CNOT3 contributes to early B cell development by controlling Igh rearrangement and p53 mRNA stability

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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–to pre–B cell transition. CNOT3 regulated generation of germline transcripts in the Iγ region of the immunoglobulin heavy chain (Iγh) locus, compaction of the locus, and subsequent Iγ 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 Iγh transgene. Thus, our data suggest that the CCR4–NOT complex regulates B cell differentiation by controlling Iγh rearrangement and destabilizing p53 mRNA.

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 (Iγh) and Ig light chain (Iγl) genes (Rajewsky, 1996; Meffre et al., 2000; Jung et al., 2006). Iγh variable region exons are assembled from variable (Vγ), diversity (Dγ), and joining (Jγ) gene segments, a recombination process that must be tightly regulated to ensure lineage and stage specificity, as well as highly ordered; Dγ4 to Jγ1 joining occurs first in pre-pro–B cells, followed by Vγ1 to Dγ1Jγ1 recombination in pre–B cells. Productive VγDγJγ rearrangements result in the expression of a μ 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 Vγ and Jγ elements in the Iγl loci, resulting in transition to the immature B cell stage, marked by the cell surface expression of an IgM BCR. Ultimately, cells expressing functional, non-self-reactive BCRs are positively selected into the peripheral pool of long-lived mature B cells.

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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 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.

Figure 1. Generation of the Cnot3 conditional knockout mice. (A) Total lysates from $1.5 \times 10^5$ sorted pre-pro–B (Lin$^-$B220$^+$IgM$^-$CD43$^+$CD25$^-$CD19$^-$) and pro–B cells (Lin$^-$B220$^+$IgM$^-$CD43$^+$CD25$^-$CD19$^-$) and 3 $\times 10^5$ pre-B (Lin$^-$B220$^+$IgM$^-$CD43$^+$CD25$^+$), immature B (Lin$^-$B220$^+$IgM$^+$), and recirculating B (Lin$^-$B220$^+$IgM$^+$) cells were separated by SDS-PAGE and immunoblotted with anti-CNOT3 antibody. Almost equal protein loading was confirmed by GAPDH. (B) Structure of the targeted Cnot3 allele. Open triangles indicate loxP sequences. Numbered boxes represent exons in the Cnot3 gene. Negative (HSV-TK) and positive (PGK-neo) selection markers are indicated by open rectangles (TK, thymidine kinase; neo, neomycin). The loxP-flanked neo-cassette was removed by transient transfection of the pIC-Cre vector into ES clones of Cnot3neo/+ mice and PCR screening. Arrowheads indicate the position of the primers used in C and D. (C and D) PCR genotyping of genomic DNA isolated from Cnot3+/+, Cnot3neo/+, Cnot3null/+, and Cnot3flox/+ mice using primers a–c (C) and primers d–f (D). (E) RNA-seq–mapped reads at the Cnot3 locus from control and bKO pro–B cells. (F) Expression of subunits of the CCR4–NOT complex in control and bKO pro–B cells. Total lysates from $1.5 \times 10^5$ sorted pro–B cells of control and bKO mice were separated by SDS-PAGE and immunoblotted with antibodies against the CCR4–NOT subunits. GAPDH, loading control. (A and F) Data are representative of two independent experiments.
(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 deleter strain (Cnot3lofl/fl; 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 (Cnot3lofl/fl;MblCre/flox/flox/+). 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\textsubscript{H}D\textsubscript{J\textsubscript{H}} recombination and the resultant production of the Ig \( \mu \) chain are essential for the pro–B to pre–B transition (Kitamura et al., 1991; Jung and Alt, 2004). We found that intracellular Ig \( \mu \) protein levels were greatly reduced in bKO pro–B cells (Fig. 2 H). To directly address whether the impaired \( \mu \) chain expression resulted from inefficient V\textsubscript{H}D\textsubscript{J\textsubscript{H}} 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\textsubscript{H} to J\textsubscript{H} and the D\textsubscript{H}-proximal V\textsubscript{H}7183 to D\textsubscript{J}1\textsubscript{H} rearrangements occur normally in bKO mice, the more distal V\textsubscript{H}Gam3.8 to D\textsubscript{J}1\textsubscript{H} and the most distal V\textsubscript{H}J558 to D\textsubscript{J}1\textsubscript{H} 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\textsubscript{H} gene segments. However, the observed defect in the distal-specific V\textsubscript{H}-D\textsubscript{J\textsubscript{H}} recombination could also be explained by a survival defect of CNOT3 deficient pro–B cells, as B cells using the distal V\textsubscript{H}J558 family members expand at later stages of B cell development compared with those using the proximal V\textsubscript{H}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-7R\( \alpha \) surface expression as well as a downstream event, the mRNA expression of Mcl1, is unaffected by the loss of CNOT3 (Fig. 3, 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 Idf4 (~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 Tgfb1 (~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− c-kit+ pro–B (Pro), B220+ IgM− CD43− CD25− pre–B (Pre), B220+ IgM+ immature B (Imm), and B220+ IgM+ recirculating B (Rec).
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 specifically 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; Sallés 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. 4 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 VH to D, D, J rearrangement abnormal. The ideal way to do this would be to restore the p53 level to the 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 pre–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 intran−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, J, and V, D, J and the resultant V, µ 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 VH to D, J recombination.

**CNOT3 is required for the efficient Igh distal VH to D, J rearrangement**

Distal VH to D, J 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 (Lu et al., 1999), and the histone modifier

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B (Rec) cells. [D and E] Flow cytometry (D) and absolute number (E) of splenic B cells (B220−CD3−) and T cells (B220−CD3+) from control (n = 8) and bKO (n = 8) mice. [F and G] Flow cytometry (F) and absolute number of B cell subsets (G) of bone marrow from Cnot3−/−Mbd−/− (n = 5) and Cnot3−/−Mbd−/− (n = 6) mice; B220+IgM− pro–B and pre–B (ProPre), B220+IgM− immature B, and B220+IgM+ recirculating B cells. Numbers in B, D, and F indicate the percentage of cells. (H) Flow cytometry of intracellular Ig µ chain (intra µ) expression in pro–B cells from control, bKO, and Rag1−/− mice. Percentages indicate the frequency of intra µ−positive cells (bracketed line). (I, top) Schematic drawing of the germline Igh locus with the relative location of the different V, H families. Bottom (bottom) PCR analysis of D, J, and V, J rearrangements with fivefold serial dilutions of genomic DNA from sorted pro–B cells of control, bKO, and Rag1−/− mice. (J, top) Schematic drawing of the rearranged D, J, and V, µ transcripts with fivefold serial dilutions of cDNA prepared from sorted pro–B cells of control, bKO, and Rag1−/− mice. Hprt, loading control; RT(−), no reverse transcription. (K) Frequency of apoptotic pro–B and pre–B cells from mice of the indicated genotypes (n = 3–5), as assessed by positive staining of active caspase-3. (L) Flow cytometry analysis of surface expression of IL-7Rx on pro–B cells from control and bKO mice. Gray histogram, isotype control signal in control mice. Numbers indicate the mean fluorescence intensity of each population. (M) Relative mRNA expression levels of Mecl in bKO pro–B cells compared with control pro–B cells, as determined by real-time qPCR. Error bars represent SD. n = 3 biological replicates. (C, E, G, and K) Each symbol represents a single mouse, and bars indicate the mean. A–M Data are representative of three (A, B, D, F, H, I, and J) or two (L and M) independent experiments or are pooled from three (C and E) or two (G and K) experiments. **, P < 0.01; ***, P < 0.001; Student’s t test.
The germline transcripts (GLTs) that initiate from the heavy chain intronic enhancer \( (I_{\text{H}}) \) and from the most 3' D segment \( (D_{Q52}) \) promoter were comparably abundant in control and bKO pro–B cells on a \( \text{Rag1}^{-/-} \) 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\( _{H} \)-D\( _{H} \)J\( _{H} \) 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\( _{H} \)7183 gene segments were somewhat decreased in bKO \( \times \text{Rag1}^{-/-} \) 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\( _{H} \) to D\( _{IH} \) rearrangement occurred normally in bKO pro–B cells, the diminished distal V\( _{H} \) to D\( _{IH} \) recombination in CNOT3-deficient pro–B cells seemed likely caused by limited accessibility of these particular V\( _{H} \) loci to the recombination machinery.

According to a recently proposed model (Verma-Gaur et al., 2012), germline transcription across the \( I_{\text{H}} \) locus (Yancopoulos and Alt, 1985) plays a key role in facilitating locus compaction, which juxtaposes the distant V\( _{H} \) genes and D\( _{IH} \)J\( _{H} \) 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 \( (D_{Q52}) \) promoter \( (\mu) \) were comparably abundant in control and bKO pro–B cells on a \( \text{Rag1}^{-/-} \) 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\( _{H} \)-D\( _{Ih} \) 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\( _{H} \)7183 gene segments were somewhat decreased in bKO \( \times \text{Rag1}^{-/-} \) 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 p53 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 bKO × B1-8 mice. Numbers indicate the mean fluorescence intensity of each population. Data are representative of three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001; Student’s t test.

antisense GLTs in the distal V\(_{\mu}\)J558 gene segments were greatly reduced in these cells (Fig. 7 A). To determine whether this decrease is caused by transcriptional or posttranscriptional events, expression levels of newly transcribed nascent RNA were quantified by pulse labeling of pro–B cells with a uridine analogue, 5-ethynyl uridine (EU), which is efficiently incorporated into the nascent RNA. Quantification of the EU-incorporated RNAs revealed that nascent V\(_{\mu}\)J558 GLTs were reduced ~60% and nascent V\(_{\mu}\)J558 GLTs were reduced more than ~95% in bKO × Rag\(^{1-/-}\) pro–B cells (Fig. 7 B). Thus, CNOT3 is highly suggested to be required for the germline transcription of the \(Igh\) V genes, particularly in the distal V\(_{\mu}\)J558 family. In contrast, nascent p53 mRNA levels were comparable between control and CNOT3-deficient cells, consistent with the unaltered p53 pre-mRNA levels in bKO pro–B cells described above (Fig. 4 A).

Next, we measured the distances between V\(_{\mu}\) probes (V\(_{\mu}\)J558 or V\(_{\mu}\)J7183) and the constant region probe (C\(_{\mu}\)) on the same \(Igh\) allele using three-dimensional DNA fluorescence in situ hybridization (3D DNA-FISH). The distribution of intralocus distances between V\(_{\mu}\)J558 and C\(_{\mu}\) probes in control × Rag\(^{1-/-}\) pro–B cells are more constrained compared with those in splenic CD4 T cells (Fig. 7, C and D), as reported previously (Sayegh et al., 2005). We found that the absence of CNOT3 resulted in a significant increase in the spatial distances between the V\(_{\mu}\)J558 and C\(_{\mu}\) loci compared with control pro–B cells, although the increase was not as extensive as seen in T cells (Fig. 7, C and D). In regard to the less extensive spatial distances compared with T cells, our data could not completely exclude the following possibility. Only a small amount of CNOT3 might be remaining, probably because of the late onset of Cnot3 deletion by mb1-cre, thereby causing the less efficient deconformation of the \(Igh\) locus in bKO × Rag\(^{1-/-}\) 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 V\(_{\mu}\)J7183 and C\(_{\mu}\) 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 V\(_{\mu}\)D\(_{\mu}\)J\(_{\mu}\) rearrangements contributes to the developmental block observed in vivo, we made use of the B1-8\(^{th}\) mouse line, which carries a pre-rearranged V\(_{\mu}\)D\(_{\mu}\)J\(_{\mu}\) segment in the endogenous \(Igh\) locus (Shih et al., 2002). As shown in Fig. 8 (A and B), compared with bKO mice, bKO × B1-8\(^{th}\) 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 V\(_{\mu}\)D\(_{\mu}\)J\(_{\mu}\) segment. Note that Cnot3\(^{fl}\) alleles were efficiently deleted in bKO and bKO × B1-8\(^{th}\) pro–B cells (Fig. 8, C and D). To address whether the only partial restoration of the pre–B cell population in bKO × B1-8\(^{th}\) mice is caused by the inefficient \(Igh\) VJ rearrangements, we analyzed the V\(_{\mu}\) to \(J_{\mu}\) rearrangements at the \(Igh\) locus in CNOT3-deficient pre–B cells. Genomic PCR and RT-PCR analyses revealed the V\(_{\mu}\)J\(_{\mu}\) rearrangements and \(k_{\mu}\) germline transcription were unaffected by the absence of CNOT3 (Fig. 8, E and F). Hence, rather, the still-augmented p53 pathway in bKO × B1-8\(^{th}\) mice (Fig. 8 C) is likely to be one of the reasons for the partial restoration of the pre–B cell population in bKO × B1-8\(^{th}\) 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 V<sub>H</sub> genes, including the V<sub>H</sub>J<sub>558</sub> family, as a result of defective V<sub>H</sub> locus contraction. The fact that the V<sub>H</sub>J<sub>558</sub> family is the largest V<sub>H</sub> gene family that occupies the 5′ distal half of the V<sub>H</sub> region explains the major reduction of intracellular <mu> chain–positive pro–B cells in the absence of CNOT3.

The mechanism by which each of the 200 V<sub>H</sub> gene segments scattered over a 2.5–Mb region has an equal opportunity to establish contact and recombine with the D<sub>H</sub>J<sub>H</sub> 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 V<sub>H</sub> 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 V<sub>H</sub>J<sub>558</sub> 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 V<sub>H</sub>J<sub>558</sub> 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 TCRβ 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 Cnot3fl/fl mice, ES clones of Cnot3neo/neo 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 loxP 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.
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 germine transmission of the targeted allele. Cnot3\textsuperscript{fl/+} F1 mice were backcrossed to the C57BL/6 background for at least 10 generations. Homozygous mutant (Cnot3\textsuperscript{fl/fl}) mice produced by crossing Cnot3\textsuperscript{fl/+} 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 of Immunology and Epigenetics; Hobeika et al., 2006), p53\textsuperscript{-/-} mice (Gondo et al., 1994), B1-8\textsuperscript{hi} IgH knock-in mice (provided by M.C. Nussenzweig, The Rockefeller University, New York, NY; Shih et al., 2002), and Rag1\textsuperscript{-/-} mice (Jax 002216; JAX Mice database) are described elsewhere and were main

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 cell type analyses. Single-cell suspensions of splenocytes and 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, Puma, Bax, and p21 in bKO × B1-8\textsuperscript{hi} pre–B cells, normalized by control × B1-8\textsuperscript{hi} pre–B cells. Cnot3 primers are designed in the flanked exons to conform 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_{\gamma-}\gamma_{1} rearrangements with fivefold serial dilutions of genomic DNA from sorted control × B1-8\textsuperscript{hi} pre–B cells, bKO × B1-8\textsuperscript{hi} pre–B cells, and Rag1\textsuperscript{-/-} pre–B cells. C\mu, loading control. (F) RT-PCR analysis of the expression of the rearranged V_{\gamma-}\gamma_{1} transcripts and C\mu GLTs with fivefold serial dilutions of cDNA prepared from sorted pre–B cells of control × B1-8\textsuperscript{hi} and bKO × B1-8\textsuperscript{hi} 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.
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5′-AAAGGGCCCCTGTCTTCTAGA-3′; p21 forward, 5′-CCGCCTTCTTGGCCCTTC-3′; p21 reverse, 5′-CATGACCCGTCCGCCTGACGCGC-3′; Cnot3 forward, 5′-CGAGGCTCCAGCTCTGATCGAGG-3′; Gapdh forward, 5′-ATGGTAGAGGTGCAGGAGTTGCCC-3′; Gapdh reverse, 5′-AGCTGCCATCTCCGCTGGACTTGTCCTG-3′; Hpt forward, 5′-TCTCTTCCTCAGACCGCTTTT-3′; Hpt reverse, 5′-GCTGGTCATCCTGCAATCTC-3′; V̴h588 GLT forward, 5′-ATGGATGAGTGCTGATCTCT-3′; V̴h588 GLT reverse, 5′-GACACATCAGAGTTGGTTTGTAG-3′; V̴h7183 GLT forward, 5′-CGGTACCAAGAASAMCCTGTGCCTGCTGCT-3′; V̴h7183 GLT reverse, 5′-GCTCTCCTGCGGCGCCCTCTGCTGCT-G3′; McI forward, 5′-TCAAGATGGCGTACAACCTCG-3′; and McI reverse, 5′-CCGTTTCTCCTFACAAGGAG-3′.

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 by using a buffer containing 20 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 50 mM Na2VO4, 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 by qPCR using a LightCycler 480 (Roche), 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). The alignment data sets were further analyzed using Cufflinks version 2.2.1 (Trapnell et al., 2010) and STAR version 2.5.2c (Dobin et al., 2013). Genes with false discovery rate (FDR) <0.05 were selected as differentially expressed ones. Four biological replicates were used in each experiment. Two biological replicates were used for each genotype. For pathway analysis 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. GSE46455.

V(DJ) 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; Fuwa et al., 2004), and PCR conditions were adjusted to be in the linear amplification range by serial dilution of template DNA. Samples from Rat 1/2 pro–B cells serve as a negative control for D3j to J558, V̴h to D3j, and V̴h to J558 experiment combinations. The PCR products were separated on agarose gels and visualized by ethidium bromide staining.

The following primers were used for V(DJ) recombination analysis: V̴h588 forward, 5′-CGAGCTTCTCCACACCCGCTGACGCTCCAGC-3′; V̴h588 reverse, 5′-CAAGGGCCCGTGTTCTGTGGA-3′; V̴h7183 forward, 5′-CGGTACCAAGAASAMCCTCTGCTGCTGCT-3′; V̴h7183 reverse, 5′-GCTCTCCTGCGGCGCCCTCTGCTGCT-G3′; McI forward, 5′-TCAAGATGGCGTACAACCTCG-3′; and McI reverse, 5′-CCGTTTCTCCTFACAAGGAG-3′.

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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)20-14 at 2°C for completion ligation. This ligated primer was subjected to primer RT using SuperScript II (Invitrogen). PCR was performed with an anchor primer (5′-GCGAGCTCCGCCGCGCCTTTTTTTTTTTTTTTT-3′) was added to the reaction to anneal at the end of poly(A) tails and incubated for 2 h at 42°C to complete ligation. The amplified product was subjected to primer extension reaction with a primer (5′-CCTGCACACATTGAAATGATCTC-3′). Specificity of the PCR reaction was confirmed by digestion of the PCR products with a restriction enzyme (Apal). The PCR products were resolved on a 14%–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 RIPA 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, CNOT7, CNOT8, and GAPDH antibodies. Images were collected at 65-nm pixels in X-Y and 200-nm steps in Z with an IX71 with an oil immersion objective (UPlanSApo 100× NA 1.40; Olympus) and a high-speed spinning disc confocal unit (CSU-X1; Yokogawa Electric Corp.).

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 C. Bosen and C. Murre, University of California, San Diego, La Jolla, CA). BAC RP24-189H12 (distal Vh locus) or RP23-404D8 (proximal Vh locus) was labeled with Cy3–dUTP (GE Healthcare), and BAC RP23-109B29 (Cy5) was labeled with Alexa Fluor 488–dUTP (Invitrogen) using a Nick Translation kit (Roche). The nuclear periphery was stained with anti–Lamin B1 antibody (M-20; Santa Cruz Biotechnology, Inc.) and Alexa Fluor 647–anti–goat antibody (Invitrogen). Image stacks were captured with an inverted microscope IX71 with an oil immersion objective (UPlanApo 100× NA 1.40; Olympus) and a high-speed spinning disc confocal unit (CSU-X1; Yokogawa Electric Corp.) equipped with a CCD camera (ORCA-AR; Hamamatsu Photonics). Images were collected at 65-nm pixels in X-Y and 200-nm steps in Z with MetaMorph software (Universal Imaging Corp.) as described previously (Isono et al., 2013). Only cells containing signals of both Vh loci were evaluated. The intracellular distances between the probes were calculated using ImageJ software (National Institutes of Health). Statistical analysis was performed with the unpaired Student’s t test using Prism software (GraphPad Software).

Statistical analysis. Statistical analyses were performed by a two-tailed unpaired Student’s t test using Prism software.

Online supplemental material. Table S1 shows the list of differentially expressed genes between control and bKO pro-B cells identified by the RNA-seq analysis. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20150384/DC1.

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