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CTNNBL1 is dispensable for immunoglobulin class switch recombination[1]

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Abstract

Immunoglobulin class switch recombination (CSR) and somatic hypermutation (SHM) require activation-induced cytidine deaminase (AID). The search for AID-interaction factors has been a major research effort in the field as the mechanism of preferential targeting of AID to immunoglobulin loci remains elusive. CTNNBL1 is one of the few identified AID-interacting factors and has been shown to affect AID-mediated mutation and gene conversion in chicken DT40 cells. CTNNBL1 was also implicated in mammalian CSR by the fact that an AID mutant that fails to interact with CTNNBL1 also fails to support CSR in AID-deficient mouse B cells. To directly assess the role of CTNNBL1 in CSR, we disrupted the CTNNBL1 gene on both alleles in mouse CH12F3 cells by gene targeting. We found normal levels of CSR in CTNNBL1-deficient cells, indicating that CTNNBL1 is dispensable for CSR.

INTRODUCTION

Antigen-stimulated mature B cell can further diversify its immunoglobulin (Ig) gene by class switch recombination (CSR), somatic hypermutation (SHM) or gene conversion (GC). CSR is a cut-and-paste chromosomal deletion event that allows a B cell to use an alternative constant region (γ, ε, α) located downstream of the default μ constant region, thereby changing the expressed Ig isotype from IgM to IgG, IgA or IgE (1). SHM introduces mutations in Ig variable regions to allow improved affinity for antigen-binding. In birds, Ig diversification occurs predominantly through templated gene conversion (2).

All three processes (CSR, SHM and GC) require local transcription and a lymphoid-specific factor called activation-induced cytidine deaminase (AID) (3,4). AID was identified in a subtractive cDNA library screening as an early up-regulated gene when a mouse B cell line (CH12F3) was induced to undergo CSR (5). Cumulative genetic and biochemical evidence indicate that AID is a cytidine deaminase that converts cytidines to uracils in DNA at specific regions (6–8). CSR, SHM and GC are all tightly associated with transcription. Purified AID deaminates cytidines only on single-stranded DNA (9,10), suggesting that the need for transcription is likely to temporarily separate the two DNA strands. The kilobase-long switch regions that are the main targets for CSR contain many GC-rich repetitive sequences. They tend to form stable secondary structure such as R-loop upon transcription (11,12). The R-loop structure could provide stable extensive single-stranded DNA region as optimal substrate for AID, which may partly explain the targeting mechanism of AID in CSR (1). However, it is more difficult to explain how AID targets to Ig variable regions,

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which do not form R-loop structures. Therefore, what distinguishes Ig loci as preferred AID targets versus other highly transcribed regions in the genome remains an enigma.

It is well known that mutations in different regions of AID differentially affect SHM or CSR, which prompted a hypothesis that AID is differentially recruited in SHM or CSR by different accessory factors (13–15). Of the few AID-interacting factors reported in the literature, *Ctnnbl1* is of particular interest because of the direct genetic evidence that *Ctnnbl1*-deficient chicken DT40 cells have a markedly reduced rate of AID-mediated mutation and gene conversion (16). In addition, an AID mutant harboring amino acid substitutions at residues 39–42 (AID39/42) that fails to interact with *Ctnnbl1*, also fails to rescue CSR in *AID*-deficient mouse primary B cells (16). Although the cellular function of *Ctnnbl1* is largely unknown, there was evidence that *Ctnnbl1* is a component of a splicesome complex (16,17). This is particularly interesting because there has been a 15-year-old mystery as to why CSR requires splicing of the non-coding switch region transcripts (18–20).

To determine whether *Ctnnbl1* is required for CSR, we knocked out both copies of *Ctnnbl1* gene in mouse CH12F3 cells by somatic gene targeting. We found that *Ctnnbl1*-deficient CH12F3 cells are normal, if not slightly more efficient in CSR to IgA, thus providing direct evidence that *ctnnbl1* is dispensable for CSR.

MATERIALS AND METHODS

Cell culture and CSR assay

CH12F3 cell line is a kind gift from Dr. T. Honjo (Kyoto University, Kyoto, Japan). Cell culture conditions, CSR and cell proliferation assays have been described previously (21).

Gene targeting

A 5.8 kb and a 1.8 kb DNA fragments were PCR amplified from CH12F3 genomic DNA and cloned into a targeting vector as homology blocks for gene targeting (Fig. 1A). Procedures of two rounds of gene targeting to knock out a gene in CH12F3 cells has been described in detail in a previous study (21).

RT-PCR

Total cellular RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instructions. One microgram of total RNA was reverse transcribed into cDNA with random hexamers and Superscript II reverse transcriptase in a 20 μl reaction (Invitrogen). Two microliters of the reverse transcription mixture was used as template to amplify the coding region of *ctnnbl1* or part of beta-actin gene as a loading control.

RESULTS AND DISCUSSIONS

Gene targeting of *ctnnbl1* gene in CH12F3 cells

Mouse *ctnnbl1* gene contains 16 exons spanning a region of approximately 150 kilobases on chromosome 2 (Fig. 1A). Little is known about the cellular function of *Ctnnbl1*, or what residues might be of any functional importance. We chose to delete exons 8 through 10, and part of exon 7 of the *ctnnbl1* gene (Fig. 1A) based on convenience of finding homology blocks that are scarce in repetitive sequences. The targeted deletion should abolish at least 60% of the coding capacity of the *ctnnbl1* gene and result in a null allele. Two rounds of gene targeting were performed using a strategy that has been described previously (21). Briefly, after targeting of the first allele (+/P), an Cre-expressing plasmid was transiently transfected to remove the Puromycin selection cassette flanked by a pair of loxP sites. The

resulting puromycin-sensitive clone (+/Δ) was re-targeted by the same targeting vector to obtain a double knockout clone (P/Δ). Cell genotypes were first screened by PCR and then confirmed by Southern blot analysis (Fig. 1B). The assays described here were carried out using a *Ctnnbl1*-haplodeficient clone (+/P) and a double knockout clone (P/Δ), which are siblings in the second round of gene targeting. Cells that are targeted on both alleles (P/Δ) lack mature *Ctnnbl1* transcript based on PCR amplification of the reverse transcribed cDNA (Fig. 1C), indicating that the targeted deletion resulted in a null allele.

Ctnnbl1-deficient cells proliferate normally (Fig. 2A) based on microscopic counting of live cells every 24 hours over a span of 3 days. When cells are induced to undergo CSR with the addition of anti-CD40 antibody, interleukin-4 and transforming growth factor beta-1, *Ctnnbl1*-deficient cells showed normal, if not slightly higher, efficiency in CSR (Fig. 2B). Identical results were obtained with *Ctnnbl1*Δ/Δ cells, which have the puromycin selection cassette removed by the Cre-LoxP reaction (not depicted). These data provide direct evidence that *Ctnnbl1* is dispensable for CSR.

Our finding that *Ctnnbl1* is dispensable for CSR appears at odds with the earlier study that identified *CTNNBL1-AID* interaction (16) with regard to the role of *Ctnnbl1* in secondary Ig gene diversification. *Ctnnbl1* was found to affect the rate of AID-mediated mutation and gene conversion in chicken DT40 cells. It was also implicated in CSR through an AID mutant (AID 39/42) that fails to interact with Ctnnbl1, as this AID mutant also fail to rescue CSR in AID knockout mouse primary B cells. However, this apparent discrepancy could be explained by a number of possibilities. First, *Ctnnbl1* might be only required for SHM/GC, but not CSR. That would explain diminished SHM/GC in *Ctnnbl1*-deficient DT40 cells, but normal CSR in *Ctnnbl1*-deficient CH12F3. Second, the CSR defect associated with AID 39/42 mutation might be attributable to an intrinsic defect in AID, rather than its interaction with *Ctnnbl1*. Finally, we couldn't rule out the possibility that there is a functionally redundant gene (or genes) in mouse that could compensate for the loss of *Ctnnbl1*. Of course in such case, the AID 39/42 mutant would have to lose the ability to interact with all of these CTNNBL1-like factors. Whether any of the above possibilities exists awaits further investigation.

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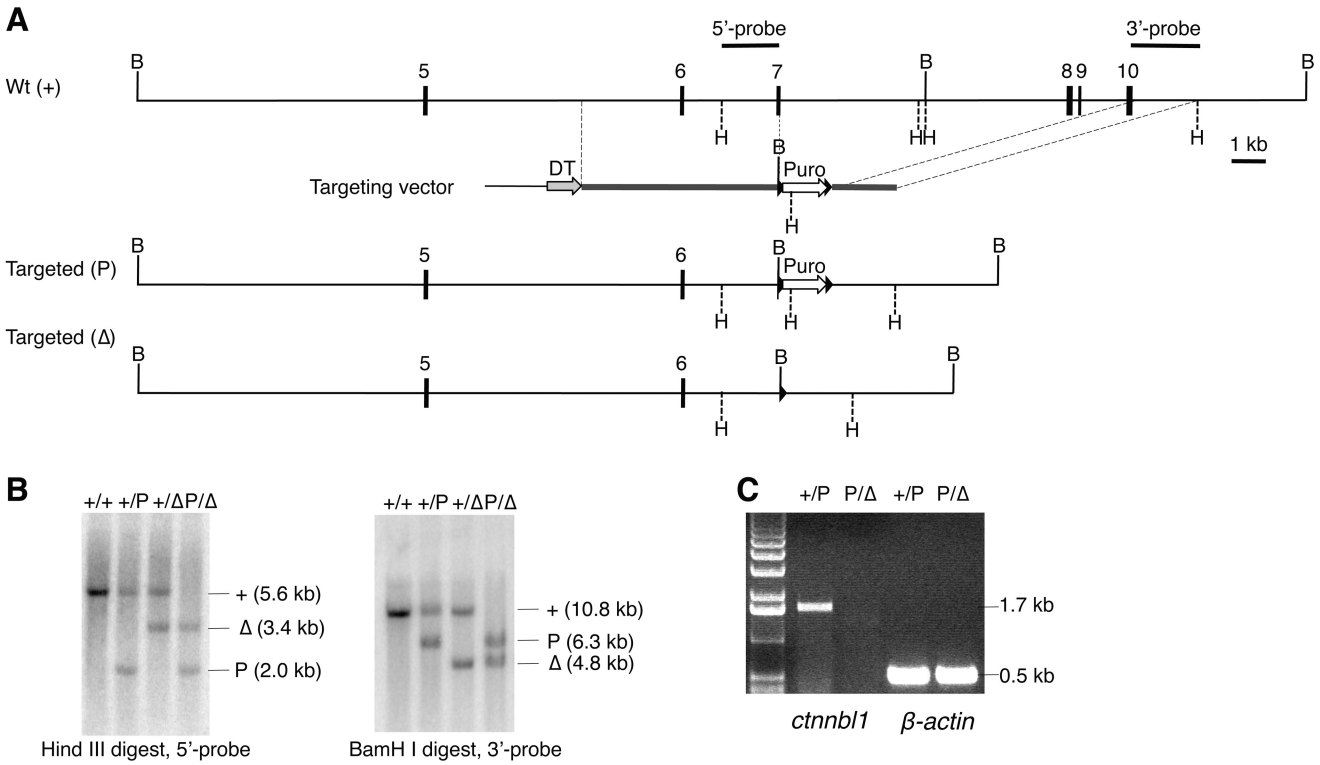

Figure 1. Gene targeting of *Ctnnbl1* in CH12F3 cells

(**A**) Genomic organization of wild type and targeted mouse *Ctnnbl1* locus. Small triangles indicate lox P sites. Restriction enzyme sites are indicated by B for BamH I and H for Hind III (shown only the relevant ones). DTA, diphtheria toxin; Puro, puromycin resistance gene. (**B**) Southern blot analysis. Left panel shows Hind III-digested genomic DNA hybridized with the 5'-probe. Right panel shows BamH I-digested genomic DNA hybridized with the 3'-Probe. Genotype symbols: +, wild-type allele; P, targeted allele with puromycin selection cassette;Δ, targeted allele with puromycin selection cassette removed. (**C**) RT-PCR. CTNNBL1 coding region and part of β-actin (as loading control) were amplified from random-primed cDNA with 30 cycles of PCR.

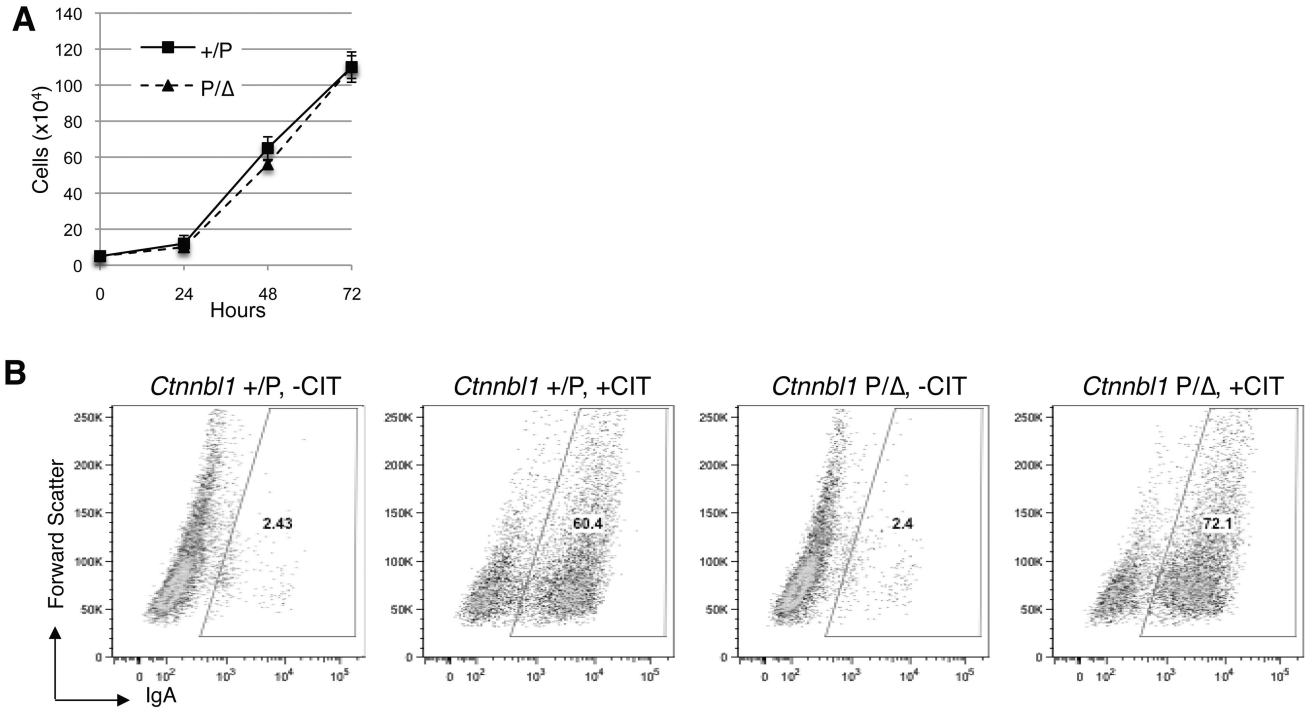

Figure 2. Normal proliferation and CSR in Ctnnbl1-deficient cells

(**A**) Cell proliferation. Cells were seeded at 5×10^4/ml and live cell numbers were counted every 24 hours over 3 days. Error bars represent standard deviations from three independent experiments. (**B**) Class switch assay. Representative FACS analysis of CSR by cell surface staining of IgA after 72 h of cell growth with or without cytokines. Numbers in boxed areas indicate percentages of IgA positive cells. CIT, anti-CD40 antibody, IL-4 and TGF- β1.