B cell development is a complex process occurring in the fetal liver and then bone marrow. It begins with the proliferative expansion of progenitor cells that undergo sequential rearrangements of the Ig heavy chain (Igh) and Ig light chain (IgL) genes (Rajewsky, 1996; Meffre et al., 2000; Jung et al., 2006). Igh variable region exons are assembled from variable (VH), diversity (DH), and joining (JH) gene segments, a recombination process that must be tightly regulated to ensure lineage and stage specificity, as well as highly ordered; DH to JH joining occurs first in pre-pro–B cells, followed by VH to DHJH recombination in pro–B cells. Productive VHDHJH rearrangement results in the expression of a heavy chain that assembles with the surrogate light chains (λ5 and VpreB) to form a pre–BCR, which defines the pre–B cell differentiation stage. After further clonal expansion, pre–B cells undergo rearrangement of VL and JL elements in the IgL loci, resulting in transition to the immature B cell stage, marked by the cell surface expression of an IgM BCR. Ultimately, cells expressing functional, nonself-reactive BCRs are positively selected into the peripheral pool of long-lived mature B cells.

The CCR4–NOT deadenylase complex plays crucial roles in mRNA decay and translational repression induced by poly(A) tail shortening. Although the in vitro activities of each component of this complex have been well characterized, its in vivo role in immune cells remains unclear. Here we show that mice lacking the CNOT3 subunit of this complex, specifically in B cells, have a developmental block at the pro–pre–B cell transition. CNOT3 regulated generation of germline transcripts in the VH region of the immunoglobulin heavy chain (Igh) locus, compaction of the locus, and subsequent Igh gene rearrangement and destabilized tumor suppressor p53 mRNA. The developmental defect in the absence of CNOT3 could be partially rescued by ablation of p53 or introduction of a pre-rearranged Igh transgene. Thus, our data suggest that the CCR4–NOT complex regulates B cell differentiation by controlling Igh rearrangement and destabilizing p53 mRNA.
early B cell developmental steps are harmoniously regulated by transcriptional networks that integrate environmental cues to evoke gene expression programs appropriate to a particular developmental stage.

Emerging evidence has demonstrated that these transcriptional regulatory mechanisms on their own are not sufficient for proper B cell development and that posttranscriptional mechanisms are also required (Koralov et al., 2008). In regard to a general posttranscriptional regulator, attention has been recently paid to the CCR4–NOT multiprotein complex, which serves as one of the major deadenylases in eukaryotes (Collart and Panasenko, 2012; Miller and Reese, 2012). Deadenylation is the initial and often rate-limiting step in mRNA decay, resulting in the repression of translation (Decker and Parker, 1993). The CCR4–NOT complex consists of two major modules: the deadenylase module composed of two subunits with deadenylation enzymatic activity (CNOT6 or CNOT6L and CNOT7 or CNOT8) and the NOT module, which minimally consists of the CNOT1 scaffold protein, CNOT2, and CNOT3. Although the precise function of the NOT module remains largely elusive, a recent study indicates that it regulates the stability and activity of the deadenylase module and participates in recruitment of the CCR4–NOT complex to its specific target mRNAs (Wahle and Winkler, 2013). To ensure the target specificity, two targeting mechanisms have been proposed: first, sequence-specific RNA-binding proteins...
(RBPs) bring the CCR4–NOT complex to sequence elements in the 3′ untranslated region (3′-UTR) of the target mRNA, and second, instead of RBPs, the microRNA (miRNA) machinery recruits the CCR4–NOT complex to its target mRNA (Wahle and Winkler, 2013). In addition to its central role in specific mRNA degradation, the CCR4–NOT complex has also been implicated in transcription initiation and elongation and protein degradation (Collart and Panasenko, 2012; Miller and Reese, 2012).

The physiological significance of CCR4–NOT-mediated regulation in mammals has been addressed by using conventional knockout mice. CNOT7 deficiency leads to defects in spermatogenesis and anomalies in bone formation (Nakamura et al., 2004; Washio-Oikawa et al., 2007) and CNOT3 ablation halts embryogenesis, whereas its haploinsufficiency results in anomalies of heart function, bone formation, and energy metabolism (Neely et al., 2010; Morita et al., 2011; Watanabe et al., 2014). Although informative, the cellular and molecular bases of these severe phenotypes remain ill defined.

Here, we explored the role of CNOT3 in B cell development and activation and how, if at all, it participates in these processes. We first show that CNOT3 deficiency results in a developmental block at the pro- to pre–B cell transition. This developmental defect is attributable primarily to impaired Igh gene rearrangement in pro–B cells and increased apoptosis in pro- and pre–B cells. Notably, our data suggest that CNOT3 contributes to these biological phenomena both transcriptionally, by regulating initiation of germline transcription of the Igh locus, and posttranscriptionally, by deadenylating mRNA encoding the tumor suppressor p53.

**RESULTS**

**CNOT3 is essential for early B cell development**

Because the CNOT3 subunit has been thought to be a key component for exerting the biological functions of the CCR4–NOT complex (Collart et al., 2013), we decided to first focus on clarifying the function of CNOT3. We confirmed that CNOT3 and other subunits of the CCR4–NOT complex are expressed in the bone marrow during early B cell development and that CNOT3 protein is up-regulated in pro-B and pre–B cells (Fig. 1 A and not depicted). To determine its possible function at these developmental stages, we conditionally deleted Cnot3 in B lineage cells by crossing with the mb1-cre delter strain (Cnot3<sup>fl/fl</sup>Mb<sup>cre/+</sup>; indicated as bKO hereafter; Fig. 1, B–D; Hobeika et al., 2006). In bKO mice, floxed exons of the Cnot3 allele and CNOT3 protein were efficiently deleted at the pro–B cell stage (Figs. 1 E and 2 A). In the absence of CNOT3, other subunits of the complex were still expressed, although at somewhat decreased levels (Fig. 1 F). In bKO mice, the number of pro–B cells was increased to some extent, whereas pre–B cells were greatly reduced compared with control mice (Cnot3<sup>+/+</sup>Mb<sup>cre/+</sup>). Immature and recirculating B cells in the bone marrow and B cells in the spleen were barely detectable (Fig. 2, B–E).

Thus, CNOT3 is essential for the development of B lymphocytes and plays its critical role during the differentiation of pro–B to pre–B cells. A single Cnot3 allele was sufficient to support normal B cell development (Fig. 2, F and G).

Successful V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> recombination and the resultant production of the Ig μ chain are essential for the pro–B to pre–B transition (Kitamura et al., 1991; Jung and Alt, 2004). We found that intracellular Ig μ protein levels were greatly reduced in bKO pro–B cells (Fig. 2 H). To directly address whether the impaired μ chain expression resulted from inefficient V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> recombination, the status of the Igh locus was examined in bKO pro–B cells. PCR analyses of genomic DNA isolated from pro–B cells clearly demonstrated that although D<sub>H</sub> to J<sub>H</sub> and the D<sub>H</sub>-proximal V<sub>H</sub>7183 to D<sub>H</sub>J<sub>H</sub> rearrangements occur normally in bKO mice, the more distal V<sub>H</sub>Gam3.8 to D<sub>H</sub>J<sub>H</sub> and the most distal V<sub>H</sub>J558 to D<sub>H</sub>J<sub>H</sub> rearrangements were greatly impaired (Fig. 2 I). Consistently, the corresponding transcript levels correlated with the genomic DNA recombination frequency (Fig. 2 J). Collectively, these findings suggest the selective involvement of CNOT3 in recombination of the Igh distal V<sub>H</sub> gene segments. However, the observed defect in the distal-specific V<sub>H</sub>-D<sub>H</sub>J<sub>H</sub> recombination could also be explained by a survival defect of CNOT3-deficient pro–B cells, as B cells using the distal V<sub>H</sub>J558 family members expand at later stages of B cell development compared with those using the proximal V<sub>H</sub>7183 family (Malynn et al., 1990). In fact, staining of active caspase-3 revealed an approximately two- and fivefold higher frequency of apoptotic pro- and pre–B cells, respectively, in bKO mice (Fig. 2 K).

Because IL-7 signaling is essential for survival of pro–B cells (Malin et al., 2010), we examined its status, demonstrating that IL-7Rα surface expression as well as a downstream event, the mRNA expression of Mcl1, is unaffected by the loss of CNOT3 (Fig. 2, L and M). Thus, apparently normal IL-7 signaling takes place in the absence of CNOT3.

**CNOT3 regulates pro–B cell survival by destabilizing p53 mRNA**

To clarify the molecular basis of the survival defect in CNOT3-deficient pro–B cells, we performed comprehensive gene expression profiling of control and bKO pro–B cells. Deep sequencing of mRNA and subsequent statistical analysis led to the identification of 79 up-regulated and 107 down-regulated genes in mutant cells (Fig. 3 A and Table S1). Bioinformatic clustering with DAVID resources and KEGG pathway analysis (Huang et al., 2009) revealed a significant enrichment of genes involved in the p53 signaling pathway among the differentially expressed genes (Fig. 3, B and C). The mRNA levels of molecules known to be important for early B cell development were not significantly affected by the loss of CNOT3 (Fig. 3 D), except for Cd79a (∼2-fold decrease) and Irf4 (∼2.5-fold increase). In addition to the p53 signaling pathway, among factors related to the cell survival, Pim1 (∼2-fold increase), Mef2a (∼2-fold decrease), and Tgfbr1 (∼2-fold decrease; Fortunel et al., 2000; McKinsey et al., 2002; Bednarski et al., 2012) were differentially expressed (Table S1), which might play a role in the survival defect of bKO pro–B cells.
Figure 2. CNOT3 is essential for early B cell development. (A) CNOT3 protein expression in sorted pre-pro–B (Lin^− B220^+ IgM^− CD43^− CD25^− CD19^− ) and pro–B (Lin^− B220^+ IgM^− CD43^− CD25^− CD19^− ) cells in the bone marrow from control and bKO mice was analyzed by Western blotting. GAPDH, loading control. (B and C) Flow cytometry (B) and absolute number of B cell subsets (C) of the bone marrow cells from control (n = 8) and bKO (n = 8) mice; B220^+ IgM^− CD43^− CD25^− CD19^− c-kit^+ pro–B (Pro), B220^+ IgM^− CD43^− CD25^− pre–B (Pre), B220^+ IgM^− immature B (Imm), and B220^+ IgM^+ recirculating.
In this study, we focused on the p53 pathway because it is known to be involved in survival of early B lymphocytes (Lu et al., 1999). Real-time quantitative PCR (qPCR) analysis revealed the significant elevation of p53 mature mRNA in bKO pro–B cells (Fig. 4 A). We next determined whether this increase was caused by transcriptional and/or posttranscriptional regulation. There was no significant difference of the level of the p53 unspliced transcript (pre-mRNA) between mutant and control pro–B cells (Fig. 4 A), indicating that CNOT3 likely down-modulates the level of mature p53 mRNA in a posttranscriptional manner. This conclusion was further substantiated by the measurement of p53 mature mRNA half-life, which was significantly longer in the absence of CNOT3 (Fig. 4 B). We next examined whether p53 mRNA is directly targeted for deadenylation by the CCR4–NOT complex. A FLAG-tagged 3′-UTR of p53 mRNA, but not of control Gapdh mRNA, associated with the CCR4–NOT complex in pro–B cell line lysates in vitro (Fig. 4 C); conversely, anti–CNOT3 antibody communoprecipitated p53 mRNA in wild-type pro–B cells (Fig. 4 D). These results indicate that physical association of the CCR4–NOT complex with the 3′-UTR of p53 mRNA occurs in pro–B cells, although our data do not distinguish whether this association is direct or indirect. The targeting of the CCR4–NOT complex to p53 mRNA resulted in shortening of the poly(A) tail; in the absence of CNOT3, the poly(A) tail length of the p53 transcripts was significantly elongated, as revealed by the ligase-mediated poly(A) test (LM-PAT) assay (Fig. 4 E; Sällès et al., 1999). A similar effect of the CNOT3 deficiency on p53 mRNA was also seen in pre–B cells (Fig. 5, A and B).

The increase in p53 mRNA in bKO pro–B cells was about twofold (Fig. 4 A), whereas the increase in p53 protein was more dramatic (Fig. 4 F). This difference could be the result of enhanced translational efficiency caused by the elongated poly(A) tail and/or by release of poly(A) tail–independent translational repression imposed by the CCR4–NOT complex (Cooke et al., 2010).

We also confirmed the up-regulation of p53 target mRNAs encoding proapoptotic factors, Puma and Bax, and a cell cycle regulator, p21, in bKO pro–B and pre–B cells (Fig. 5, A and C). However, in contrast to the case of p53 (Fig. 5 B), these mRNAs were not stabilized in mutant pro–B cells (Fig. 5 D); the Puma mRNA even appeared to be somewhat destabilized in the absence of CNOT3. Because the elevated Puma, Bax, and p21 mRNA levels in mutant cells were normalized by additional deletion of the p53 gene (Fig. 5 C), their up-regulation appears to be primarily caused by p53-mediated transcriptional regulation.

**Genetic ablation of p53 rescues the survival defect but not the impaired Igh gene rearrangement**

Having demonstrated the activated p53 pathway in CNOT3-deficient pro–B cells, we wished to test whether this indeed results in a survival defect, causing the distal V_{H} to D_{H}J_{H} recombination anomaly. The ideal way to do this would be to restore the p53 level to near normal, but not null, level in the CNOT3-knockout background. For this purpose, we considered using bKO × p53^{+/−} mice; however, their pro–B cells were found to express higher levels of p53 protein and its target mRNAs such as Puma compared with control pro–B cells (Fig. 5, E and F). Therefore, instead, bKO × p53^{−/−} mice were used to address the putative cause–effect relationship. As shown in Fig. 2 K, the proportion of apoptotic cells in bKO pro–B/pre–B cells was restored to the level of control cells by genetic ablation of p53. Moreover, the reduced pro–B cell numbers in bKO mice were significantly restored, albeit incompletely, upon loss of p53 (Fig. 6, A and B). Importantly, we found that the decreased percentage of intra−μ-positive cells among bKO pro–B cells could not be complemented by p53 deletion (Fig. 6 C). Consistent with this result, rearrangement of distal V_{H}J_{S58} and V_{H}Gam3.8 to D_{H}J_{H} and the resultant V_{H}μCμ transcripts could hardly be detected in pro–B cells deficient in both CNOT3 and p53 (Fig. 6, D and E). Based on these data, we conclude that CNOT3 is a novel regulator of IgH distal V_{H} to D_{H}J_{H} recombination.

**CNOT3 is required for the efficient Igh distal V_{H} to D_{H}J_{H} rearrangement**

Distal V_{H} to D_{H}J_{H} rearrangement is known to be selectively affected by deficiencies in several molecules such as in the B cell commitment factor Pax5 (Fuxa et al., 2004), the general transcription factor YY1 (Liu et al., 2007), and the histone modifier...
We found that the germline transcripts (GLTs) that initiate from the heavy chain intronic enhancer (I\(_{\text{H}}\)) and from the most 3' D segment (DQ52) promoter (\(\mu\)) were comparably abundant in control and bKO pro–B cells on a Rag1\(^{-/-}\) background (Fig. 7 A). The levels of intergenic antisense transcripts from Pax5-associated intergenic repeat (PAIR) elements, PAIR4 and PAIR6, which are the major intergenic antisense transcripts induced by Pax5 and are thought to be involved in the regulation of distal V\(_{\text{H}}\)-DH\(_{\text{JH}}\) recombination (Ebert et al., 2011; Verma-Gaur et al., 2012), were also unaffected by the absence of CNOT3. In contrast, the GLTs in the proximal VH7183 gene segments were somewhat decreased in bKO pro–B cells. Strikingly, both sense and Ezh2 (Su et al., 2003); however, all of these genes were expressed at comparable levels in bKO and control pro–B cells (Fig. 3 D). Given that proximal V\(_{\text{H}}\) to DH\(_{\text{JH}}\) rearrangement occurred normally in bKO pro–B cells, the diminished distal V\(_{\text{H}}\) to DH\(_{\text{JH}}\) recombination in CNOT3-deficient pro–B cells seemed likely caused by limited accessibility of these particular V\(_{\text{H}}\) loci to the recombination machinery.

According to a recently proposed model (Verma–Gaur et al., 2012), germline transcription across the \(I_{\text{gh}}\) locus (Yancopoulos and Alt, 1985) plays a key role in facilitating locus compaction, which juxtaposes the distant V\(_{\text{H}}\) genes and DH\(_{\text{JH}}\) segments, allowing access to these distal genes for efficient recombination (Kosak et al., 2002; Sayegh et al., 2005; Jhunjhunwala et al., 2008). We found that the germline transcripts (GLTs) that initiate from the heavy chain intronic enhancer (I\(_{\text{H}}\)) and from the most 3' D segment (DQ52) promoter (\(\mu\)) were comparably abundant in control and bKO pro–B cells on a Rag1\(^{-/-}\) background (Fig. 7 A). The levels of intergenic antisense transcripts from Pax5-associated intergenic repeat (PAIR) elements, PAIR4 and PAIR6, which are the major intergenic antisense transcripts induced by Pax5 and are thought to be involved in the regulation of distal V\(_{\text{H}}\)-DH\(_{\text{JH}}\) recombination (Ebert et al., 2011; Verma–Gaur et al., 2012), were also unaffected by the absence of CNOT3. In contrast, the GLTs in the proximal V\(_{\text{H}}\)7183 gene segments were somewhat decreased in bKO × Rag1\(^{-/-}\) pro–B cells. Strikingly, both sense and
assay. Wild-type pro–B cell lysates were immunoprecipitated with anti-CNOT3 antibody or control IgG, and the immunoprecipitates were analyzed by RT-PCR with primers specific to p53 and Gapdh mRNA. (E) Poly(A) tail length of Gapdh mRNA in pre-pro–B and pro–B cells of control and bKO mice was determined by the LM-PAT assay. (F) Flow cytometry of intracellular p53 protein expression in pro–B cells from control, bKO, and p53−/− mice. (A) Relative expression levels of spliced p53 mature mRNA and unspliced pre-mRNA in bKO pro–B cells compared with control pro–B cells, as determined by real-time qPCR. Error bars represent SD. n = 4 biological replicates. RT(−), no reverse transcription. (B) Stability of p53 mature mRNA. Pro–B cells from control and bKO mice were treated with actinomycin D for the indicated periods of time, and the relative mRNA levels compared with time 0 were determined by real-time qPCR. Data are shown as mean ± SD of n = 3 biological replicates. (C) Association of CNOT3 and CNOT3 with the 3′-UTR of p53 mRNA. FLAG-tagged 3′-UTRs of p53 and Gapdh mRNA were incubated with lysates of an Abelson virus–transformed pro–B cell line and immunoprecipitated with anti-FLAG antibody, followed by Western blotting. (D) Association of CNOT3 with p53 mRNA by RIP assay. Wild-type pro–B cell lysates were immunoprecipitated with anti-CNOT3 antibody or control IgG, and the immunoprecipitates were analyzed by Real-time qPCR, which measures the amount of CNOT3 that might be remaining, probably because of the late onset of Cnot3 deletion by mb1-cre, thereby causing the less efficient decontraction of the Igh locus in bKO × Rag1−/− pro–B cells compared with T cells. In this regard, our deletion detection system might not have sufficed to detect such small changes. We also analyzed the distances between proximal Vh7183 and CcH probes, showing they were not significantly affected in the absence of CNOT3 (Fig. 7, C and E). These results suggest that CNOT3 is required for the regulation of efficient compaction of the Igh locus in pro–B cells.

To test whether the inability of CNOT3-deficient pro–B cells to perform distal Vh-DJcH rearrangements contributes to the developmental block observed in vivo, we made use of the B1/8hi mouse line, which carries a pre-rearranged VHDJH segment in the endogenous Igh locus (Shih et al., 2002). As shown in Fig. 8 (A and B), compared with bKO mice, bKO × B1/8hi mice displayed increased numbers of pre–B and immature B cells in the bone marrow, indicating that the developmental block imposed by CNOT3 ablation was partially alleviated by the introduction of the pre-rearranged Vh-DJcH segment. Note that Cnot3hi alleles were efficiently deleted in bKO and bKO × B1/8hi pro–B cells (Fig. 8, C and D). To address whether the only partial restoration of the pre–B cell population in bKO × B1/8hi mice is caused by the inefficient decontraction of the Igh locus in pre–B cells, we analyzed the Vh to Jc rearrangements at the Igh locus in CNOT3-deficient pro–B cells. Genomic PCR and RT-PCR analyses revealed the Vh-Jc rearrangements and κc germline transcription were unaffected by the absence of CNOT3 (Fig. 8, E and F). Hence, rather, the still-augmented p53 pathway in bKO × B1/8hi mice (Fig. 8 C) is likely to be one of the reasons for the partial restoration of the pre–B cell population in bKO × B1/8hi mice.
DISCUSSION

In the present work, we addressed the physiological function of CNOT3 and its action mechanisms by B cell–specific deletion of the Cnot3 gene and by identifying functionally critical CNOT3 targets in the in vivo context. One of the causes of impaired B cell development in the absence of CNOT3 lies in reduced rearrangement of distal VH genes, including the VHJ558 family, as a result of defective VHI locus contraction. The fact that the VHJ558 family is the largest VH gene family that occupies the 5′ distal half of the VH region explains the major reduction of intracellular μ chain–positive pro–B cells in the absence of CNOT3.

The mechanism by which each of the ∼200VHI gene segments scattered over a 2.5-Mbp region has an equal opportunity to establish contact and recombine with the DHI element was a mystery until it was realized that large-scale locus contraction could occur through chromatin looping (Kosak et al., 2002; Fuxa et al., 2004; Sayegh et al., 2005; Jhunjhunwala et al., 2008). Recent chromosome conformation capture sequencing experiments (4C-seq) have revealed the existence of two layers of chromatin contraction, local and large scale (Medvedovic et al., 2013). The local chromatin loops, ranging in length from 0.5 to 1.3 Mb, as well as VHJ GLTs are observed even in the absence of Pax5 (Hesslein et al., 2003). Subsequently, Pax5 and YY1 activate PAIRs, particularly PAIR4 and PAIR6, by inducing noncoding intergenic antisense transcripts, thereby promoting PAIRs to form the base of large-scale chromatin loops. Our data suggest that, similar to noncoding intergenic antisense transcripts, the VHJ558 sense and antisense GLTs are likely to be required for the local chromatin looping, which, in turn, could be a prerequisite for subsequent large-scale looping mediated by Pax5 and YY1.

The mechanism by which CNOT3 induces VHJ558 germ-line transcription remains elusive. The presence of defective nascent transcripts in the mutant pro–B cells makes the involvement of CNOT3 in the transcription processes by itself likely, although its participation in destabilization of mRNAs encoding suppressor-type transcription factors could not be completely excluded. In support of the transcriptional regulation model, recent genome-wide RNAi screening experiments identified CNOT3 as one of the molecules that form a unique module in the transcription network required for mouse embryonic stem (ES) cell self-renewal (Hu et al., 2009). Assuming that CNOT3 participates in transcription processes,
pro–B cells, which may, at least to some extent, contribute to the defective pro– to pre–B cell transition.

p53 contributes to the suppression of pro–B cell expansion during the checkpoint associated with Igh locus recombination and the subsequent formation of the pre–BCR (Lu et al., 1999). This process requires the creation of DNA double-strand breaks and is thought to activate the p53 response, which initiates transcription of genes that arrest cell cycle and induce apoptosis (Khanna and Jackson, 2001). Therefore, a reasonable speculation is that CNOT3-mediated suppression of p53 expression may allow pro–B cells to survive the physiological genomic stress required for proper recombination at the Igh locus and to subsequently facilitate the expansion of successfully recombined cells. Up-regulation of some subunits of the CCR4–NOT complex, including CNOT3, in pro– and pre–B cell stages may support this speculation.

Because miRNAs are involved in the recruitment of the CCR4–NOT complex to the target mRNAs (Wahle and Winkler, 2013), we anticipated that the phenotypes of three possibilities could be envisaged: (1) regulating transcription initiation by its interaction with transcription factors (Badarinarayana et al., 2000; Lemaire and Collart, 2000; Deluen et al., 2002), (2) controlling transcription elongation by its binding to Pol II elongation factor Paf1c (Kruk et al., 2011), and (3) controlling histone modifications (Laribee et al., 2007; Mulder et al., 2007; Neely et al., 2010).

The CCR4–NOT complex acts as one component for the nonsense-mediated mRNA decay (NMD) mechanism; aberrant mRNAs containing premature translation termination codons are detected by the NMD mechanism, thereby being degraded by the action of the CCR4–NOT complex (Loh et al., 2013). For instance, in early B lymphocytes, nonsense Igh mRNA from a nonproductively rearranged allele is rapidly degraded by this mechanism (Li and Wilkinson, 1998). Given the recent evidence for the importance of the NMD mechanism in development of early lymphocytes (Frischmeyer-Guerrero et al., 2011; Lutz et al., 2011), it is possible that the NMD pathway is perturbed in the CNOT3-deficient pro–B cells, which may, at least to some extent, contribute to the defective pro– to pre–B cell transition.

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Such CNOT3-associated RBPs are thought to function by destabilizing on p53 mRNA. Counteracting this action are stabilizing RBPs, for example HuR, which was initially identified as an AU-rich element-binding protein with a potential to stabilize mRNA (Barreau et al., 2005). By using Lck-Cre–dependent deletion of HuR, one study demonstrated that the transition through the β-selection checkpoint during T cell development (the TCR β locus recombination stage) was promoted and that protein expression of p53 was reduced in HuR-deficient thymocytes (Papadaki et al., 2009), suggesting that HuR functions as a positive regulator of p53 mRNA stability in pro– and pre–T cell stages. Hence, it is reasonable to anticipate that p53 expression is regulated posttranscriptionally, mediated by the balance of opposing RBPs, thereby contributing significantly to early lymphocyte development.

**MATERIALS AND METHODS**

**Mice.** To generate Cnot3−/− mice, ES clones of Cnot3neo/+ mice (Morita et al., 2011) were transiently transfected with a pIC-Cre vector and screened by PCR and Southern blot analyses to select the clones in which the loxpP CNOT3- and Dicer-deficient mice might have some overlap. However, key biological targets differ in these two mutant mice. Bim expression was dramatically increased in Dicer-deficient pro–B cells (Koralov et al., 2008) but was normal in the absence of CNOT3 (Fig. 3 D and not depicted). Given that CNOT1 interacts with the miRNA machinery–associated proteins GW182/TNRC6 (Braun et al., 2011), one straightforward explanation among many is that even in the absence of the CNOT3 subunit, the CCR4–NOT complex, including the CNOT1 subunit, is recruited to the miRNA machinery, thereby functioning as a posttranscriptional regulator. In contrast, our data clearly demonstrate that CNOT3 is required for targeting the CCR4–NOT complex to p53 mRNA. Considering the previous evidence that NOT3, a Drosophila melanogaster homologue of CNOT3, directly interacts with Bic–C, an RBP, thereby recruiting the CCR4–NOT complex to target mRNAs (Chicoine et al., 2007), we would propose that the interaction between RBPs and CNOT3 for recruitment of the CCR4–NOT complex to p53 mRNA is a possible mechanism operating in pro- and pre–B cells.

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flanked neo-cassette was correctly removed (Fig. 1, B–D). Positive ES clones were used for microinjection to obtain chimeric mice. These chimeric mice were then crossed with C57BL/6 mice to obtain animals with germline transmission of the targeted allele. Cnot3<sup>fl/fl</sup> F1 mice were backcrossed to the C57BL/6 background for at least 10 generations. Homozygous mutant (Cnot3<sup>fl/fl</sup>) mice produced by crossing Cnot3<sup>fl/fl</sup> heterozygotes were born at Mendelian ratios, were fertile, and had no obvious aberrant phenotype.

Flow cytometry analysis. 7–12-wk-old experimental and control mice were used for cell type analyses. Single-cell suspensions of splenocytes and bone marrow cells from two tibiae and femurs lysed of red blood cells were used for microinjection to obtain chimeric mice. These chimeric mice were used for cell type analyses. Single-cell suspensions of splenocytes and bone marrow cells from two tibiae and femurs lysed of red blood cells were used for microinjection to obtain chimeric mice. These chimeric mice were then crossed with C57BL/6 mice to obtain animals with germline transmission of the targeted allele. Cnot3<sup>fl/fl</sup> F1 mice were backcrossed to the C57BL/6 background for at least 10 generations. Homozygous mutant (Cnot3<sup>fl/fl</sup>) mice produced by crossing Cnot3<sup>fl/fl</sup> heterozygotes were born at Mendelian ratios, were fertile, and had no obvious aberrant phenotype.

Mb1-cre mice (provided by E. Hobeika and M. Reth, Max Planck Institute at Mendelian ratios, were fertile, and had no obvious aberrant phenotype. Mb1-cre mice (provided by E. Hobeika and M. Reth, Max Planck Institute at Mendelian ratios, were fertile, and had no obvious aberrant phenotype.

Figure 8. Partial rescue of the differentiation defect in bKO mice by the introduction of pre-rearranged V<sub>D</sub>D<sub>B</sub> segment. (A and B) Flow cytometry (A) and absolute number of B cell subsets (B) of the bone marrow cells from the indicated mice (n = 3–6) using the same gating strategy as in Fig. 2 (B and C). (E) Each symbol represents a single mouse, and bars indicate the mean. (C) Real-time qPCR to analyze the relative mRNA expression levels of Cnot3, p53, Puma, Bax, and p21 in bKO × B1-8<sup>hi</sup> pre–B cells, normalized by control × B1-8<sup>hi</sup> pre–B cells. Cnot3 primers are designed in the flanked exons to confirm efficient deletion of Cnot3. Error bars represent SD. n = 3 biological replicates. (D) PCR genotyping of pre–B cells of the indicated mice. The PCR fragments corresponding to wild-type, floxed, or deleted (Δ) alleles are indicated to the right. (E) PCR analysis of V<sub>C</sub>-J<sub>C</sub> rearrangements with fivefold serial dilutions of genomic DNA from sorted control × B1-8<sup>hi</sup> pre–B cells, bKO × B1-8<sup>hi</sup> pre–B cells, and Rag1<sup>−<sup>−</sup>−</sup> pro–B cells. Cμ, loading control. (F) RT-PCR analysis of the expression of the rearranged V<sub>C</sub>-C<sub>μ</sub> transcripts and κ, GLTs with fivefold serial dilutions of cDNA prepared from sorted pre–B cells of control × B1-8<sup>hi</sup> and bKO × B1-8<sup>hi</sup> mice. Hprt, loading control; RT(−), no reverse transcription. (A–F) Data are representative of three (A) or two (C–F) independent experiments or are pooled from two experiments (B). *, P < 0.05; **, P < 0.01; ***, P < 0.001; Student's t test. 

RNA analysis. Total RNA was extracted from purified cell populations using TRizol reagent (Invitrogen). For RT-PCR, DNase I (Invitrogen)-treated RNA was reverse transcribed with a mixture of random primers and bubble of Immunology and Epigenetics; Hobeika et al., 2006), Mb1-cre mice (provided by E. Hobeika and M. Reth, Max Planck Institute at Mendelian ratios, were fertile, and had no obvious aberrant phenotype.

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FLAG-tagged RNA-based immunoprecipitation assay. Preparation of FLAG peptide–tagged RNAs was performed as described previously (Adachi et al., 2014). The PD31 Abelson virus–transformed pro–B cell line (provided by M. Schlissel, University of Michigan, Ann Arbor, MI) was expanded as described previously (Muljo and Schlissel, 2003) and lysed with a buffer containing 20 mM Hepes–NaOH, pH 7.5, 150 mM NaCl, 50 mM Na2VO3, 1% digitonin, protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail (Nacalai Tesque). FLAG-tagged RNA (10 pmol) was mixed with anti–FLAG M2 agarose (Sigma–Aldrich) and subjected to immunoprecipitation with the cell lysate. The immunoprecipitates eluted with 3× FLAG peptide (Sigma–Aldrich) were analyzed by Western blotting.

RNA-seq and pathway analysis. Total RNA was extracted from sorted cells by using TRIzol reagent. The DNA library for RNA-seq analysis was constructed with a TruSeq RNA sample prep kit (Illumina) as instructed by the supplier. The size range of the resulting DNA library was estimated on a 2100 Bioanalyzer (Agilent Technologies). After checking the molar concentration, the DNA library was subjected to sequencing on a HiSeq 1000 sequencer (Illumina) in a 100-bp single-end read mode. The raw data were processed with CASAVA 1.8.2 (Illumina) to generate fastq files. The sequence reads were aligned to the Mus musculus reference genome (Build 37) using TopHat version 2.0.13 (Trapnell et al., 2010). According to the mapped data, Cufflinks version 2.2.1 (Trapnell et al., 2010) was used to calculate the fragments per kilobase of exon per million reads (FPKM) with the Mus musculus genome annotation NCBI build 37.2 downloaded from its website. The statistical significance of the differential gene expression between cell populations was evaluated using the Cuffdiff software version 2.2.1. (Trapnell et al., 2010). Genes with false discovery rate (FDR) <0.05 were selected as differentially expressed ones. Four biological replicates were used in each genotype. For pathway analysis of the differentially expressed genes, we used DAVID resources version 6.7. The RNA-seq data are available at Gene Expression Omnibus database under accession no. GSE64455.

V(D)j recombination analysis. Genomic DNA was isolated from sorted pro–B or pro–B cells by phenol extraction and ethanol precipitation. PCR analyses were performed using published primers as described previously (Schlissel et al., 1991; Fukuyama et al., 2004), and PCR conditions were adjusted to be in the linear amplification range by serial dilution of template DNA. Samples from Rag1−/− pro–B cells serve as a negative control for D5 to JH5. V5 to D5 and V5 to J5 recombination experiments. The PCR products were separated on agarose gels and visualized by ethidium bromide staining.

The following primers were used for V(D)J recombinatorial analysis (V5): 5′-CCAGCTCTCCTACAGCTCCTACACTC-3′, V5Gam3.8 forward, 5′-CAAGGACGCTTGCCTAGATG-3′, VH7183 GLT reverse, 5′-TCTGCAGGTGTGAGGTCCTT-3′, V5GLT reverse, 5′-AAACACGCGCGCCGCGCGGACGAGG-3′, Bax forward, 5′-GGAGACGCTTGGGAGCG-3′, Bax reverse, 5′-AAAGGGCCCTGCTTCTATGAG-3′, p21 forward, 5′-CCGGTTGTCCTGGGCA-3′, p21 reverse, 5′-CATGAGGCGCTCAGC-3′, Ctno3 forward, 5′-GGGAAGAAGGGCGACAGG-3′, Ctno3 reverse, 5′-GGAGGCGCTTGGGCA-3′, p21 forward, 5′-ATGGTGAAGGTCGTTGAGGGATTGCTG-3′, Gapdh reverse, 5′-AGCTTCACCTTGCCTCGAGTCTG-3′, Hpt forward, 5′-CTCTGCACAGGCGCTTCTT-3′, Hpt reverse, 5′-CTCTGGTGTCACTGCAATCTC-3′, V5GLT forward, 5′-ATGGATGAGCTGATCGTCTG-3′, V5GLT reverse, 5′-GACACATCAGGTGGTTGTGTAG-3′, V5GLT forward, 5′-CCGATTCAAGAASAMCCTGGTCTCGTCAATGACG-3′, V5GLT reverse, 5′-GTCCTTCGCCGCTCCTGCTG-3′, McI forward, 5′-TCAAGAATGGTGATACACAC-3′, and McI reverse, 5′-CCGTTTCTGCCTACAAAGGAGA-3′.
LM-PAT assay. LM-PAT assay was performed as described previously (Sallès et al., 1999) with some modifications. In brief, the poly(A) tails of total RNAs (500 ng) were first saturated with 5'-phosphorylated oligo(dT)20-30 at 42°C in the presence of T4 DNA ligase. Then, the excess amount of oligo(dT) anchor primer (5'-GGCGAGCTCCGGCCGCGCTTTTTTTTTTTTTT-3') was added to the reaction to anneal at the end of poly(A) tails and incubated for 2 h at 12°C to complete ligation. This ligated primer was subjected to primer RT using SuperScript II (Invitrogen). PCR was performed with an anchor primer (5'-GGCGAGCTCCGGCCGCGCTTTTTTTTTTTTTT-3') and a p53 3'-UTR-specific sense primer (5'-CTGGCACCTACATGAATCTCCTC-3'). Specificity of the PCR reaction was confirmed by digestion of the PCR products with a restriction enzyme (ApaI). The PCR products were resolved on a 10–20% gradient polyacrylamide gel, stained with ethidium bromide, and visualized with an ImageQuant LAS 4000 mini imager (GE Healthcare).

Western blot. Cells were lysed in RIP assay buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, and 0.05% SDS) containing protease inhibitor cocktail and phosphatase inhibitor cocktail. Equal amounts of each sample were separated by SDS-PAGE and transferred to PVDF membranes (EMD Millipore). Immunoblotting was performed using the following antibodies: anti–CNOT1, CNOT6, CNOT6L, CNOT8, CNOT9, and CNOT10 antibodies described previously (Moriya et al., 2007; Chen et al., 2011; Ito et al., 2011); and commercially obtained anti-CNOT2 (Cell Signaling Technology), CNOT3 (clone 4B8; Abnova), CNOT7 (clone 2F6; Abnova) and GAPDH (clone 6C5; EMD Millipore) antibodies.

3D DNA-FISH. 3D DNA-FISH was performed essentially as described previously (Sayegh et al., 2005). In brief, 4 × 10⁴ sorted pro–B cells were attached to poly-L-lysine–coated coverslips. Cells were fixed in paraformaldehyde, permeabilized, and hybridized with fluorescently labeled BAC probes (provided by Hybridoma Bank; University of Iowa) per the 3D DNA-FISH protocol and providing BAC clones, P.D. Burrows for critical review. Mice were generated using the 3320 Cre mice, M. Schlissel for the PD31 pro–B cell line, C. Bossen and C. Murre for sharing reagents, A. Hatzfeld, and J.A. Hatzfeld. T. Inoue performed most of the experiments with the assistance of K. Isono, T. Ikawa, H. Kawamoto, and H. Koseki; M. Morita, S. Adachi, T. Natsume, and T. Yamamoto contributed to new reagents; A. Hikijata, Y. Fukuda-Yuzawa, T. Fukao, and O. Ohara helped with transcriptome analyses; and T. Inoue and T. Kurosaki designed the research and wrote the paper.

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