissues were analyzed by flow cytometry. Only chimeras in which ≥98% of cells in the spleen were donor derived were included in the analysis.

PCR assays for Ig gene recombinations. PCR reactions were performed in a final volume of 25 µl containing 4, 1, and 0.25 ng of genomic DNA using the primers listed in Table S3. Specific probes were also generated by PCR using the primers listed in Table S4, gel purified using the QiAquick Gel Extraction Kit (QIAGEN), and radioactively labeled using the Prime-It Kit (Agilent Technologies). Samples were amplified for 25 cycles of 94°C for 1 min, 60°C for 30 s, and 72°C for 1.5 min. PCR products were electrophoresed on 1.5% (0.8% for Ig V-J) agarose gels, blotted on nylon membranes (Schleicher & Schuell), and hybridized with radioactive probes. Signals were detected with a PhosphorImager (GE Healthcare) and analyzed using ImageJ software (available at http://rsweb.nih.gov/ij/).

ChIPs. ChIP assays were performed as previously described (Agata et al., 2007). In brief, B cells were fixed with 1% formaldehyde for 10 min at room temperature, lysed, and sonicated. Samples were precleared for 1 h with protein G beads, and ChIP was performed overnight using anti-E2A (anti-E12 V18; Santa Cruz Biotechnology, Inc.), anti-H3K4me3 (Millipore), anti-acetyl H3K9/14 (Millipore), or control normal rabbit IgG (Santa Cruz Biotechnology, Inc.). After washing and elution of the beads, cross-linking was reversed and protein was digested by proteinase K treatment. ChIP DNA was isolated using QiAquick PCR Purification Kit (QIAGEN) and analyzed by quantitative PCR using Brilliant SYBR Green Master Mix on an MX3000P and the gene-specific primers listed in Table S5.

In vitro HEL-Tg B cell cultures. HEL-Tg mice, which bear rearranged IgH and Igλ light chain genes that encode an HEL-specific BCR, were obtained from the Jackson Laboratory. Whole bone marrow was isolated from 4–6-wk-old HEL-Tg mice, and red blood cells were lysed and cultured in RPMI 1640 medium with 10% (vol/vol) FCS, 100 U/ml penicillin-streptomycin, 2 mM l-glutamine, and 50 µM 2-mercaptoethanol in the presence of 1% conditioned supematant of rIL-7–producing J558L cells for 4 d at 37°C in 5% CO₂. Remaining myeloid cells as well as surface IgD+ B cells were depleted of T cells by treatment with biotinylated anti-Thy1.2 antibodies and anti-biotin microbeads (Miltenyi Biotec). 10 million cells were transferred i.v. per recipient. After 6 wk, recipients were killed and their lymphoid tissues were analyzed by flow cytometry. Only chimeras in which ≥98% of cells in the spleen were donor derived were included in the analysis.
were depleted using magnetic cell sorting (Miltenyi Biotec), and purified B cells were cultured in the absence of IL-7. Cells were harvested at the time points indicated in the figures and used for quantitative PCR.

In vitro B cell cultures. Total B cells from bone marrow were isolated using B220–coupled magnetic beads (Miltenyi Biotec) and cultured in OptiMEM medium with 10% (vol/vol) FCS, 100 U/ml penicillin-streptomycin, 2 nM l-glutamine, and 50 µM 2-mercaptoethanol in the presence of IL-7 and stem cell factor for 8 d at 37°C in 5% CO2. The remaining non-pro-B cells were depleted using magnetic cell sorting (Miltenyi Biotec), and purified B cells were cultured in the absence of IL-7 for 18 h before they were used for ChIP.

Statistical analysis. Statistical significance was calculated using the two-tailed Student’s t test. P < 0.05 was considered significant.

Online supplemental material. Fig. S1 shows c-kit staining in the bone marrow of E12- and E47-deficient mice. Fig. S2 shows E12 and E47 expression levels in LMPP and CLP cells. Fig. S3 is the analysis of bone marrow chimeras generated by transplanting wild-type and E12-deficient bone marrow cells into κ-microself transgenic mice. Fig. S4 shows IgH and Igk gene rearrangement. Fig. S5 shows RAG1 and RAG2 expression levels in pre-B cells. Tables S1–S5 list primer sequences used for PCR. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090756/DC1.

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