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**Identification of the transgenic integration site in 2C T cell receptor transgenic mice**

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*Abbreviations:* TCR, T cell receptor; pMHC, peptide/major compatibility complex; DC-PCR, Digestion-circularization polymer chain reaction; cPLA2, cytosolic phospholipase A2 group IVA; CTL, cytotoxic T lymphocyte; P13, Vβ13 promoter deficient mice; ZBED4, zinc finger domain containing 4; PBMC; peripheral blood mononuclear cell; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

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## Abstract

2C T cell receptor (TCR) transgenic mice have been long used to study the molecular basis of TCR binding to peptide/major compatibility complexes (pMHC) and the cytotoxicity mechanism of cytotoxic T lymphocytes (CTLs). To study the role of variable gene promoters in allelic exclusion, we previously constructed mutant mice in which the V $\beta$ 13 promoter was deleted (P13 mice). Introduction of 2C transgene into P13 mice accelerated the onset of systemic CD8 T cell lymphoma between 14 to 27 weeks of age, although parental P13 mice appeared to be normal. This observation suggests that the lymphoma development may be linked to features of 2C transgene. To identify the integration site of 2C transgene, Southern blotting identified a 2C-specific DNA fragment by 3' region probe of 2C TCR  $\alpha$  transgene, and digestion-circularization-polymerase chain reaction (DC-PCR) amplified the 2C-specific DNA fragment with inverse primers specific to the southern probe. Sequence analysis revealed that DC-PCR product contained the probe sequences and the junction sequences of integration site, indicating that 2C TCR  $\alpha$  transgene is integrated into chromosome 1. Further genomic analysis revealed cytosolic phospholipase A2 group IVA (cPLA2) as the nearest gene to the integration site. cPLA2 expression was upregulated in the normal thymi and T cell lymphomas from 2C transgenic mice, although it was not altered in the lymph nodes of 2C transgenic mice. The result is the first report demonstrating the integration site of 2C TCR transgene, and will facilitate the proper use of 2C transgenic mice in studies of CTLs.

*Keywords:* 2C TCR transgenic mice, T cell lymphoma, Integration site, DC-PCR, cPLA2.

## Introduction

The 2C T cell receptor (TCR) clone was generated to study the molecular basis of TCR binding to peptide/MHC (pMHC) complexes (Kranz, et al. 1984). The 2C clone is a CD8<sup>+</sup> mouse T cell clone (H-2<sup>b</sup>) that expresses a MHC-I-restricted  $\alpha\beta$  TCR (Kranz, et al. 1984). 2C transgenic mice were also generated to investigate the cytotoxicity mechanism of cytotoxic T lymphocytes (CTLs) of a given specificity (Sha, et al. 1988). 2C transgenic mice (type A) have approximately 2 copies of  $\beta$  transgene and 4 copies of  $\alpha$  transgene (Sha, et al. 1988). In addition to a fully rearranged TCR V $\beta$ 8.2-D $\beta$ 2-J $\beta$ 2.4 gene, 2C  $\beta$  construct has germline TCR V $\beta$ 5.2, V $\beta$ 8.3, V $\beta$ 5.1, and V $\beta$ 14 genes additionally (Sha, et al. 1988). 2C  $\alpha$  construct contains a productive V $\alpha$ 3.1-J $\alpha$  rearrangement and additional joining segment genes (Sha, et al. 1988). 2C transgenic mice express the same  $\alpha\beta$  antigen receptor from the CTL clone 2C, and the 2C TCR is expressed on 20-95% of peripheral blood T lymphocytes in the transgenic mice (Saito, et al. 1984; Sha, et al. 1988). The 2C TCR clones from the transgenic mice is able to lyse targets with the same specificity as the original 2C clone (Saito, et al. 1984; Sha, et al. 1988). Multiple pMHC ligands to 2C TCR have been identified. 2C TCR interacts with an endogenous K<sup>b</sup> MHC-I when it is bound to dEV8 (EQYKFYSV) (Tallquist, et al. 1996). 2C TCR interacts with the alloantigen L<sup>d</sup> when it is bound to the synthetic peptide QL9 (QLSPFPFDL) (Sykulev, et al. 1994). 2C TCR also recognizes K<sup>b</sup> when it is bound to SIY (SIYRYGYL) (Udaka, et al. 1996). Furthermore, there are high-resolution crystal structures of both 2C TCR and 2C TCR-pMHC complex (Colf, et al. 2007; Degano, et al. 2000; Garcia, et al. 1996). Thus, 2C transgenic mice have been extensively utilized over 30 years to understand the molecular biology of CTLs.

A previous study reported that all 2C transgenic mice develop T cell lymphomas when they are crossed to *Tp12* proto-oncogene-deficient mice, although parental mouse strains (*Tp12*<sup>-/-</sup> and 2C transgenic mice) show no signs of T-cell malignancy (Tsatsanis, et al. 2008). Most of the tumor cells are 2C CD8 single positive (SP) T cells in 2C*Tp12*<sup>-/-</sup> mice, suggesting that 2C TCR-positive T cells turn into CD8 SP T cell lymphomas when the cells are chronically stimulated in *Tp12*<sup>-/-</sup> background. The results suggest the possibility that the lymphoma development may be linked to features of 2C transgene. Previously, we constructed mutant mice in which a 1.2-kb region of the V $\beta$ 13 promoter was either deleted (P13<sup>-/-</sup>) or replaced with the simian virus 40 minimal promoter plus five copies of Gal4 DNA sequences (P13<sup>R/R</sup>) (Ryu, et al. 2004). When 2C TCR transgenic mice were crossed to both P13<sup>-/-</sup> and P13<sup>R/R</sup> mice (both called P13 mice), in this study, we also found that all 2CP13 mice spontaneously developed systemic T cell lymphomas while parental P13 mice appeared to be normal. The result also suggests that 2C TCR transgenic mice are susceptible to the T cell lymphoma development. However, the integration site of 2C TCR transgene has not been reported until now. Here, we report for the first time the integration site of 2C TCR transgene, which is probably associated with the onset of systemic CD8 T cell lymphoma.

## Materials and Methods

**Mice and genotyping** 2C and F5 TCR transgenic mice were described previously (Mamalaki, et al. 1993; Sha, et al. 1988). P13<sup>-/-</sup> and P13<sup>R/R</sup> mice were also described previously (Ryu, et al. 2004). The 2C transgenic RAG1<sup>-/-</sup> mice (2CRAG<sup>-/-</sup>) (Manning, et al. 1997) were backcrossed to the C57BL/6 (B6, H-2<sup>b</sup>) background, and 2CP13<sup>+/-</sup> mice were primarily generated by mating 2CRAG1<sup>+/-</sup> mice with P13<sup>-/-</sup> mice. Studies were carried out according to institutional guidelines for animal use and care. Mice were genotyped for 2C expression by a flow cytometric assay of peripheral blood mononuclear cells (PBMCs) with the clonotypic antibody 1B2. PCR genotyping assays for P13 mice were performed as described previously (Maurice, et al. 2001; Ryu, et al. 2004). The PCR conditions were performed as follows: 95°C for 5min followed by 30 cycles of 95°C for 1min, 60°C for 1min, 72°C for 3min, and final extension at 72°C for 10 min. To identify 2C transgene junction in 2C transgenic mice, 6R primer (5'-GCTCATCAACTAAACACTGAA-C-3') and 2C-333/2C-333-nested primers (5'-ATA-CATCCGGTGAAGTCT-3'/5'-TCAGTTGGTCTTTAAAACAG-3') were used for 2C genotyping PCR. As a control,  $\beta$ -actin gene was also amplified by PCR with two primers (5'-GACATCCGTAAAGACCTCTAT-3' and 5'-TTGATCTTCATGGTCCTACGA-3').

**Cell culture** Two thymic lymphoma cell lines P101 and P99 were derived from p53<sup>-/-</sup> mice and cultured in DMEM (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum and antibiotics (Haines, et al. 2006). Other thymic lymphoma cells were derived from 2CP13<sup>+/-</sup> (P13) and 2CP13<sup>R/R</sup> (R18) mice and were grown in the same medium. The medium was replaced every 3 days.

**Southern blot analysis** To detect the 2C TCR-specific DNA fragment, 2C TCR genomic DNAs were analyzed

by Southern blot analysis. Genomic DNAs from wild type and 2C transgenic mice were digested with EcoRI or BamHI, fractionated, and transferred to Nylon membrane (Biorad, Seoul, Korea). The subsequent fragments were detected by hybridization using 3' region probe of 2C TCR  $\alpha$  construct (3' TCR  $\alpha$  probe in Fig. S1). The 3' TCR  $\alpha$  probe was obtained using the primers TSP68 (5'-CTCTGCACTCTGCCGCCAT-3') and TSP67 (5'-TGACAACCTGCGCTCCAT-C-3') in a PCR reaction. P<sup>32</sup>-radiolabeled DNA probe was generated using the random primer DNA labeling kit (Roche, Seoul, Korea).

**Digestion-circularization PCR (DC-PCR)** Genomic DNAs of 2CP13<sup>+/-</sup> and P13<sup>+/-</sup> mice were digested with EcoRI or BamHI (Takara, Shiga, Japan). The purified DNAs were then self-circularized using T4 DNA ligase (New England Biolabs, Beverly, MA, USA) at 16°C for 16 h. The circularized DNAs were heated at 65°C for 20 min and purified by QIAEX II Extraction Kit (Qiagen, Hilden, Germany). The amplification was performed in a 50  $\mu$ l reaction mixture containing 500 ng template DNAs, 17.5 nmole each deoxynucleotide triphosphates, 10 pmole 6F and 6R primers, 5  $\mu$ l of 10 $\times$  ExTaq buffer containing 2 mM MgCl<sub>2</sub>, and 2.5 unit of ExTaq (Takara). The gradient PCR conditions included initial denaturation at 94°C for 2 min, 10 cycles of 94°C for 30 sec, 48-63°C for 30 sec and 72°C for 5 min, 20 cycles of 94°C for 30 sec, 48-63°C for 30 sec and 72°C for 5 min, and a final extension step at 72°C for 7 min. The amplified products were fractionated and visualized by ethidium bromide staining. The PCR products were further confirmed by nested PCRs with nested primers 1F and 2F, specific to sequences internal to 6F primer. The final PCR products were purified from the gel for cloning. The primer sequences of 6F, 6R, 1F and 2F are 5'-CAGGTAACCTTCTTTATGATTGGT-3', 5'-GCTCATCAACTAAACAC-TGAAC-3', 5'-TGAAGGCAGAGAGCTAGACAGA-3' and 5'-GCAGATGATC-CAAGGACAATGT-3', respectively.

**Cloning and sequencing** DC-PCR Products were amplified by PCR with 6F-SalI and 6R-XbaI primers and cloned into the SalI and XbaI sites of pBluescript KS+ vector. The primer sequences of 6F-SalI and 6R-XbaI are 5'-CCAGTCGACCAGGTAACCTTCTTTATGATTGG-3' and 5'-GTGTCTAG-AGCTCATCAACTAAACACT-GAA-3', respectively. Cloned products were sequenced commercially (Cosmo Genetech, Seoul, Korea). Alignment of the flanking sequence to the mouse genome was performed by the BLAT genome browser of University of California Santa Cruz (<http://genome.ucsc.edu/cgi-bin/hgBlat>).

**Semi-quantitative Reverse transcriptase-PCR (RT-PCR)** Total RNAs were isolated from the thymi and lymph nodes of 5-8 week-old-wildtype, P13<sup>-/-</sup>, 2C, and 2CP13<sup>+/-</sup> mice using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNAs were also isolated from lymphoma cells of 2CP13<sup>+/-</sup> and 2CP13<sup>+R</sup> mice. One-Step RT-PCR premix kit (Intron, Seoul, Korea) was mixed with specific primers for cPLA2. The PCR conditions were performed as follows: one cycle at 45 °C for 60 min and at 94°C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50°C for 1 min and 72 °C for 3 min, and a final extension step at 72°C for 10 min.  $\beta$ -actin was used as a loading control. The cPLA2 primers used were 5'-TGTTCAACAGAGTTTTGG-3' and 5'-ACAGAGCAACGAGATGG-3'. The signal intensities of cPLA2 expression were analyzed using the Image J.

**Flow cytometry** Flow cytometry was performed after the isolation of PBMCs from mouse tail vein. First, red blood cells were lysed by resuspending cell pellets with ammonium chloride buffer (0.15 M NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA) for 5 min at room temperature. The remaining cells were washed with ice-cold FACS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 1% bovine serum albumin, 0.02% NaN<sub>3</sub>). One million cells were then resuspended in FACS buffer. The antibodies used were as follows: 1B2 (specific for 2C TCR), fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8a (BD Biosciences, Seoul, Korea), phycoerythrin (PE)-conjugated anti-mouse CD4 (BD Biosciences). Propidium iodide (PI)-negative cells were analyzed using a FACSCalibur cytometer (BD Biosciences).

## Results

### Southern blot analysis of 2C TCR genomic DNA

To study the role of variable gene promoters in allelic exclusion, previously, we constructed P13 mice where the V $\beta$ 13 promoter was deleted (Ryu, et al. 2004). When 2C TCR transgenic mice were crossed to P13 mice, interestingly, all 2CP13 mice died from systemic CD8 T cell lymphoma between 14 to 27 weeks of age while

parental 2C and P13 mice appeared to be normal (Fig. 1a). Most of lymphoma cells found in thymus and spleen were CD8 SP lymphocytes (Fig. 1b). To see whether accelerated lymphoma development in 2CP13 mice was due to some general effect of transgenic TCR expression, P13 mice were crossed with another transgenic TCR  $\alpha\beta$  mouse termed F5 (Mamalaki, et al. 1993). Neither F5 nor F5P13<sup>+/-</sup> mice succumbed to disease over the course of one year (Fig. 1a). Furthermore, some 2C TCR transgenic mice themselves spontaneously developed T cell lymphomas when they were aged (Fig. 1a). The results suggest that 2C TCR transgenic mice are susceptible to the T cell lymphoma development. To detect the integration site of 2C TCR transgene, therefore, we did Southern blot analysis with wildtype and 2C TCR genomic DNAs. To generate a Southern probe specific to 2C TCR transgene, thymic DNA from 2C TCR transgenic mice was amplified by PCR with TSP67 and 68 primers matched to 3' region of 2C TCR  $\alpha$  construct (3'TCR  $\alpha$  probe, Fig. S1). Genomic DNAs from wild type and 2C TCR transgenic mice were digested with the indicated restriction enzyme, and were hybridized with P<sup>32</sup>-radiolabeled 3' TCR  $\alpha$  probe. The 5.5 and 2.0 kb DNA fragments were only detected in EcoRI-digested 2C DNA lane (Fig. 2a). A 2.8 kb DNA fragment was also specifically detected in BamHI-digested 2C DNA lane, suggesting that the 5.5, 2.0 and 2.8 kb fragments may contain a part of 2C transgene and the flanking sequences of the integration sites.

#### PCR amplification of 2C transgene integration site

To determine whether the 2C-specific fragments contain 2C transgene junction, we conducted DC-PCR with two primers (6F and 6R) specific to the 2C-specific DNA fragment (Fig. S2a). Genomic DNAs were isolated from 2C<sup>+</sup>P13<sup>+/-</sup> (2C+) and 2C<sup>-</sup>P13<sup>+/-</sup> (2C-) mouse thymi and cleaved with EcoRI or BamHI. The DNAs were self-ligated and then subjected to DC-PCR with 6F and 6R primers. DC-PCR with BamHI-digested DNAs did not produce any PCR products. DC-PCR with EcoRI-digested DNAs did not produce 5.5 kb PCR products, either. However, DC-PCR with EcoRI-digested DNAs produced approximately 2 kb PCR products at 59.8 and 56.9 °C of annealing temperature in 2C+ lanes but not in 2C- lanes (Fig. 2b). To prove whether the 2 kb PCR product is 2C TCR-specific DNA fragment, the DC-PCR product was isolated and subjected to nested PCR with 1F and 2F primers (Fig. S2a). Nested PCRs also produced the expected DNA fragments (Fig. S2b). The result suggests that the amplified 2 kb fragment has 2C-specific DNA fragment and the flanking sequences of integration site.

#### Sequencing of 2C transgene junction reveals the integration site of 2C transgene

To identify the flanking sequence of 2C transgene, both the DC-PCR product and cloned DC-PCR product were subjected to sequence analysis. The results showed that both the primary DC-PCR product and the cloned DNA product have the same sequences (Fig. 3a). The full size of the DC-PCR product consisted of 1,934 bp, and contained the fragment (535 bp) of 3'TCR  $\alpha$  probe and unknown flanking sequences (1399 bp) (Fig. 3a). By using BLAT genome browsers (<http://genome.ucsc.edu/cgi-bin/hgBlat>), the unknown flanking sequences were aligned to mouse chromosome 1. Further analysis revealed that the 2C transgene was located between cytosolic phospholipase A2 group IVA (cPLA2) and Zinc finger domain containing 4 (ZBED4) at chromosome 1 (Fig. 3b).

#### Confirmation of 2C transgene junction

2C transgenic mice generally have CD4<sup>+</sup>CD8<sup>+</sup> T lymphocytes in the peripheral blood (Sha, et al. 1988). PBMCs were isolated from the tail blood of 2C and wildtype mice with various genotypes, such as 2CWt, Wt, 2CP13<sup>+/-</sup>, and 2CP13<sup>+/-</sup>. As expected, flow cytometric analysis with anti-CD4 and CD8 antibodies showed that 2C-positive mice only had CD8 SP lymphocytes in the PBMCs (Fig. S3a). Based on the sequence information of 2C transgene junction, we designed 2C-333 and 2C-333-nested primers complementary to the flanking sequences in chromosome 1 (Fig. 3a). When these primers were paired with 6R specific to 2C transgene, 333 bp-sized PCR product was expected. Therefore, genomic DNAs were isolated from the tail tips of 2C-positive and negative pups in the same litter, and subjected to PCR genotyping with transgene junction-specific primers. 2C-transgene junction-specific 333 bp bands were amplified by PCR with 6R and 2C-333 primers from 2C transgenic DNAs, whereas the transgene junction DNAs were not amplified from wildtype DNAs (Fig. S3b). The results confirmed the integration site of 2C transgene in chromosome 1.

#### Expression of the nearest cPLA2 gene

To examine whether 2C transgene integration affects the expression of the nearest gene cPLA2 of 2C TCR  $\alpha$  transgene, total RNAs were isolated from thymic lymphoma cells of 2CP13<sup>+/-</sup> (P13), 2CP13<sup>+R</sup> (R18) and p53<sup>-/-</sup> (P101 and P99) mice, and the RNAs were subjected to semi-quantitative RT-PCR analysis. As compared with wildtype, cPLA2 expression was increased by approximately 25-28-fold in 2C-derived lymphoma cells (P13 and R18) while it was not detected in p53<sup>-/-</sup>-derived lymphoma cells (P101 and P99) (Fig. 4), suggesting that increased expression of cPLA2 is associated with 2C transgene integration. To further figure out whether 2C transgene integration affects lymphoma development, total RNAs were isolated from the normal thymi and lymph nodes of wildtype, P13<sup>-/-</sup>, 2C, and 2CP13<sup>+/-</sup> mice before lymphoma development. cPLA2 expression was just slightly increased by approximately 1.3-1.5-fold in the lymph nodes of 2C-positive mice (Fig. 4b left panels). However, cPLA2 expression was significantly increased by approximately 8.7-11.1-fold in the thymi of 2C-positive mice (Fig. 4b right panels). The results suggest the possibility that the integration of 2C transgene is associated with T cell lymphoma development through altered expression of cPLA2 in the thymi of 2C-positive mice.

## Discussion

2C transgenic mice develop CD8 SP T cell lymphomas when they have *Tpl2*<sup>-/-</sup> background, although parental mouse strains (*Tpl2*<sup>-/-</sup> and 2C transgenic mice) show no signs of T-cell malignancy (Tsatsanis, et al. 2008). In this study, we also found that 2C transgenic mice developed CD8 SP T cell lymphomas when they were crossed to P13 mice, and both parental mice did not develop any lymphomas until 23 weeks of age (Fig. 1a,b). It seems that the acceleration of T cell lymphomagenesis in 2C<sup>+</sup>P13<sup>-/-</sup> and 2C<sup>+</sup>P13<sup>R/R</sup> mice is due to the presence of 2C transgene. In the previous study, we cloned and sequenced V $\beta$ 13 signal ends during V(D)J recombination (Ryu, et al. 2004). Interestingly, we found that all V $\beta$ 13 DNA cleavage products (100%) in P13<sup>-/-</sup> and P13<sup>R/R</sup> mice were derived from aberrant cleavages in the presence of 2C transgene, whereas the aberrant cleavages were observed in a small fraction of wildtype (13%), P13<sup>-/-</sup> (18%) and P13<sup>R/R</sup> mice (47%) in the absence of 2C transgene. Therefore, it is possible to speculate that a continuation of the aberrant cleavages is the origin of genome instability and ends up with the development of T cell lymphoma in 2C<sup>+</sup>P13<sup>-/-</sup> and 2C<sup>+</sup>P13<sup>R/R</sup> mice.

In this study, we also found that some of 2C transgenic mice also spontaneously developed T cell lymphoma when they were aged (Fig.1a), suggesting that 2C transgene-containing mice may be prone to the development of T cell lymphoid malignancies because of 2C transgene itself. To generate 2C transgenic mice, 2C  $\alpha$  and  $\beta$  constructs (approximately 40 kb, respectively) were digested with the same Not I restriction enzyme and injected into fertilized eggs simultaneously (Sha, et al. 1988). Therefore, it is highly likely that both 2C  $\alpha$  and  $\beta$  constructs form a concatemer that contains multiple copies of the 2C  $\alpha$  and  $\beta$  constructs linked in series in the same chromosome. As expected, 2C transgenic mice used in this study (type A) have 2 copies of the  $\beta$  transgene and 4 copies of the  $\alpha$  transgene (Sha, et al. 1988). In addition to a fully rearranged TCR V $\beta$ 8.2 gene, 2C  $\beta$  construct also has germline TCR V $\beta$ 5.2, V $\beta$ 8.3, V $\beta$ 5.1, and V $\beta$ 14 genes additionally (Sha, et al. 1988). 2C  $\alpha$  construct also has additional joining segment genes (Sha, et al. 1988). Therefore, the multiple copies of the additional TCR segment genes in 2C transgenic mice may be exposed to DNA cleavages during V(D)J recombination, and the DNA cleavages may be not strictly controlled, suggesting that the uncontrolled DNA cleavages in 2C-positive P13 mice cause genome instability, and 2C-positive P13 mice end up with the development of T cell lymphomagenesis. Further investigation is necessary to find the cause of T cell lymphomagenesis in 2C-positive P13 mice.

To figure out why 2C transgene accelerates T cell lymphomagenesis in 2C-positive P13 mice, in this study, we tried to clone the integration site of 2C transgene. We were not able to clone the integration site of 2C  $\beta$  transgene. Instead, we were able to clone the integration site of 2C  $\alpha$  transgene, which was integrated into chromosome 1 (Fig. 3). The location of 2C  $\beta$  transgene has not been confirmed in this study. However, there is a high possibility that both 2C  $\alpha$  and  $\beta$  transgenes are integrated into the same chromosome 1 as a concatemer, because both constructs were digested with the same Not I and injected into fertilized eggs simultaneously (Sha, et al. 1988). As 2C transgene has enhancer and promoters for TCR gene expression, one thinks that the integration of 2C transgene may affect the expression of near genes. Actually, cPLA2, the nearest gene of the integration site, was abundantly detected in T cell lymphoma cells and normal thymic cells from 2C-positive P13 mice, whereas it was not detected in p53<sup>-/-</sup> mice-derived T cell lymphoma cells (Fig. 4a). Interestingly, cPLA2 expression was also increased in the thymi of 2C-positive P13 mice before the development of T cell lymphomas, while it was not significantly increased in the lymph nodes of 2C-positive P13 mice (Fig. 4b). The results suggest that cPLA2 expression is induced by TCR  $\alpha$  and  $\beta$  enhancers of 2C transgenes, which are

preferentially active in immature thymocytes (Bouvier, et al. 1996; Carvajal and Sen 2000; Krangel, et al. 2000). Overexpression of cPLA2 has been identified in a variety of cancers including non-small cell lung cancer, cholangiosarcomas, esophageal cancers, and cancers of the colon, ovarian, and small intestine (Nakanishi and Rosenberg 2006; Yarla, et al. 2016). cPLA2 specifically recognizes the sn-2 acyl bond of phospholipids and catalytically hydrolyzes the bond, releasing arachidonic acid (AA) and lysophosphatidic acid. AA is further modified into eicosanoids, which are categorized as anti-inflammatory and inflammatory mediators (Yarla, et al. 2016). Thus, cPLA2 can play a key role in pathophysiology of various cancers and inflammation. cPLA2 is also an important factor for the function of CTLs. cPLA2 is required for CTL-mediated immunopathology independently of their TCR via NKG2D and IL-15 (Tang, et al. 2009; Tang, et al. 2013), suggesting that altered expression of cPLA2 dysregulates activity of CTLs through induction of inflammation. Furthermore, Vβ13 promoter knockout in P13 mice shows increased levels (18-47%) of aberrant DNA cleavages, as compared with that (13%) of wildtype mice (Ryu, et al. 2004). Vβ13 promoter knockout also depletes a Vβ13-positive T cell subset from T cell population in P13 mice (Ryu, et al. 2004). Therefore, immature thymocytes may initially undergo programmed cell death because of AA release and genome instability in 2C-positive P13 mice. However, some malignant T cell clones may be derived from 2C TCR-positive T lymphocytes through 2C TCR-mediated chronic stimulation on the situation of continuous induction of inflammation and genome instability in 2C-positive P13 mice. Therefore, it is tempting to speculate that combination of 2C transgene and P13 mutation gives synergistic effects on T cell lymphomagenesis. However, it still needs more studies to understand the exact correlation between T cell lymphomagenesis and combination of 2C transgene and P13 mutation.

The present study also brings up another interesting point. A recent study reported that although B16.SIY melanoma cells are triggered to express K<sup>b</sup>, they are not well recognized by primed 2C<sup>+</sup>/RAG2<sup>-/-</sup>CD8 T lymphocytes (Blank, et al. 2004). However, PD-1-depleted 2C<sup>+</sup>/RAG2<sup>-/-</sup> T lymphocytes show augmented cytokine production and cytolytic activity against the melanoma cells compared to wildtype 2C lymphocytes (Blank, et al. 2004), suggesting that PD-1 expression and activity may be altered in 2C transgenic mice. As both the 2C integration site and PD-1 gene are located in the same chromosome 1, it is possible that 2C integration makes 2C CTLs sensitive to PD-1-associated cell death. Actually, annexin V binding were upregulated in 2C thymocytes as compared with wildtype thymocytes (data not shown). Therefore, it could be interesting to study the relationship between PD-1 and 2C TCR integration site during T cell lymphomagenesis in 2C transgenic mice.

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#### Compliance with ethical standards

This article does not contain any studies with human participants performed by any of the authors. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

**Conflict of interest** The authors declare that they have no conflict of interest.

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## Figure legends

**Fig. 1** 2CP13 mice develop CD8 SP T cell lymphoma between 14 to 27 weeks of age. **a** Kaplan-Meier survival curves for various genotypes are expressed as percentage lymphoma-free survival as a function of time (weeks). Controls indicate mice including Wt, P13<sup>-/-</sup>, P13<sup>+/-</sup>, P13<sup>R/R</sup>, P13<sup>+R</sup>, F5, and F5P13<sup>+/-</sup> genotypes (n >30 for all). Genotypes bearing lymphomas include 2C (n=37), 2CP13<sup>-/-</sup> (n=27), 2CP13<sup>+/-</sup> (n=33), and 2CP13<sup>+R</sup> (n=38). **b** CD4 and CD8 cell surface phenotype of systemic lymphoma cells from a representative 15 week-old terminally ill 2CP13<sup>+/-</sup> mouse. Thymic and spleen origin of lymphoma cells were analyzed by flow cytometry with CD4 and CD8 antibodies.

**Fig. 2** Southern blot analysis of Wt and 2C thymic DNAs and amplification of 2C TCR integration site. **a** 2C and Wt genomic DNAs were digested with the indicated restriction enzymes, and transferred to Southern membrane after fractionation. 2C-specific fragments were detected by hybridization with 3' TCR  $\alpha$  probe. 2C-specific DNA fragments were indicated with arrowheads. **b** DC-PCR amplification of 2C transgene junction. Genomic DNAs of 2C<sup>+</sup> and 2C<sup>-</sup> mice were digested with EcoRI and self-ligated with T4 DNA ligase. DC-PCR was performed by 6F and 6R primers at various annealing temperatures. 2C-specific PCR products (2.0 kb) were indicated with arrows. The larger products were considered to be irrelevant because they were also present in the 2C<sup>-</sup> samples.

**Fig. 3** Sequence analysis of the integration site of 2C TCR  $\alpha$  construct. **a** Sequence analysis of DC-PCR product. 2C TCR transgene is shown in black-colored letters with PCR primers used. The flanking sequence of 2C TCR  $\alpha$  is shown in red-colored letters with PCR primers used. **b** Schematic diagram of 2C-TCR  $\alpha$  transgene in chromosome 1. Transgene junction is indicated with an arrow. Newly designed PCR primers to amplify the integration junction is also shown as three arrows. Alignment of the flanking sequence to the mouse genome was performed with the BLAT genome browser of University of California Santa Cruz (UCSC).

**Fig. 4** Expression analysis of cPLA2, which is located in the nearest site of 2C transgene integration site. **a** Total RNAs were isolated from thymic lymphoma cells of 2CP13<sup>+/-</sup> (P13), 2CP13<sup>+R</sup> (R18) and p53<sup>-/-</sup> (P101 and P99) mice. **b** Total RNAs were also extracted from the normal thymi and lymph nodes of 2C, Wt, 2C<sup>+</sup>P13<sup>+/-</sup> and 2C<sup>-</sup>P13<sup>+/-</sup> mice. The RNAs were served for RT-PCR analysis with specific primers for cPLA2. To analyze the expression of cPLA2 quantitatively, three 3-fold dilution series of the RNA templates were used. The measurable intensities of cPLA2 expression were analyzed using the Image J, and relative intensities are presented after dividing the intensity of each sample by the intensity of wildtype thymus or lymph node.  $\beta$ -actin expression was used as a loading control.

## Supplementary information

**Fig. S1** 2C TCR  $\beta$  and  $\alpha$  constructs. 3' TCR  $\alpha$  probe is shown.

**Fig. S2** Schematic diagram of DC-PCR. **a** Genomic DNA was digested with EcoR, self-circularized with T4 DNA Ligase and subjected to DC-PCR with 6F and 6R. Nested PCR was carried out with 6R and 1F/2F. Final PCR products were either directly sequenced or cloned. The flanking sequences of 2C transgene were searched by the BLAST and BLAT servers. **b** Nested PCRs for confirmation of DC-PCR product. To confirm whether DC-PCR product contains 2C transgene, nested PCR was conducted using 6R and 1F/2F primers. The eluted DC-PCR product and amplified PCR products were indicated with arrows.

**Fig. S3** Identification of 2C transgene junction by PCR with two primers specific to transgene and the flanking sequences. **a** PBLs were isolated from 2C, Wt, 2C<sup>+</sup>P13<sup>+/-</sup> and 2C<sup>-</sup>P13<sup>+/-</sup> mice and stained with CD8-FITC and CD4-PE. **b** PCR genotyping to confirm the integration site. Tail genomic DNAs from 2C, Wt, 2C<sup>+</sup>P13<sup>+/-</sup> and 2C<sup>-</sup>P13<sup>+/-</sup> mice were amplified by PCR with 6R and 2C-333/2C-333-nested primers. Amplified products (333 bp) were indicated by arrows.  $\beta$ -actin PCR was used as a control PCR.

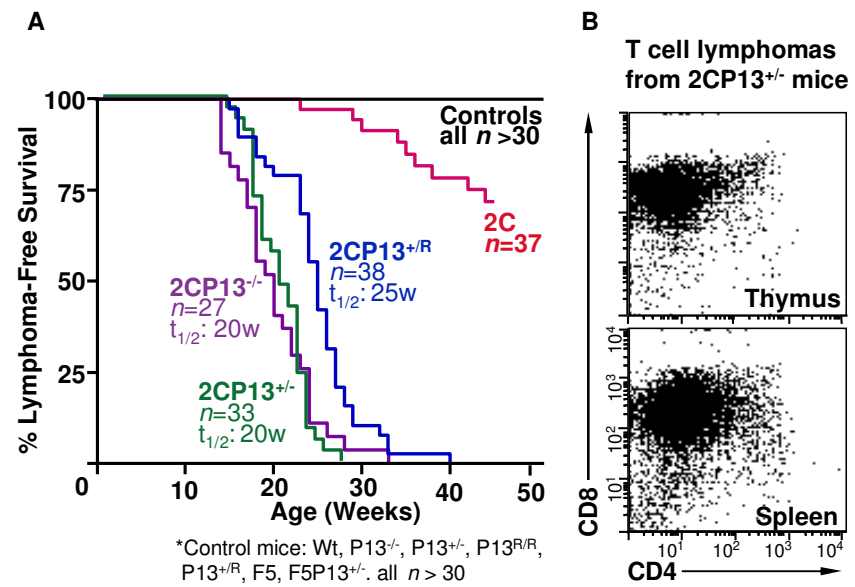


Fig 1.  
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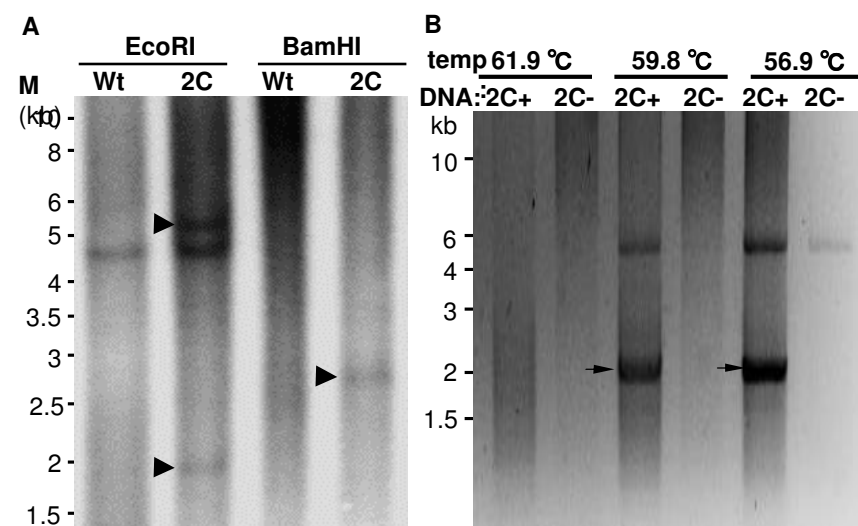


Fig.2  
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**A**

2C TCR  $\alpha$  (Chromosome 14, 535 bp)  
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 GAACCTGGGCCACAGAAACAGACTATCTCAAAAAAAAAAAAAAAAAAGAGCTGGATCAGTGGGTACTCCTGCTACTCC  
 TGACAACCTGGCGTCCATCCCCAGGACCCACACGGTGGAAAGGAAGGAGAGAGCAACCCCGAACATTGTCTTGG  
 ATCATCTGCCATGCTGCCATATCCACAGACCCCCCCCCACCCACCCACACACACACACACGAATAATC  
 ←2F  
 TAACTGCACTCTGTCTAGCTCTCTGCCCTTCAAAGACCAACCAGAATAATTACAGCCACAAATTAAGAATCAATT  
 ←1F  
 AATGCTTTTAAAGACTATTAAAGACCAATCATAAAGAAAGTTACCTGACTTTCAGCCCCCTGAGGGCTGAGGCAC  
 ←6F  
 CGAGATACAGTTAGGGGACCCACTCGCTCATCAACTAAACACTGAACAGTATTGCCAGGGGGCACTCCGAGCTGG  
 6R→  
 Chromosome 1 (1399 bp)  
 AGGCAATAAAAtgaagatttggtagtatgtcacttttctaaccagatagttagggtccgcccagcattgttattg  
 tgaggagatggaaatttacagaggtgaagcacttacaggtgcagtgagcactcaggcatgctcagtaataatta  
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 gtttcttgaggaaatttgggacttagaaaattaaagcaactgttttgaagaccaactgaaagcagttcaccggatg  
 ←2C-333-nested  
 tatgtttacatgtatgttcacagaaccacaaaatgaaatccacttcggatatgtcactccttaaataggcaacgg  
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**B**

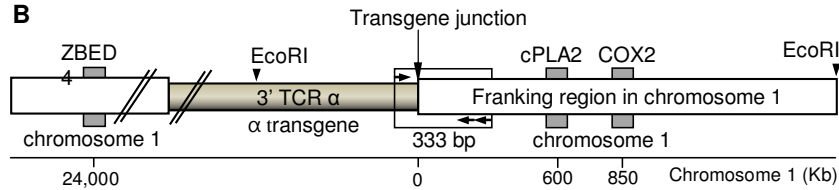


Fig. 3  
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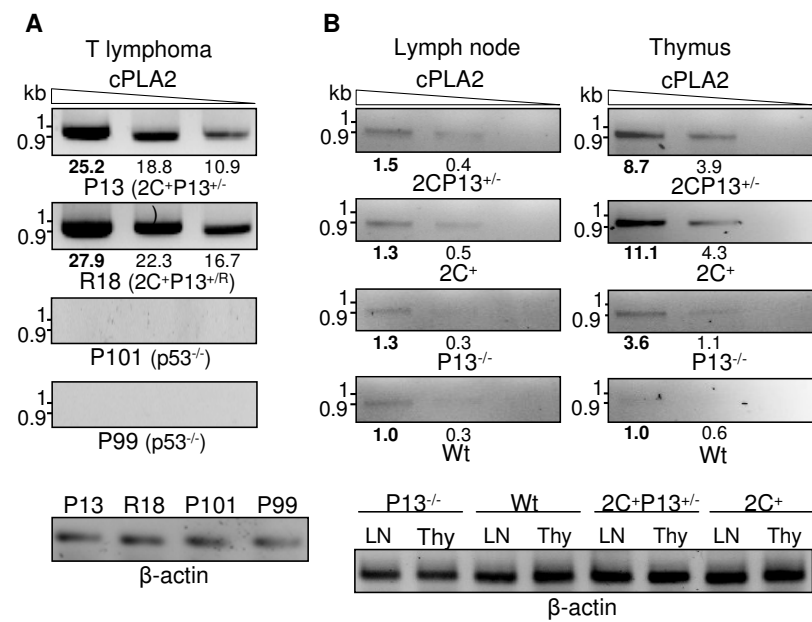


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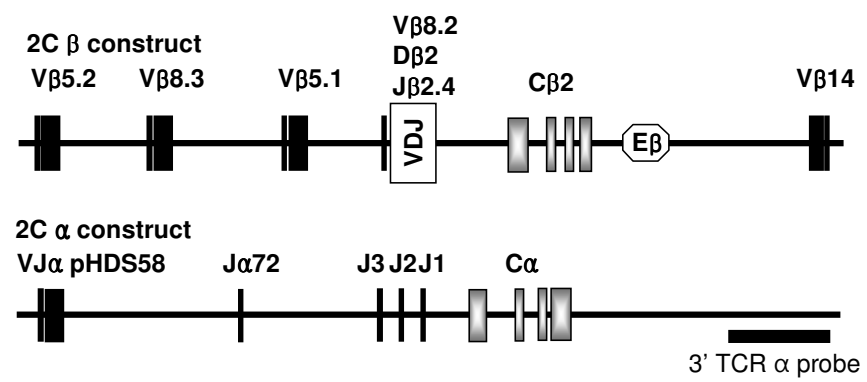


Fig. S1  
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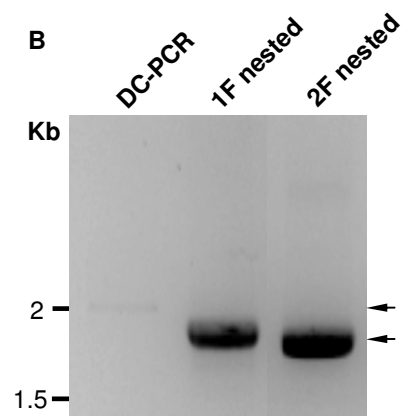
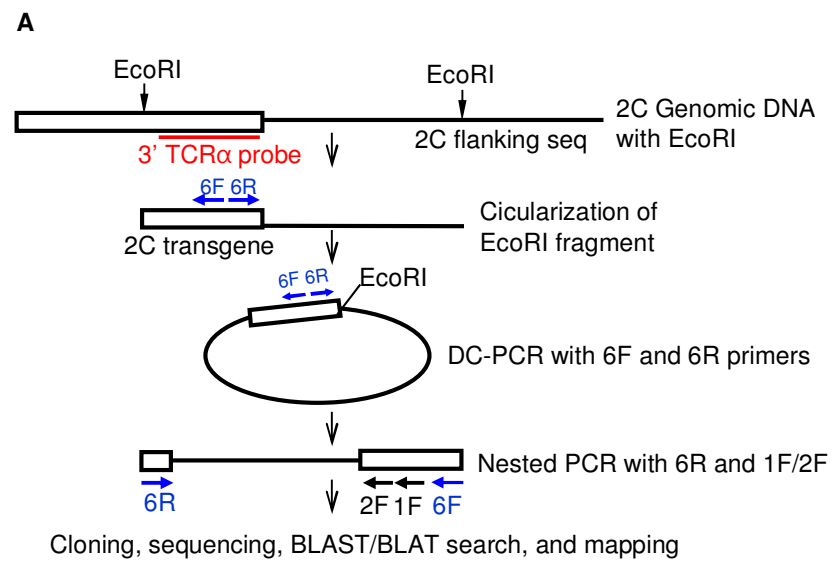


Fig. S2  
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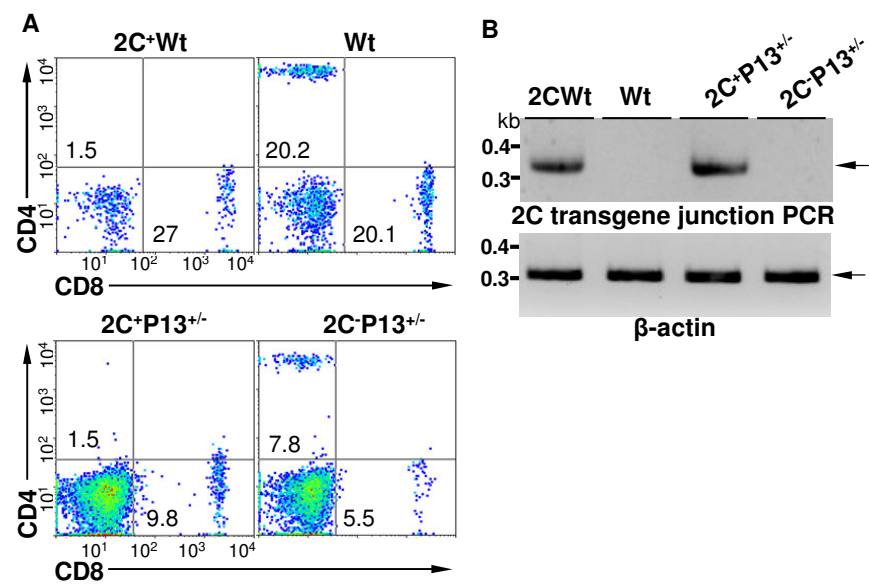


Fig. S3  
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