GA binding protein (GABP) consists of GABPα and GABPβ subunits. GABPα is a member of the Ets family transcription factors and binds DNA via its conserved Ets domain, whereas GABPβ does not bind DNA but possesses transactivation activity. In T cells, GABPα has been demonstrated to regulate the gene expression of interleukin-7 receptor α chain (IL-7Rα) and postulated to be critical in T cell development. To directly investigate its function in early thymocyte development, we used GABPα conditional knock-out mice where the exons encoding the Ets DNA-binding domain are flanked with LoxP sites. Ablation of GABPα with the Lck-Cre transgene generally diminished thymic cellularity, blocked thymocyte development at the double negative 3 (DN3) stage, and resulted in reduced expression of T cell receptor (TCR) β chain in DN4 thymocytes. By chromatin immunoprecipitation, we demonstrated in DN thymocytes that GABPα is associated with transcription initiation sites of genes encoding key molecules in TCR rearrangements. Among these GABP-associated genes, knockdown of GABPα expression by RNA interference diminished expression of DNA ligase IV, Artemis, and Ku80 components in DNA-dependent protein kinase complex. Interestingly, forced expression of prearranged TCR but not IL-7Rα can alleviate the DN3 block in GABPα-targeted mice. Our observations collectively indicate that in addition to regulating IL-7Rα expression, GABPα is critically required for TCR rearrangements and hence normal T cell development.

T cells are derived from multipotent lymphoid progenitors in the bone marrow and differentiate into mature T cells as a result of a series of lineage commitment steps occurring in the thymus and periphery (1, 2). The co-receptor molecules CD4 and CD8 can be used to distinguish four different populations of developing thymocytes, with the most immature thymocytes being double negative for CD4 and CD8 expression (termed DN thymocytes).² DN cells then become double positive (DP) thymocytes expressing both CD4 and CD8 that undergo vigorous positive and negative selections, with subsequent differentiation into single positive (SP) thymocytes expressing either CD4 or CD8. The SP thymocytes leave the thymus and populate peripheral lymphoid organs, where they can be activated upon encountering foreign antigens. During their early developmental stages, DN thymocytes can be subdivided further into four sequential stages of differentiation, which are identified by their surface expression of CD44 and CD25: DN1 (CD44⁺CD25⁻), DN2 (CD44⁺CD25⁺), DN3 (CD44⁻CD25⁺), and DN4 (CD44⁻CD25⁻). Both DN1 and DN2 subsets retain the potential to become natural killer cells and dendritic cells, and complete commitment of early thymocytes to the T cell lineage occurs at the DN3 stage (3). Rearrangement of T cell receptor β (TCRβ) locus is initiated in DN3 thymocytes, and productive recombination and proper expression of TCRβ form a major checkpoint known as β-selection. At this selection stage, TCRβ pairs with pre-Tα chain and forms pre-TCR. Deficiency in critical components of V(D)J recombination machinery such as Rag recombinases blocks T cell development at the DN3 stage.

Successful commitment and further development of T lineage cells are orchestrated by a number of transcription factors, as revealed by germ line and conditional gene knock-out experiments. These include Ikaros family factors, the “E proteins” E2A and HEB, Runx family factors, TCF family transcription factors activated by Wnt signaling, RBPShu activated by Notch-Delta interaction, GATA-3, Myb, Gfi-1, and the Ets family transcription factor PU.1 (2, 3). Ikaros family factors and PU.1 are critical in specifying hematopoietic stem cells (HSCs) to lymphoid lineages (4, 5). Notch molecules and their direct transcriptional effector RBPShu as well as E2A and HEB and their antagonists Id2 and Id3 have critical roles in establishing T cell identity and affecting successive T-lineage subspecializations (6–8). The T cell-specific transcription factors GATA3 and TCF-1 have nonredundant roles at multiple stages during T cell development (9, 10). In addition to PU.1, other Ets factors, including Ets-1, Fli-1, Tel, Elf-1, and GA binding protein (GABP), are expressed throughout most stages of T cell development (11). Ets factor functions may be partially redundant; for example, no lymphoid developmental phenotype has been observed in Elf-1 knock-out mice even though Elf-1 has been shown to bind to immunologically important genes (12).

Among more than 30 Ets factors, GABP is the only one that functions as a heterodimer of GABPα and GABPβ (13). GABPα contains the DNA-binding Ets domain that is conserved among all Ets factors, whereas GABPβ cannot bind DNA but has trans-

² The abbreviations used are: DN, double negative; DP, double positive; SP, single positive; GABP, GA binding protein; IL-7Rα, interleukin-7 receptor α chain; TCR, T cell receptor; HSC, hematopoietic stem cells; ChIP, chromatin immunoprecipitation; TIS, transcription initiation site; DNA-PK, DNA-dependent protein kinase; EYFP, enhanced yellow fluorescent protein; SISsRs,
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activation activities. GABP proteins are ubiquitously expressed and known to regulate genes that control basic cellular functions such as cell cycle progression (14, 15). Interestingly, GABP also participates in the regulation of tissue/cell type-specific genes, such as nicotinic acetylcholine receptor subunits δ and ε in neuromuscular synapses (16) and CD18 in myeloid cells (13, 17). In the immune system, we have demonstrated that GABP is critically required for normal expression of Pax5 and thus B cell development and that diminished GABP expression causes severe defects in humoral responses (18). In T cells, GABP is critical for the expression of IL-7 receptor α chain (IL-7Rα) (19). Because IL-7Rα is indispensable for T cell development, survival, and homeostasis of naive T cells, as well as for the maintenance and possibly the generation of memory T cells (20), ablation of GABP is postulated to have profound impact on multiple aspects in T cell biology. In this report, we investigated the roles of GABP in T cell development by targeting the GABPα subunit in developing thymocytes.

EXPERIMENTAL PROCEDURES

Mice—GABPα+/+ and GABPα+/- mice were kindly provided by Dr. Steven Burden (Skirball Institute, New York University Medical School) (21). The targeting strategy was summarized in Fig. 1A, and the genotyping primers are as follows: Acondi1, 5'-cagccaagacagatgtgag; Acondi2, 5'-tagggcatcataagagctg; and Acondi3, 5'-caagagaggaatcctttcc. Lck-Cre transgenic mice were from Taconic, and OT-1 TCR transgenic mice were from The Jackson Laboratory. IL-7Rα transgenic mice were previously described (22). All of the mice were handled in accordance with protocols approved by the Institutional Animal Use and Care Committee of the University of Iowa.

Flow Cytometry—Single cell suspensions were prepared from thymi and stained with fluorochrome-conjugated antibodies, as described (19). All fluorochrome-conjugated antibodies were from either eBioscience or BD Biosciences. For intracellular staining, the cells were sequentially surface-stained, fixed, and permeabilized with Cytofix/Cytoperm solution (BD Biosciences), followed by staining with anti-TCRβ. All of the data were acquired on a BD FACSCalibur flow cytometer and analyzed using FlowJo software (Tree Star Inc.).

Analysis of ChIP-Seq Data Using Site Identification from Short Sequence Reads (SISSRs) Algorithm—Genome-wide mapping of GABP binding locations in human Jurkat T cell lymphoma cells was performed using chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-Seq) (23). The sequence tag reads from anti-GABPα antibody and control IgG samples were downloaded and analyzed using the SISSRs application (24). SISSRs v1.4 was run with option a (which retains one read/genomic position even if multiple reads were mapped to that position and thus avoids overrepresentation of one position caused by PCR amplification-generated bias) with fragment length F set to 200, p value set to 0.01, and the remaining parameters set to their default values. Each identified binding site is associated with a fold enrichment score, which is the ratio of the normalized number of GABP sequence tags supporting the inferred binding site to the normalized number of control IgG tags supporting the exact same site. Genome-wide distribution of GABPα-binding sites was determined with reference to RefSeq genes downloaded from the UCSC genome browser.

Chromatin Immunoprecipitation—DN thymocytes were isolated by depleting lineage-positive cells from total thymocytes using biotinylated antibodies and Dynabeads M280 Streptavidin (Invitrogen). Chromatin fragments of DN cells were prepared as previously described and immunoprecipitated with anti-GABPα antibody (H180; Santa Cruz Biotechnologies) or rabbit IgG using Dynabeads Protein A (Invitrogen) (18). To assess enrichment of selected chromatin fragments by the GABPα antibody, primers were designed to amplify the conserved region of a gene in the mouse genome based on GABP binding locations in the same gene in the human genome as revealed by ChIP-Seq in Jurkat cells. Each primer set was tested for linear amplification range with input DNA using SYBR Advantage qPCR premix (Clontech) on ABI 7300 Real Time PCR System (Applied Biosystems). For Rag1 and Rag2 gene loci where no GABP binding was found in ChIP-Seq, the primers were designed to amplify their promoter regions as negative controls. For calculation of enrichment of each selected gene/region, 2−ΔΔCt was used, where ΔCt is the difference of Ct (cross-over threshold) values detected in GABPα antibody- and IgG-precipitated samples. Primer sequences for the amplification of each genomic segment are as follows: Rag1, 5′-AGCTATACCTGCGAGGCGAGCAAGTCCAGACAG; Rag2, 5′-CACTCTACCTGCGAGCCTTC and 5′-TCTGCCCTCTTGTAGCCAGT; Atm, 5′-ATGCGACCTTCTTCTTTGG and 5′-TGGAGAAAGATGGCTGAGA; Atg, 5′-TTTGTTGGTGTCGTCAGGTC and 5′-GAAATCCCCAGGCGCACAGACG; Prkd, 5′-CTCCGCATGGTTAGACTGGT and 5′-CTAGGCAAAGTGGTCCTTCTGA; Xrcc6, 5′-GGCGTGTCCGTTAATAGGT and 5′-AGGGTGAGGCTGGAGAAG; Xrc5, 5′-CTGATGCGGCGACGAA and 5′-TCACCCAGGCCGTTCC; and Lig4, 5′-CAGCCGTGCCGATACAACCTAA and 5′-CTGCTAAGCGAAGTGGTTG.

Knockdown of GABPα Expression in EL-4 Cells and Quantitative Reverse Transcription-PCR—Small interfering RNA constructs targeting GABPα (siGABPα) and control vector pBS/U6 were described previously (19). Murine thymoma EL-4 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and penicillin/streptomycin. EL-4 cells were transiently transfected by electroporation as described (19). In brief, 4 × 10⁶ EL-4 cells were suspended in RPMI 1640 containing 25 mM HEPEs, mixed with 9 μg of pBS/U6 or siGABPα along with 1 μg of PEYFP-N1 (Clontech) in a GenePulser cuvette (4-mm gap; Bio-Rad) and then electroporated at 250 V using a GenePulser Xcell electroporator (Bio-Rad). Forty-eight hours later, EYFPhigh cells were isolated by cell sorting, and total RNA was extracted and reverse-transcribed using a Quantitech reverse transcription kit (Qiagen). The resulting cDNA was then analyzed for expression of different genes using quantitative PCR as described above. Relative expression levels of genes of interest were normalized to that of a housekeeping gene, hypoxanthine phosphoribosyltransferase 1.
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**RESULTS**

Ablation of GABPα Blocks T Cell Development at DN3 Stage—In our previous studies, we have generated GABPα-deficient mice using a gene trap strategy, yielding hypomorphic GABPα alleles (GABPα<sup>tp/tp</sup>) (19). In contrast to preimplantation lethality resulting from a complete GABPα-null mutation, hypomorphic expression of GABPα prolonged the survival of GABPα<sup>tp/tp</sup> embryos up to embryonic day 14.5 (19). By transferring GABPα<sup>tp/tp</sup> fetal liver cells, which contain hematopoietic stem cells, into sublethally irradiated Rag2<sup>−/−</sup> mice, we observed partial blocks of T cell development at DN or DP stages. These data were presented as supplemental information in a previous report (18). The incomplete developmental block may be accounted for by the hypomorphic nature of the GABPα<sup>+/tp</sup> allele, where the levels of leaky expression of the WT GABPα protein may vary. To circumvent this caveat, we used conditionally targeted GABPα<sup>+/FL</sup> mice, in which exons 8 and 9 (encoding the DNA binding Ets domain) were flanked with LoxP sites (Fig. 1A) (21). The floxed GABPα allele was converted to a deleted allele (GABPα<sup>+/−</sup>) by crossing to Ela-Cre transgenic mice.

To inactivate GABPα in early developing thymocytes, we crossed GABPα<sup>+/FL</sup> and GABPα<sup>+/−</sup> to Lck-Cre transgenic mice to obtain LckCre-GABPα<sup>FL/+</sup> and LckCre-GABPα<sup>FL/−</sup> progeny. LckCre-GABPα<sup>FL/−</sup> mice showed expected normal DN, DP, and SP subsets. In contrast, LckCre-GABPα<sup>FL/−</sup> mice manifested a block at the DN stage in thymocyte development, with concomitant reduction of DP and SP cells (Fig. 1B). In some of the LckCre-GABPα<sup>FL/−</sup> mice, DP and SP thymocytes can be detected as more discrete populations. Regardless of the presence of DP and SP cells, all LckCre-GABPα<sup>FL/−</sup> mice had

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**FIGURE 1. Ablation of GABPα blocks T cell development at DN stage.** A, GABPα targeting strategy. In the Gabpa gene, exons 8 and 9, which encode most of the Est DNA-binding domain, were flanked with 2 LoxP sites (open triangles). The relative locations of genotyping primers are indicated. With these primer combinations, filled triangles, Frt sites. A paired of Frt sites were used to flank a neomycin-resistant gene, which was excised by a Flippase excision.

B, Representative flow cytometric profile of thymocytes. LckCre-GABPα<sup>FL/−</sup> mice were analyzed at an age of 6–8 weeks. The thymocytes from mice of indicated genotypes were stained with anti-CD4 and anti-CD8 antibodies. The percentages of each population, all LckCre-GABPα<sup>FL/−</sup> mice showed expected normal DN, DP, and SP subsets. In contrast, LckCre-GABPα<sup>FL/−</sup> mice manifested a block at the DN stage in thymocyte development, with concomitant reduction of DP and SP cells (Fig. 1B). In some of the LckCre-GABPα<sup>FL/−</sup> mice, DP and SP thymocytes can be detected as more discrete populations. Regardless of the presence of DP and SP cells, all LckCre-GABPα<sup>FL/−</sup> mice had

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(HPRT1). Primer sequences for each transcript are as follows: Gabpa, 5′-CCGCTACAACCGACTACGATT and 5′-ACCTTCATCACCAACCCAAG; II7r, 5′-AAAGCATGATGTTGCCATTACC and 5′-GACTCCACTGGCTCCAGAAAG; Tuns, 5′-GCCAGTTTGGTTCTCAAA and 5′-CCAGGCACATGATGATTC; Pola1, 5′-TGAACTGGAAGTGCTGCTG and 5′-CAGATATCGGCTCCAGAAAG; Atn, 5′-AGTGTTGACATGAGACAGCACA and 5′-CGGAATATGGATCAGCCTCAG; Atr, 5′-TGTTGGAGATGCTGCTAC and 5′-ATAGCGTGGTGAACGTC; Prkdc, 5′-AAGGCAGAACGCTGGCACAAG and 5′-ATCCGCCCAGTATGGTCAATGCG; Xrcc6, 5′-GATCAGAAGCATTCAGTGACTCC and 5′-GCC-
similar greatly diminished thymocyte cellularity (Fig. 1C). Despite the increased frequency of DN thymocytes, their absolute numbers were still significantly reduced (Fig. 1D), suggesting an absolute requirement of GABP for normal thymocyte maturation.

We next examined early T cell development at the DN stages. LckCre-GABPα<sup>FL/−</sup> mice all exhibited increased frequency of DN3 subsets and a corresponding reduction in DN4 cells compared with LckCre-GABPα<sup>FL/+</sup> controls (Fig. 2, A and B). The absolute counts of both DN3 and DN4 thymocytes were diminished in LckCre-GABPα<sup>FL/−</sup> mice, despite the increased DN3 frequency (Fig. 2C). The Lck-Cre transgene initiates excision of the floxed sequences at the DN2 stage and completes the excision at the DN3 stage (25). To determine the excision efficiency, we sorted DN3 and DN4 thymocytes from LckCre-GABPα<sup>FL/+</sup> and LckCre-GABPα<sup>FL/−</sup> mice and used PCR to detect the conversion of a floxed allele to a deleted allele. In LckCre-GABPα<sup>FL/−</sup> DN3 and DN4, the excision of the floxed allele was almost complete as expected, indicating the accessibility of the floxed GABPα allele and efficiency of LckCre-mediated excision (Fig. 2D). Contrary to the expectation that the floxed allele in LckCre-GABPα<sup>FL/−</sup> DN3 and DN4 cells should be converted to a deleted allele, it was still detectable, although the signal was weaker in DN4 cells. Our observations suggest that the excision of the floxed sequence is efficient in the presence of protection by a WT allele, converting GABPα<sup>FL/−</sup>/DN3 and DN4 thymocytes to GABPα<sup>−/−</sup>. On the other hand, in LckCre-GABPα<sup>FL/−</sup> DN3 and DN4 subsets, there is strong selection against “double deleted” GABPα<sup>−/−</sup> cells, leading to greatly diminished thymic cellularity. As a result, a considerable portion, if not all, of the remaining DN3 and DN4 thymocytes in LckCre-GABPα<sup>FL/−</sup> mice is an accumulation of those that escaped the complete excision and were detected as “undeleted” cells. This is not an unprecedented scenario. For example, when Mcl-1, a pro-survival Bcl-2 family member, was conditionally inactivated, profound reduction of thymocytes and peripheral T cells was observed, and the residual T cells found in the mice all contained a protective undeleted allele, indicating that the double deleted cells are not viable (26). Strong selection against Mcl-1-null cells also occurred when it was inactivated in B lineage cells (26) or HSCs (27). Similarly, lymphoblastoid leukemia 1 (Ly11) and T cell acute lymphocytic leukemia 1 (Tal1/Scl), members of the basic helix-loop-helix class transcription factors, are recently shown to be absolutely required for HSC survival, and Tal1<sup>−/−</sup>/Ly11<sup>FL/FL</sup> HSC-derived myeloid colonies always contained at least one Ly11-floxed allele after type I interferon-induced, Mx1-Cre-mediated excision (28).

We previously demonstrated that GABP critically regulates the expression of IL-7Rα in developing and mature T cells (19). Although LckCre-GABPα<sup>FL/−</sup> DN3 thymocytes expressed similar levels of IL-7Rα compared with LckCre-GABPα<sup>FL/+</sup>
DN3 cells, the IL-7Rα expression was reduced in LckCre-GABPαFL−/− DN4 cells (Fig. 2E). β-Selection occurs at the DN3 stage, which requires productive rearrangements of the TCRβ locus and successful pre-TCR signaling. The block at the DN3 stage in LckCre-GABPαFL−/− mice is reminiscent of failed TCRβ rearrangements, for example, because of deficiency in Rag recombinases. To determine whether GABPα has IL-7Rα-independent roles in early T cell development, we measured TCRβ expression by intracellular staining. As shown in Fig. 2F, although reduction of intracellular TCRβ was small in DN3 cells, LckCre-GABPαFL−/− DN4 thymocytes exhibited more apparent decrease in intracellular TCRβ expression. These observations are consistent with the notion that DN3 and DN4 thymocytes in LckCre-GABPαFL−/− mice may consist of a mixture of undeleted (FL−/) and double deleted (−/−) cells. The lack of apparent defects in IL-7Rα and TCRβ expression in DN3 might be explained by the fact that there are fewer double deleted cells because of stronger selection at this developmental stage. On the other hand, DN4 thymocytes might be less stringently dependent on GABPα activity for survival, and thus more double deleted DN4 thymocytes are present, allowing a window for observation of the effects derived from GABPα deficiency and thus revealing critical roles for GABP in supporting the normal expression of IL-7Rα and TCRβ in developing thymocytes.

GABP Directly Regulates Expression of Genes Critical for V(D)J Recombination—Diminished TCRβ expression in LckCre-GABPαFL−/− DN4 thymocytes raised the intriguing possibility that GABP may contribute to the regulation of V(D)J recombination at the TCR loci in addition to its direct role in activating IL-7Rα transcription. High throughput analyses of GABP-associated genes have been performed on human Jurkat T cell lymphoma cells using ChIP coupled with DNA microarrays (ChIP-on-chip) (29) and more recently ChIP coupled with massive parallel sequencing (ChIP-Seq) (23). The ChIP-Seq approach covers the entire genome and has enhanced sensitivity, and we therefore downloaded the ChIP-Seq data of GABPα antibody or control IgG-enriched sequence tags and reanalyzed the data using the SISRSs algorithm (24). Our analysis revealed ∼13,000 GABP binding locations where GABPα-associated sequences are significantly enriched. Focusing on protein-coding genes that harbor GABP-binding sites within 2 kb of their transcription initiation sites (TISs), we found that GABP is associated with 8308 unique RefSeq genes. It was reported that GABP has a unique role in inducing cell cycle re-entry through direct regulation of thymidylate synthase and p180 catalytic subunit of DNA polymerase α (encoded by TYSM and POLA1, respectively) (14). These genes were found to have enriched GABPα binding at their TISs, validating the usefulness of the ChIP-Seq data (GABP binding at the POLA1 locus was shown as an example in Fig. 3A).

Based on the phenotypic analysis of LckCre-GABPαFL−/− mice, we hypothesized that GABPα may have critical roles in TCRβ rearrangement, which is initiated by expression of RAG1 and RAG2 recombinases that introduce DNA double-strand breaks (DSBs). The DSBs are dangerous lesions that can be sensed and amended in cells with great efficiency to avoid possible chromosomal abnormalities, cell death, or neoplastic transformation. Sensing the DSBs leads to activation of at least three phosphatidyl-inositol 3 kinase-like protein kinases, ATM, ATR, and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (30). Repair of the DSBs is achieved by nonhomologous end joining (NHEJ) mechanism, which involves Ku70- and Ku80-containing DNA-PK complex, Artemis, Cernunnos, and DNA ligase IV (31). Analysis of these gene loci in the Jurkat ChIP-Seq data revealed that whereas no GABP binding was associated with the RAG1, RAG2, and NHEJ1 (encoding Cernunnos) loci, enriched GABP binding was found at the TISs of ATM, ATR, DCLRE1C (encoding Artemis), LIG4 (encoding DNA ligase IV), PRKDC, XRCC6, and XRCC5 (encoding the catalytic subunit, Ku70, and Ku80 components in the DNA-PK complex, respectively) genes (actual GABP binding at the LIG4 TIS shown in Fig. 3A, and fold enrichment of GABP binding summarized in Fig. 3B). To determine whether such GABP binding occurs in developing thymocytes, we isolated WT DN thymocytes and performed ChIP using an anti-GABPα antibody. The direct binding of GABPα was confirmed in the TISs of Atm Prkdc, Xrcc5, Dclre1c, and Lig4 genes (Fig. 3C), supporting a potential role for GABP in regulating key factors involved in the rearrangements of TCR loci.

It has been known that binding of a transcription factor to gene regulatory regions as found in ChIP-Seq or ChIP-on-chip is not necessarily correlated with its direct activation/regression of the bound genes, as seen previously with Foxp3 and in yeast (32, 33). We next investigated whether the expression levels of GABP-associated genes were altered by diminished GABPα expression. As discussed above, LckCre-GABPαFL−/− DN4 thymocytes contained double deleted cells exhibiting reduced levels of IL-7Rα and intracellular TCRβ (Fig. 2E), but they are also mixed with nondeleted FL−/− cells that escaped excision because of the selection pressure against the double deleted cells (Fig. 2D). In sorted DN4 thymocytes from LckCre-GABPαFL−/− and LckCre-GABPαFL−/− mice, with the exception that GABPα transcript was modestly decreased in LckCre-GABPαFL−/− DN4 cells, all other gene transcripts examined did not show consistent changes (data not shown), which is likely explained by obscuration caused by those nondeleted cells. To circumvent this problem, we used siGABPα to knock down its expression in EL-4 thymoma cells (19). After co-transfection of siGABPα or control pBS/U6 vector along with pEYFP-N1 expressing enhanced yellow fluorescent protein into EL-4 cells, we sorted for EYFP-positive cells 48 h later and analyzed gene expression by quantitative reverse transcription-PCR. Consistent with our previous findings (19), the siGABPα constructs effectively reduced the Gabpa transcript and its T cell target gene Il7r (Fig. 3D). In addition, knockdown of Gabpa resulted in decreased expression of Tymis and Pola1, other known GABP targets in regulating cell cycle progression (14) (Fig. 3D). Among the V(D)J recombination-related genes, Lig4 expression was most reduced, and Xrcc6, Xrcc5, and Dclre1c exhibited moderate but consistent reduction upon knockdown of Gabpa, whereas Atm, Atr, and Prkdc expression was not affected (Fig. 3D). Coupled with validated direct binding of GABPα at the TISs in DN thymocytes, our observations suggest that GABP directly regulates the expression of DNA ligase IV (Lig4), Ku80 (Xrcc5), and Artemis (Dclre1c), highlighting its direct involve-
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FIGURE 3. GABP directly regulates key genes involved in V(D)J recombination. A, GABP binding at selected gene loci inferred from ChIP-Seq. Sequence tags enriched by GABPα antibody in ChIP-Seq of Jurkat T cells were mapped to human genome on the UCSC genome browser. The raw data are displayed as accumulative tag numbers within a 200-bp-wide window as shown in the lower part of each panel, and the tag numbers are marked on the left. The ChIP-Seq data with GABPα and control IgG samples were further analyzed using the SISSR algorithm to calculate the relative enrichment in individual locations. The data are displayed as enrichment peaks in the lower part of each panel, with their heights (y axis) corresponding to fold enrichment of GABP sequence tags over control tags, as marked on the right of each panel. The gene symbols are as marked, and the gene structure and direction of transcription are shown under each panel, with vertical lines/boxes denoting exons. B, summary of GABPα binding at the TISs of genes involved in V(D)J recombination, including gene symbols, commonly used gene names, and fold enrichment by the GABPα antibody versus control IgG. The results from C and D were also summarized in the last two columns. C, validation of GABP binding in DN thymocytes. Chromatin fragments were prepared from murine DN thymocytes and immunoprecipitated with either anti-GABPα antibody or a control IgG. Gene regulatory regions overlapping with TISs of indicated genes were detected by quantitative PCR using primers designed based on the Jurkat ChIP-Seq results. The relative enrichment by the GABPα antibody versus control IgG was shown for each gene. D, effect of knocking down GABPα on the expression of GABP-bound genes. EL-4 cells were transfected with siGABPα or control pBS/U6 vector alone with pEYFP-N1, and EYFP-positive cells were sorted, RNA extracted, and reverse-transcribed. Expression of genes of interest was measured by quantitative PCR. The data are the means ± S.D. of four samples from two independent experiments. *, p < 0.05; **, p < 0.01 by t test.
Interestingly, TCR expression alleviated DN3 block in LckCre-DN3 frequencies (Fig. 6A). In the DN4 compartment, whereas the IL-7Rα transgene did not improve its frequency and numbers, the OT-I TCR increased DN4 frequency as well as numbers (Fig. 6, C and D). It should be noted that early expression of a prearranged TCRα chain has been reported to impact the proliferation and survival of DN4 thymocytes and partially block their development to the DP stage (40, 41). Although OT-I TCR had the smallest impact compared with other TCR transgenes such as C2 and HY (41), these side effects should be taken into account for the interpretation of increased DN4 subset. Nonetheless, our observations using IL-7Rα and TCR transgenes collectively provided corroborating evidence for a critical role of GABPα in TCRβ rearrangements.

**DISCUSSION**

The GABPα/β complex has versatile roles in regulating basic cellular functions as well as tissue-specific genes (13, 42, 43). Gene targeting studies of the GABPα DNA-binding subunit have revealed its critical roles during embryogenesis (44), during re-entry of cell cycle (14), and at the neuro-muscular junction (16, 45). Our previous studies on the gene regulation of IL-7Rα have identified GABP as a nonredundant factor in supporting transcription of IL-7Rα in T cells (19). In this study, we found that ablation of GABPα in developing T cells by Lck-Cre-GABPα-null mice to IL-7Rα alleles alleviated the DN3 block caused by GABPα inactivation. These findings revealed an unexpected contribution of GABPα to normal TCRβ rearrangements, in addition to its direct regulation of IL-7Rα, which provides survival signals to developing thymocytes.

Assessment of conversion of the floxed GABPα allele to a deleted one in DN3 and DN4 thymocytes of LckCre-GABPα-F/+ mice revealed a strong selection against double deleted, i.e. GABPα-null cells. Such strong selection against cells lacking genes that are essential for cell survival and/or proliferation have been observed in multiple lineages including T and B lymphocytes as well as HSCs when Mcl-1, a pro-survival Bcl-2 family member, was targeted (26, 27). Similar selection against double deleted cells was also observed in HSCs lacking another Ets family transcription factor Etv6 (46) or Tal1/Scl and Lyl1 (28). The strong selection against GABPα-null cells thus suggests an essential role for GABPα in thymocyte survival and/or proliferation. Indeed, it has been demonstrated in murine embryonic fibroblasts that GABPα directly regulates gene expression of Pola1 and Tym5 (14). Binding of GABPα to these gene loci was found in Jurkat T cells (23) and validated in DN thymocytes in this study. We further showed decreased expression of Pola1 and Tym5 upon knockdown of GABPα in EL-4 cells. Because DN3 and DN4 thymocytes are highly proliferative, their expansion is conceivably severely impaired when GABPα is ablated, thus at least partly explaining the greatly reduced thymic cellularity. The requirement of GABPα in
cell proliferation may be extended to DP thymocytes and later stages, which may explain why forced expression of OT-I TCR, which consists of both prearranged TCRα/H9251 and TCRβ/H9252 chains, failed to promote further thymocyte maturation and improve overall thymic numbers. It should be noted that the role of GABP in promoting cell proliferation is more general in various cell types, unlike its more T cell-specific regulation of IL-7Rα/H9253 and V(D)J recombination at the TCR loci. Because of the strong selection against double deleted cells, DN3 and DN4 thymocytes in LckCre-GABPαFL/H11002 mice con-

FIGURE 5. Effect of IL-7Rα or OT-I TCR transgene on early thymocyte development. A, representative flow cytometric profiles of DN thymocytes based on CD25 and CD44 fractionation in mice of indicated genotypes. The percentages of each subset are shown. B, detection of cell surface IL-7Rα and intracellular TCRβ expression in the presence or absence of transgenes. Percentages of the positive populations are indicated. The data are representative from two independent experiments with three to eight animals analyzed.
Development of B cells from pluripotent hematopoietic precursors is guided by three transcription factors, E2A, early B cell factor, and Pax5, and these factors act in a relatively simple gene regulatory cascade in B lineage specification and commitment (52, 53). In contrast, no equivalent T cell-specific transcription factors have unequivocal positive roles in instructing T lineage commitment and/or maintaining T cell identity. Rather, multiple transcription factors, T cell-specific or nonspecific, act in concert to direct the progressive commitment and maturation of T cells (2). Systematic analysis of transcription factors, with demonstrated essential roles or as yet undefined function in T cell development, identified different expression patterns throughout early T cell developmental stages (11). Along with
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Myb, Ikaros, Gfi-1, Stat5b, Oct1, and TOX, GABPα was classified in the group of "legacy" transcription factors, showing minimally changing transcript levels. Their stable expression during early T cell development contrasts remarkably with the massive changes in T-lineage gene expression. It is plausible to assume that their stable expression forms a critical platform for modulation of and/or interaction with "up-regulated" or "down-regulated" transcription factors, thus creating a genetic environment optimal for T cell commitment. The molecular details and dynamic changes in the regulatory circuit at different thymocyte developmental stages await further investigation. Nonetheless, our current studies revealed GABP as a key component in such a regulatory circuit, which has essential and multifaceted roles in programming normal T cell development.

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