

## TECHNOLOGY REPORT

# Generation of Dhx9-Deficient Clones in T-Cell Development With a Mitotic Recombination Technique

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**Summary:** Mitotic recombination is an effective tool for generating mutant clones in somatic tissues. Because of difficulties associated with detecting and quantifying mutant clones in mice, this technique is limited to analysis of growth-related phenotypes induced by loss function of tumor suppressor genes. Here, we used the polymorphic CD45.1/CD45.2 alleles on chromosome 1 as pan-hematopoietic markers to track mosaic clones generated through mitotic recombination in developing T cells. We show that lineage-specific mitotic recombination can be induced and reliably detected as CD45.1 or CD45.2 homozygous clones from the CD45.1/CD45.2 heterozygous background. We have applied this system in the analysis of a lethal mutation in the Dhx9 gene. Mosaic analysis revealed a stage-specific role for Dhx9 during T-cell maturation. Thus, the experimental system described in this study offers a practical means for mosaic analysis of germline mutations in the hematopoietic system. *genesis* 50:543–551, 2012. © 2011 Wiley Periodicals, Inc.

**Key words:** mitotic recombination; CD45.1/CD45.2; T lymphocyte; Dhx9; Cre/lox

Mutational analysis in the mouse represents one of the most effective approaches in understanding gene functions relevant to human biology and human diseases. The mouse genome encodes ~30,000 transcribed units (based on NCBI Build m37), 99% of which have homologous counterparts in the human genome (Waterston *et al.*, 2002). Mouse mutations have been produced at a rapid pace during the past two decades, covering a significant percentage of protein-coding genes (Gondo, 2008). However, a significant fraction of germline mutations results in

embryonic or neonatal lethality, thus preventing functional studies in postnatal life. Therefore, a straightforward characterization of germline mutations is not always sufficient to fully understand gene function involving different developmental stages and different tissue types. Cre/lox-mediated conditional knockout provides an alternative solution (Wirth *et al.*, 2007). Although increased effort has been put into the generation of conditional alleles in ES cells (Skarnes *et al.*, 2011), much more work need to be done before these alleles are converted into live strains to replace the existing mutant stains.

Mitotic recombination-mediated mosaic analysis represents a relatively new and under-used genetic tool in the mouse. Cre/lox- or flp/FRT-mediated recombination has been successfully used in mice to induce recombination between homologous chromosomes during mitosis (Liu *et al.*, 2002; Wang *et al.*, 2007; Zong *et al.*,

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2005). The daughter cells resulting from mitotic recombination inherit either the homozygous maternal or paternal allele of the test chromosome. The feasibility of mosaic analysis in mice was further demonstrated in loss-of-heterozygosity assays of tumor suppressor genes p27 and p53 (Muzumdar *et al.*, 2007; Wang *et al.*, 2007). More recently, a mitotic recombination system has also been used to study the initiation and progression of gliomas induced by concomitant loss of tumor suppressor genes p53 and NF1 (Liu *et al.*, 2011). However, these applications are limited to tumor studies in which the growth advantage of mutant clones facilitates the assessment of growth-related phenotypes. A major limitation of this technique is that overall efficiency of mitotic recombination is at or below  $10^{-3}$  per cell generation (Sun *et al.*, 2008a). Therefore, the recombination events are infrequently detected in most somatic tissues reaching homeostasis.

The lymphoid system represents one of the somatic tissue types suitable for mitotic recombination tests. Extensive cell proliferation associated with lymphocyte development and immune activation provides two windows of opportunities for mitotic recombination (Sun *et al.*, 2008a). We developed a mitotic recombination system for efficient and accurate detection of mutant clones in hematopoietic system. We tested this mitotic recombination system in T-cell development by generating homozygous mutations in mice carrying a heterozygous mutation in the *Dhx9* gene. A direct comparison between homozygous wild-type clones and homozygous mutant clones revealed a novel function of *Dhx9* during T-cell maturation in the thymus. Because the allelic markers used in this recombination system are expressed in all hematopoietic cells, the system should be generally applicable for mosaic analysis of relevant mutations throughout the hematopoietic system.

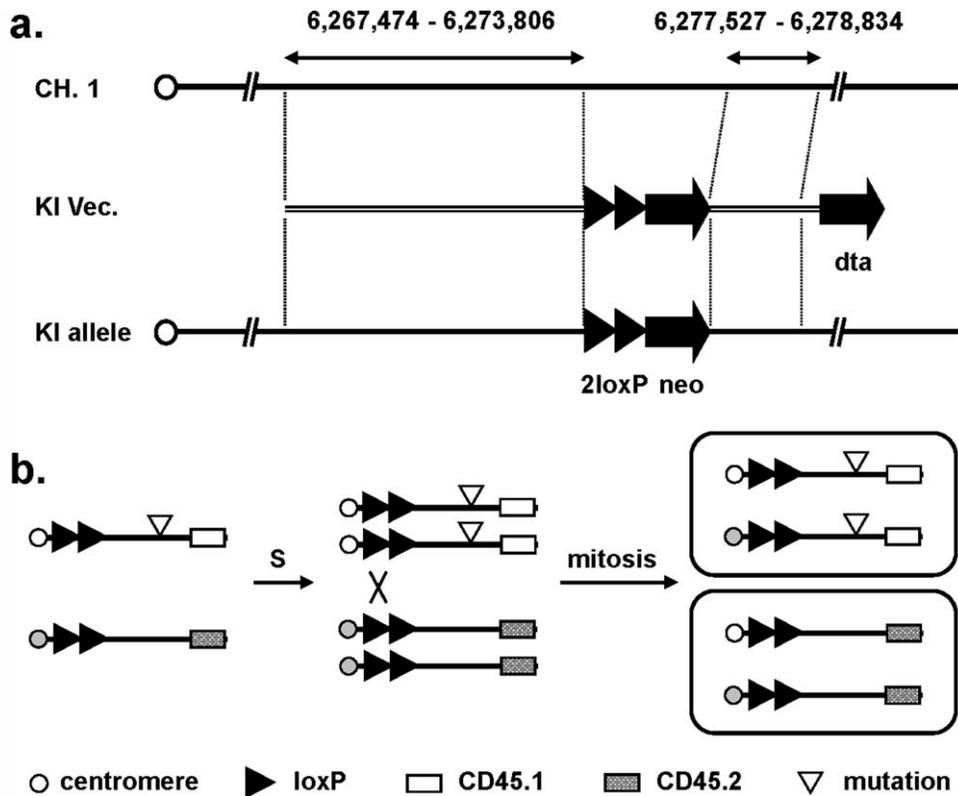
## RESULTS

Given the availability of a broad array of tissue-specific Cre transgenic lines, we chose the Cre/lox technique to build mitotic recombination system. We selected mouse chromosome 1 in this study, because it offers the pre-existing CD45.1 and CD45.2 allelic markers (Zebedee *et al.*, 1991). A pair of loxP sites was introduced into chromosome 1 at the position  $\sim 6$  Mb away from the centromere end through gene targeting (Fig. 1a). More than 99% known protein-coding genes on this chromosome sit distal to the integration site. The targeted allele was successfully generated on mixed genetic background of C57/BL6 and 129/SvEV, both of which express CD45.2. The CD45.1 allele from the SJL/J strain was crossed with the targeted allele to produce a recombinant chromosome 1 carrying both the loxP tag and the CD45.1 marker (Fig. 1b). Mice homozygous for the loxP tag but transheterozygous for CD45.1 and

CD45.2 were produced as the base strain for subsequent mitotic recombination tests. This mitotic recombination system would allow us to track the segregation pattern of any mutation on chromosome 1 based on CD45.1 and CD45.2 expression during mosaic analysis (Fig. 1b). FACS analysis showed that these mice express both CD45.1 and CD45.2 markers on the surface of T cells (Fig. 2a) and other hematopoietic cells (data not shown). A low percentage ( $\sim 0.03\%$ ) of CD45.1 and CD45.2 single positive T cells was detected in these CD45.1/CD45.2 heterozygous mice (Fig. 2), suggesting the presence of a low level of Cre-independent mitotic recombination events.

To evaluate the efficiency of Cre-dependent mitotic recombination in T-cell development, we introduced an LckCre transgene (Pan *et al.*, 2002) into the loxP-tagged CD45.1/CD45.2 mice. Both CD45.1 and CD45.2 single positive cells were detected in DP thymocytes and lymph node T cells at a level significantly higher ( $\sim 10$ -fold) than background (Fig. 2). T-cell development in the thymus progresses through several discrete developmental stages, in the order of DN, DP, to SP before the mature SP cells enter peripheral lymphoid organs and tissues. LckCre is activated at the DN stage of thymocyte development when T-cell progenitors undergo proliferation. Most T cells have ceased proliferation when they reach the DP stage of development and remain in a non-proliferating state in naïve animals. Therefore, we anticipate that the majority of mitotic recombination must have occurred before the DP stage. Both CD45.1 and CD45.2 single positive cells were identified among DP thymocytes at an equal frequency of 0.29% (SD equals 0.08 and 0.07 for CD45.1 and CD45.2 cells, respectively; Fig. 2a,b), confirming that mitotic recombination gives rise to equal numbers of the two recombinant daughter populations. The relative percentage of CD45.1 and CD45.2 single positive cells remains stable from the DP to SP stage in the thymus (Fig. 2a). A similar frequency of CD45.1 or CD45.2 single positive cells was detected in lymph node CD4 and CD8 T cells (Fig. 2c).

One of the most common applications of mitotic recombination system is mosaic analysis of germline mutations. Toward this goal, we selected a germline mutation from the recently established *piggyBac* insertional library (Sun *et al.*, 2008b) for mosaic analysis. The *Dhx9*<sup>PB</sup> mutation was generated through transposition of the PB[Act-RFP] transposon reporter (Ding *et al.*, 2005) into the fourth intron of the *Dhx9* gene (Fig. 3a). The *Dhx9* gene encodes an RNA helicase that participates in multiple biological processes, including transcriptional and translational regulation, micro-RNA processing, and RNA virus sensing (Fuller-Pace, 2006; Kim *et al.*, 2010; Ranji *et al.*, 2011; Robb and Rana, 2007). To explore the mutagenic effect of the inserted transposon, we used RT-PCR to search for alternative

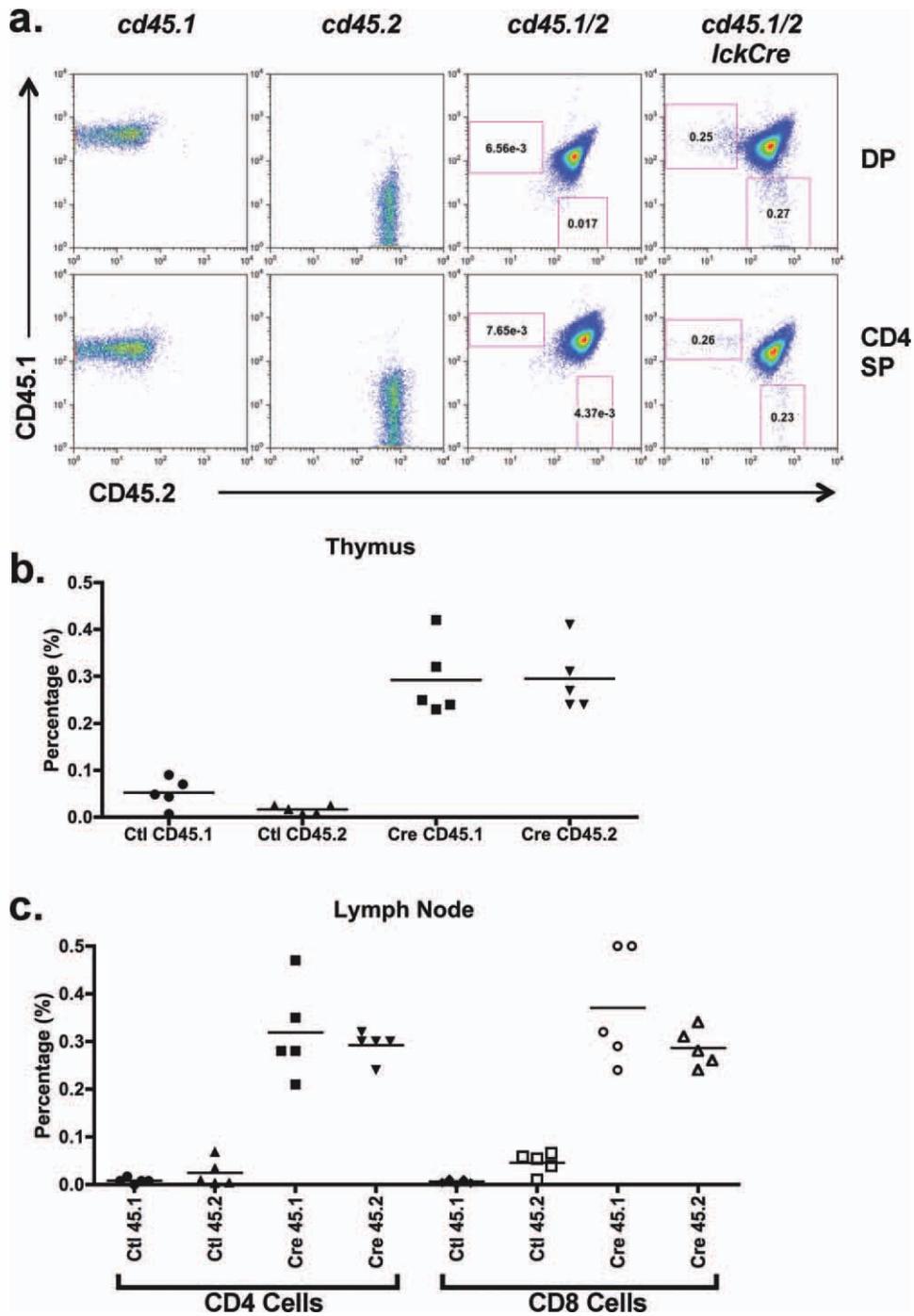


**FIG. 1.** Construction of mitotic recombination system on mouse chromosome 1. (a) Knock-in strategy for loxP integration. Two arrow-ended bars mark the positions of genomic regions for long-arm and short-arm sequences in the targeting vector. neo and dta are used for positive and negative selection in gene targeting, respectively. The tandem loxP sites are indicated with black arrows between the long arm and the neo cassette. Hollow circles indicate the centromere. (b) Schematic view of Cre/lox mediated mitotic recombination. The centromere origin of the two homologous chromosomes is differentially marked with either an open or shaded circle. The open circle chromosome is linked with the  $Dhx9^{PB}$  mutation (open triangle) and CD45.1 (open box). The shaded circle chromosome is linked with CD45.2 (shaded box). During the S-phase of cell cycle, each homologous chromosome is duplicated. Cre/lox-mediated recombination (indicated by the X sign) between homologous chromosomes during mitosis can lead to segregation of the  $Dhx9$  mutant and wild-type allele along with the linked CD45 markers in daughter cells.

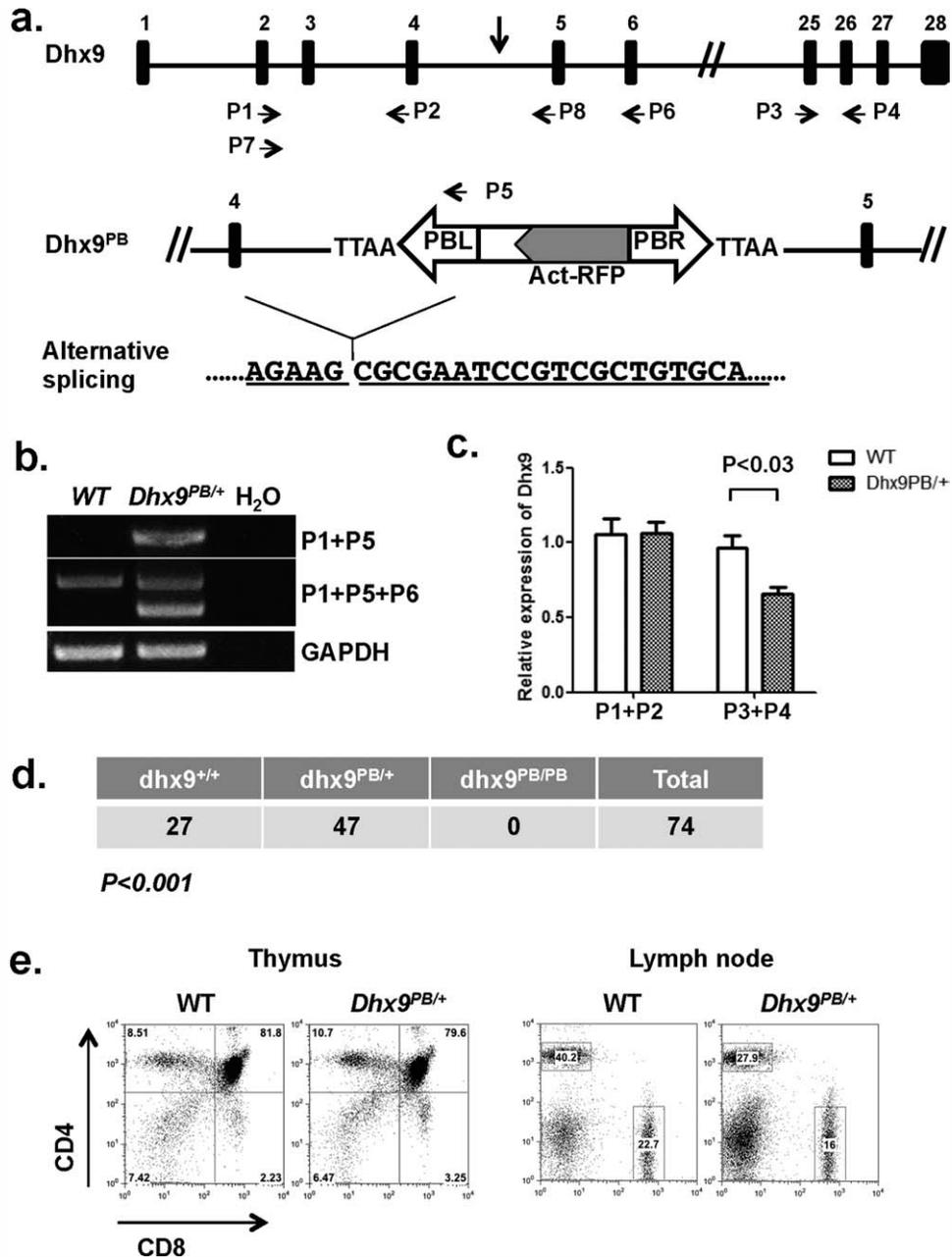
splicing involving the transposon sequence. An alternative spliced product was detected from total thymocyte RNA of  $Dhx9^{PB}$  heterozygous mice with an upstream exon primer and a transposon-specific primer (Fig. 3a,b). This particular splice site in the *piggyBac* left terminal repeat region (PBL) has been previously reported in *piggyBac* insertion involving the Hprt locus in ES cells (Wang *et al.*, 2008). Quantitative real-time RT-PCR analysis of  $Dhx9^{PB/+}$  mice with primers downstream of the insertion site revealed that the levels of wild-type transcripts are reduced in comparison with the wild-type littermate controls ( $P < 0.029$ ). However, the overall RNA message levels containing the upstream exons are equal between  $Dhx9^{PB/+}$  and wild-type controls. To further evaluate the effect of the transposon insertion on the function of the  $Dhx9$  gene, we attempted to produce  $Dhx9^{PB}$  homozygous offspring from an intercross between  $Dhx9^{PB}$  heterozygous mice. This cross failed to generate  $Dhx9^{PB}$  homozygous offspring, confirming an essential function for  $Dhx9$  in embryonic development (Fig. 3d; Lee *et al.*, 1998).  $Dhx9^{PB/+}$  mutant mice exhib-

ited relatively normal T-cell development and homeostasis (Fig. 3e). Because embryonic lethality associated with homozygous mutation, it is not known whether  $Dhx9$  plays any significant role in T-cell development.

We introduced the  $Dhx9^{PB}$  allele into the chromosome carrying the loxP tag and the CD45.1 marker (Fig. 1b) through breeding. The wild-type  $Dhx9$  gene was kept on the chromosome carrying the loxP tag and the CD45.2 marker. In the presence of LckCre, both  $Dhx9^{PB/PB}$  (CD45.1) and  $Dhx9$  wild-type (CD45.2) cells were detected in the thymus and spleen on the  $Dhx9^{PB/+}$  background (Fig. 4a). We used the ratio of CD45.1 mutant cells to CD45.2 wild-type cells to evaluate any potential effect of the  $Dhx9^{PB}$  mutation on T-cell development. The ratio for  $Dhx9^{PB/+}$  mice was found to be  $\sim 1.0$  at the DP stage (mean 0.95, SD 0.11) and reduced to  $\sim 0.5$  at the SP stage (CD4 cells: mean 0.50, SD 0.07; CD8 cells: mean 0.54, SD 0.08; Fig. 4b). The difference in the ratio was attributed to a decrease in CD45.1 frequency without any change in CD45.2 frequency (Fig. 4a). After mature SP cells exit the thymus, this ratio



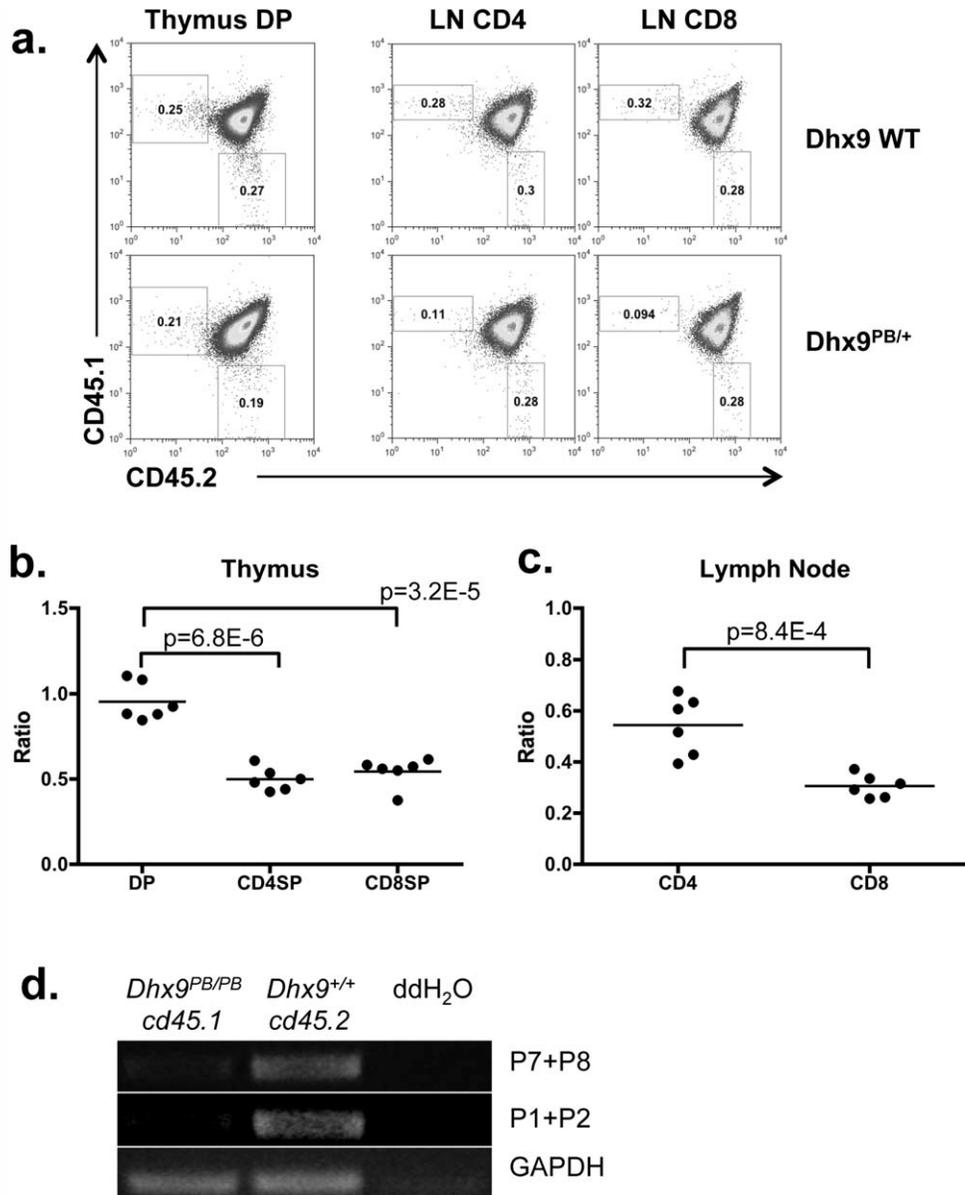
**FIG. 2.** LckCre mediated mitotic recombination during T lymphocyte development. (a) Representative FACS plots of CD45.1 and CD45.2 expression in DP (top panel) and CD4 SP (bottom panel) thymocytes from mice of indicated genotypes on the top. DP and CD4SP cells are displayed in two-D plots for CD45.1 and CD45.2 expression. The relative percentages of CD45.1 and CD45.2 single positive clones from CD45.1/2 heterozygous mice with or without the LckCre transgene are shown in the gated areas. (b) Dot graph summary of mitotic recombination efficiency in DP thymocytes. Percentages of CD45.1 or CD45.2 single positive cells from control mice (Ctl) or Cre-positive mice (Cre) are shown. Genotype for control mice is *loxP;cd45.1/loxP;cd45.2* and for Cre-positive mice is *loxP;cd45.1/loxP;cd45.2;LckCre*. Horizontal bars indicate mean values. *P* values of two-tailed Student's *t*-test are 0.0002 between Ctl CD45.1 and Cre CD45.1 groups, 0.0001 between Ctl CD45.2 and Cre CD45.2 groups, and 0.98 between Cre CD45.1 and Cre CD45.2 groups. (c) Dot graph summary of recombination efficiency for CD4 and CD8 T cell subsets in lymph nodes from the same mice as in (b).



**FIG. 3.** Description of the *Dhx9*<sup>PB</sup> mutation. (a) Mouse *Dhx9* gene contains 28 exons on chromosome 1 (ENSMUSG00000042699, NCBI m37). The *piggyBac* Act[RFP] insertion in the *Dhx9*<sup>PB</sup> allele was mapped to the fourth intron of the *Dhx9* gene (nucleotide position 155327336 of chromosome 1, NCBI m37) with the RFP transcript in the opposite direction of the *DHX9* transcript. Positions of primers used in RT-PCR analysis are indicated in the diagram. The sequence of the alternative splice site from the *Dhx9*<sup>PB</sup> allele is shown on the bottom. (b) RT-PCR detection of an alternative splice product from the *Dhx9*<sup>PB</sup> allele. PCR reaction with total thymocyte RNA from wild type or *Dhx9*<sup>PB</sup> heterozygous mice were performed with either two primers (P1 + P5) or three primers (P1 + P5 + P6). GAPDH was included as a loading control. (c) Real-time RT-PCR test of *Dhx9* transcripts. Results from two separate primer pairs representing 5' (P1 + P2) and 3' (P3 + P4) of the insertion site are based on three wild type and three *Dhx9*<sup>PB</sup> heterozygous mice. (d) Genotyping results were obtained from offspring of *Dhx9*<sup>PB/+</sup> intercross. *P* value was derived from the chi-square test. (e) FACS analysis of CD4 and CD8 T cells in the thymus and lymph node from wild type (wt) and *Dhx9*<sup>PB/+</sup> mice. Plots are representative of three pairs.

remained stable in CD4 T cells (mean 0.54, SD 0.11) and dropped further in CD8 T cells (mean 0.31, SD 0.04) in the lymph nodes (Fig. 4c). We further verified the expression status of *Dhx9* among the CD45.1

mutant cells and CD45.2 wild-type cells isolated by FACS sorting. RT-PCR analysis was carried out with two separate primer pairs covering the region upstream of the transposon insertion site and in the region spanning



**FIG. 4.** Mosaic analysis of Dhx9 during T lymphocyte development. (a) FACS analysis of Dhx9 wild type and mutant clones from Dhx9<sup>PB/+</sup> mice. Dhx9 wild-type mice (top panel) were included as controls in this analysis. Data are representative of thymus DP cells and lymph-node CD4 and CD8 cells from samples summarized in (b) and (c). CD45.1 and CD45.2 single positive cells are defined in the rectangle gates. (b) Dot graph analysis of ratios of CD45.1-CD45.2 single positive cells in mosaic thymi. Relative percentage of CD45 single positive cells was calculated using the same gating criteria in Figure 2a. Ratios between these two populations within DP, CD4SP, and CD8SP subsets are shown. (c) Dot graph analysis of lymph-node CD4 and CD8 T cells from the same groups of mice as in (b). (d) RT-PCR analysis of Dhx9 transcripts in the sorted CD45.1 and CD45.2 single-positive T cells with primers spanning the exons either cross or upstream the insertion site. The purity for CD45.1 and CD45.2 cells are 93% and 91%, respectively, based on postsorting FACS analysis. Result is representative of two independent sorts.

the insertion site. Under this experimental condition, Dhx9 transcripts were found dramatically diminished among CD45.1 mutant cells in comparison with those of CD45.2 wild-type cells (Fig. 4d). We conclude from this mosaic analysis that Dhx9 plays a unique role during the transition from DP to SP and an additional role among CD8 cells in the periphery.

## DISCUSSION

We established a mitotic recombination system on mouse chromosome 1 by introducing a pair of loxP sites close to the centromere end. Mouse chromosome 1 carries the pre-existing CD45.1 and CD45.2 allelic markers, which are ubiquitously expressed in hemato-

poietic cells. This mitotic recombination system is specifically designed to reveal gene function in the hematopoietic system and is particularly useful in the characterization of lethal mutations. We have successfully used this system to generate and analyze homozygous wild-type and mutant T cells from Dhx9<sup>PB</sup> heterozygous mice. Our analysis showed that the Dhx9<sup>PB</sup> mutant allele appears to affect Dhx9 function through disruption of normal splicing. Interestingly, RT-PCR analysis revealed a dramatic reduction of Dhx9 message among homozygous mutant T cells, a phenomenon that cannot be easily explained by the splicing defect. This outcome raised the possibility that Dhx9 may be involved in regulation of its own transcripts. Given the demonstrated roles of Dhx9 in transcriptional regulation (Nakajima *et al.*, 1997) and micro-RNA processing (Robb and Rana, 2007), this phenomenon deserves additional investigation. Our study also detected stage-specific loss of Dhx9 homozygous mutant cells among CD4 and CD8 SP cells in the thymus and CD8 cells in the lymph nodes. Although the mechanism underlying the cell loss is not known based on current phenotyping result, the mitotic recombination system provides a means to retrieve the mutant cells from live animals for functional studies. This mitotic recombination system may be applied to mosaic analysis of other existing mutations on mouse chromosome 1.

The overall efficiency of mitotic recombination observed in this experiment is similar to those reported for other cell types and our previous studies involving chromosome 17 in T and B cells (Sun *et al.*, 2008a). One of the advantages of using this technique in the lymphoid system is that lymphocytes naturally undergo clonal expansion during immune responses to pathogens. Thus, mitotic recombination events could be further enriched or selected during clonal expansion. Although the current analysis of Dhx9 mutation is limited to characterization of developmental phenotypes, this approach can be further extended to the analysis of immune functions. Clonal expansion during an immune response presents an opportunity for amplification of mutant cells in a way similar to the effect caused by loss function of tumor suppressor genes. Therefore, the method described in this study provides a means to apply mitotic recombination techniques beyond tumor studies.

## METHODS

### Mice

The long arm and short arm of the targeting vector was PCR amplified from mouse BAC clone library (RP24, <http://bacpac.chori.org/>). A pair of loxP sites (2loxP, neo-F/MOCK-B) was cloned into the vector between the arms. Neomycin (neo) resistance gene and diphtheria

toxin A (dta) gene were used as positive and negative selection markers, respectively. Gene targeting was conducted in the W4 ES cell line. The chimeric mouse was crossed with SJL/J congenic strain to generate loxp,cd45.1/loxp,cd45.2 mouse. The Dhx9<sup>PB/+</sup> strain (071225026-HRA, Dhx9PB-F/B) was established in the Institute of Developmental Biology and Molecular Medicine at Fudan University. The detailed information of the strain can be accessed through the PBmice database (<http://idm.fudan.edu.cn/PBmice/>). Genotyping primers are given as follows: neo-F: TCGCAGCGCATCGCC TTCTA, MOCK-B: ATATATCCATGAGAATGTGTC, cd45-F: GTTCCATCCCCCTCATTTTT, cd45.1-B: TGAGCCTGTATCTAACCTGAGTC, cd45.2-B: TGAGCCTGCATCTAACCTGATCC, Dhx9PB-F: ACTTTCCTCCAAAGCTGGGCTTCAGG, Dhx9PB-B (P5): CTGAGATGTCTAAATGCACAGCG, lck-P: GCAGGAAGTGGGTAAGTACTAGACTAAC, mx-3: TCTCCCACCGTCAGTACGTGAGATATC.

All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Institute of Developmental Biology and Molecular Medicine at Fudan University.

### FACS Analysis and Antibodies

Single-cell suspensions were prepared from freshly isolated thymi, spleens, and lymph nodes in FACS buffer (PBS, 5% FBS). Cells were stained with antibodies (eBioscience or Caltag Laboratories) and 7-aminoactinomycin D (Sigma-Aldrich) on ice for 20 min and washed once with FACS buffer before being analyzed on FACSCalibur (BD Biosciences). Statistical analyses were performed using two-tailed Student's *t* test with Prism (GraphPad Software).

### RT-PCR and Primers

Total RNAs were isolated from thymi and spleens of wide type and Dhx9<sup>PB/+</sup> adult mice using TRIzol (15596-026, Invitrogen). cDNAs were reverse transcribed from 2 µg total RNAs using RNA PCR Kit Ver.3.0 (RR019A, TaKaRa). Real-time PCR was carried out using MX3000P Multiplex Quantitative PCR System (Stratagene, La Jolla, CA) and Brilliant II SYBR Green QPCR Master Mix kit (Stratagene). The reactions were incubated in a 96-well plate at 95°C for 15 min followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 40 sec. The level of mRNA expression was measured using Ct (threshold cycle). A pool of six samples was used for the standard curve calculation. The mRNA of GAPDH was used to normalize the relative amount of Dhx9 mRNA. Conventional RT-PCR was performed in the analysis of sorted mutant cells. About 10,000 cells of CD45.1 or CD45.2 single positive T cells were obtained from each FACS sorting of pooled samples of three mice and used to produce cDNA. PCR was performed by 40 cycles of 93°C for 3 min, 57°C for

30 sec, and 72°C for 1 min. RT-PCR primers are given as follows: P1: CTGGTGTGGCAAAAAGGAAGATG, P2: TGTGCTATCAGAGGTGTCGCTAAG, P3: TATCCACAAGTCATCTGTGAAC, P4: GCAAAGAGAAGTAGCTGTAAGG, P5: CTGAGATGTCCTAAATGCACAGCG, P6: AAAGCTCCTGTTGTGGTCCG, P7: GCAAAGGAAGATGACTCCA GCC, P8: GAGAGCCATAGCCAGAAGACTCAAC, GAPDH-F: TGCACCACCAACTGCTTAGC, GAPDH-B: GGCATGGAC TGTGGTCATGAG.

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