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Generation of Dhx9 deficient clones in T cell development with a mitotic recombination technique

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Abstract

Mitotic recombination is an effective tool for generating mutant clones in somatic tissues. Due to 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.

Keywords

Mitotic recombination; CD45.1/CD45.2; T lymphocyte; Dhx9; Cre/lox

Introduction

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 approximately 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 result 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

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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 utilized 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., 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 approximately 6Mb 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 stain 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 hemotopoietic cells (data not shown). A low percentage (~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 (~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 gaol, we selected a germline mutation from the recently established *piggyBac* insertional library (Sun et al., 2008b) for mosaic analysis. The $Dhx9^{PB}$ 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 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 *piggBac* 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 exhibited relatively normal T cell development and homeostasis (Fig. 3e). Due to 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 ~1.0 at the DP stage (Mean 0.95, SD 0.11) and reduced to ~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 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 FASC 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 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 hematopoietic 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 $Dhx9^{PB}$ heterozygous mice. Our analysis showed that the $Dhx9^{PB}$ 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 which can not 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 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 stain to generate loxp,cd45.1/loxp,cd45.2 mouse. The Dhx9$^{PB/+}$ 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 as following. neo-F: TCGCAGCGCATCGCCTTCTA, MOCK-B: ATATATCCATGAGAATGTGTC, cd45-F: GTTCCATCCCCCTCATTTTT, cd45.1-B: TGAGCCTGTATCTAAACCTGAGTC, cd45.2-B:TGAGCCTGCATCTAAACCTGATCC, Dhx9PB-F:ACTTTCCCAAAGCTGGGCTTCAGG, Dhx9PB-B (P5):CTGAGATGTCCTAAATGCACAGCG, lck-P: GCAGGAAGTGGGTAACTAGACTAAC, 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 (7AAD) (Sigma-Aldrich) on ice for 20min, 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$^{PB/+}$ adult mice using TRIzol (15596-026, Invitrogen). cDNAs were reverse transcribed from 2ug 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 30sec, 55°C for 30sec and 72°C for 40sec. 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 3min, 57°C for 30sec, and 72°C for 1min. RT-PCR primers are as following. P1: CTGGTGTGGCAAAAGGAAGATG, P2: TGTGCTATCAGAGGTGTCGCTAAG, P3: TATCCACAAGTCATCTGTGAAC, P4: GCAAAGAGAAGTAGCTGTAAGG, P5: CTGAGATGTCCTAAATGCACAGCG, P6: AAAGCTCCTGTTGTGGTCGG, P7: GCAAAAGGAAGATGACTCCAGCC, P8: GAGAGCCATAGCCAGAAGACTCAAC, GAPDH-F: TGCACCACCAACTGCTTAGC, GAPDH-B: GGCATGGACTGTGGTCATGAG.

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References

Ding S, Wu X, Li G, Han M, Zhuang Y, Xu T. Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. Cell. 2005; 122:473–483. [PubMed: 16096065]

Fuller-Pace FV. DExD/H box RNA helicases: multifunctional proteins with important roles in transcriptional regulation. Nucleic acids research. 2006; 34:4206–4215. [PubMed: 16935882]

Gondo Y. Trends in large-scale mouse mutagenesis: from genetics to functional genomics. Nat Rev Genet. 2008; 9:803–810. [PubMed: 18781157]

Kim T, Pazhoor S, Bao M, Zhang Z, Hanabuchi S, Facchinetti V, Bover L, Plumas J, Chaperot L, Qin J, et al. Aspartate-glutamate-alanine-histidine box motif (DEAH)/RNA helicase A helicases sense microbial DNA in human plasmacytoid dendritic cells. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107:15181–15186. [PubMed: 20696886]

Lee CG, da Costa Soares V, Newberger C, Manova K, Lacy E, Hurwitz J. RNA helicase A is essential for normal gastrulation. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95:13709–13713. [PubMed: 9811865]

Liu C, Sage JC, Miller MR, Verhaak RG, Hippenmeyer S, Vogel H, Foreman O, Bronson RT, Nishiyama A, Luo L, et al. Mosaic analysis with double markers reveals tumor cell of origin in glioma. Cell. 2011; 146:209–221. [PubMed: 21737130]

Liu P, Jenkins NA, Copeland NG. Efficient Cre-loxP-induced mitotic recombination in mouse embryonic stem cells. Nature genetics. 2002; 30:66–72. [PubMed: 11740496]

Muzumdar MD, Luo L, Zong H. Modeling sporadic loss of heterozygosity in mice by using mosaic analysis with double markers (MADM). Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:4495–4500. [PubMed: 17360552]

Nakajima T, Uchida C, Anderson SF, Lee CG, Hurwitz J, Parvin JD, Montminy M. RNA helicase A mediates association of CBP with RNA polymerase II. Cell. 1997; 90:1107–1112. [PubMed: 9323138]

Pan L, Hanrahan J, Li J, Hale LP, Zhuang Y. An analysis of T cell intrinsic roles of E2A by conditional gene disruption in the thymus. J Immunol. 2002; 168:3923–3932. [PubMed: 11937548]

Ranji A, Shkriabai N, Kvaratskhelia M, Musier-Forsyth K, Boris-Lawrie K. Features of double-stranded RNA-binding domains of RNA helicase A are necessary for selective recognition and translation of complex mRNAs. The Journal of biological chemistry. 2011; 286:5328–5337. [PubMed: 21123178]

Robb GB, Rana TM. RNA helicase A interacts with RISC in human cells and functions in RISC loading. Mol Cell. 2007; 26:523–537. [PubMed: 17531811]

Skarnes WC, Rosen B, West AP, Koutsourakis M, Bushell W, Iyer V, Mujica AO, Thomas M, Harrow J, Cox T, et al. A conditional knockout resource for the genome-wide study of mouse gene function. Nature. 2011; 474:337–342. [PubMed: 21677750]

Sun L, Wu X, Han M, Xu T, Zhuang Y. A mitotic recombination system for mouse chromosome 17. Proceedings of the National Academy of Sciences of the United States of America. 2008a; 105:4237–4241. [PubMed: 18326030]

Sun LV, Jin K, Liu Y, Yang W, Xie X, Ye L, Wang L, Zhu L, Ding S, Su Y, et al. PBmice: an integrated database system of piggyBac (PB) insertional mutations and their characterizations in mice. Nucleic acids research. 2008b; 36:D729–D734. [PubMed: 17932058]

Wang W, Lin C, Lu D, Ning Z, Cox T, Melvin D, Wang X, Bradley A, Liu P. Chromosomal transposition of PiggyBac in mouse embryonic stem cells. Proc Natl Acad Sci U S A. 2008; 105:9290–9295. [PubMed: 18579772]

Wang W, Warren M, Bradley A. Induced mitotic recombination of p53 in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:4501–4505. [PubMed: 17360553]

Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P, et al. Initial sequencing and comparative analysis of the mouse genome. Nature. 2002; 420:520–562. [PubMed: 12466850]

Wirth D, Gama-Norton L, Riemer P, Sandhu U, Schucht R, Hauser H. Road to precision: recombinase-based targeting technologies for genome engineering. Curr Opin Biotechnol. 2007; 18:411–419. [PubMed: 17904350]

Zebedee SL, Barritt DS, Raschke WC. Comparison of mouse Ly5a and Ly5b leucocyte common antigen alleles. Dev Immunol. 1991; 1:243–254. [PubMed: 1822988]

Zong H, Espinosa JS, Su HH, Muzumdar MD, Luo L. Mosaic analysis with double markers in mice. Cell. 2005; 121:479–492. [PubMed: 15882628]

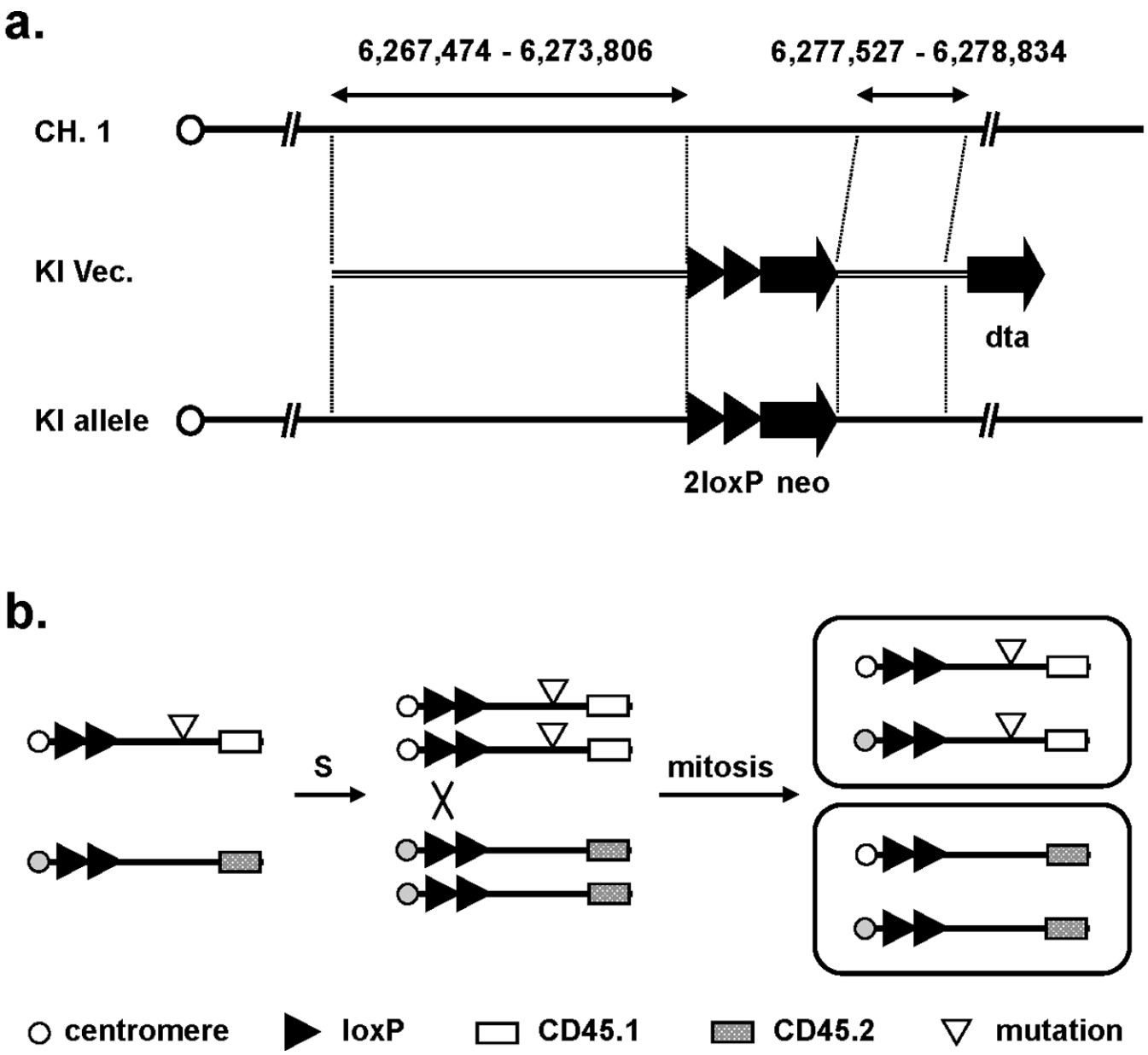

Fig. 1.

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 (indicted 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.

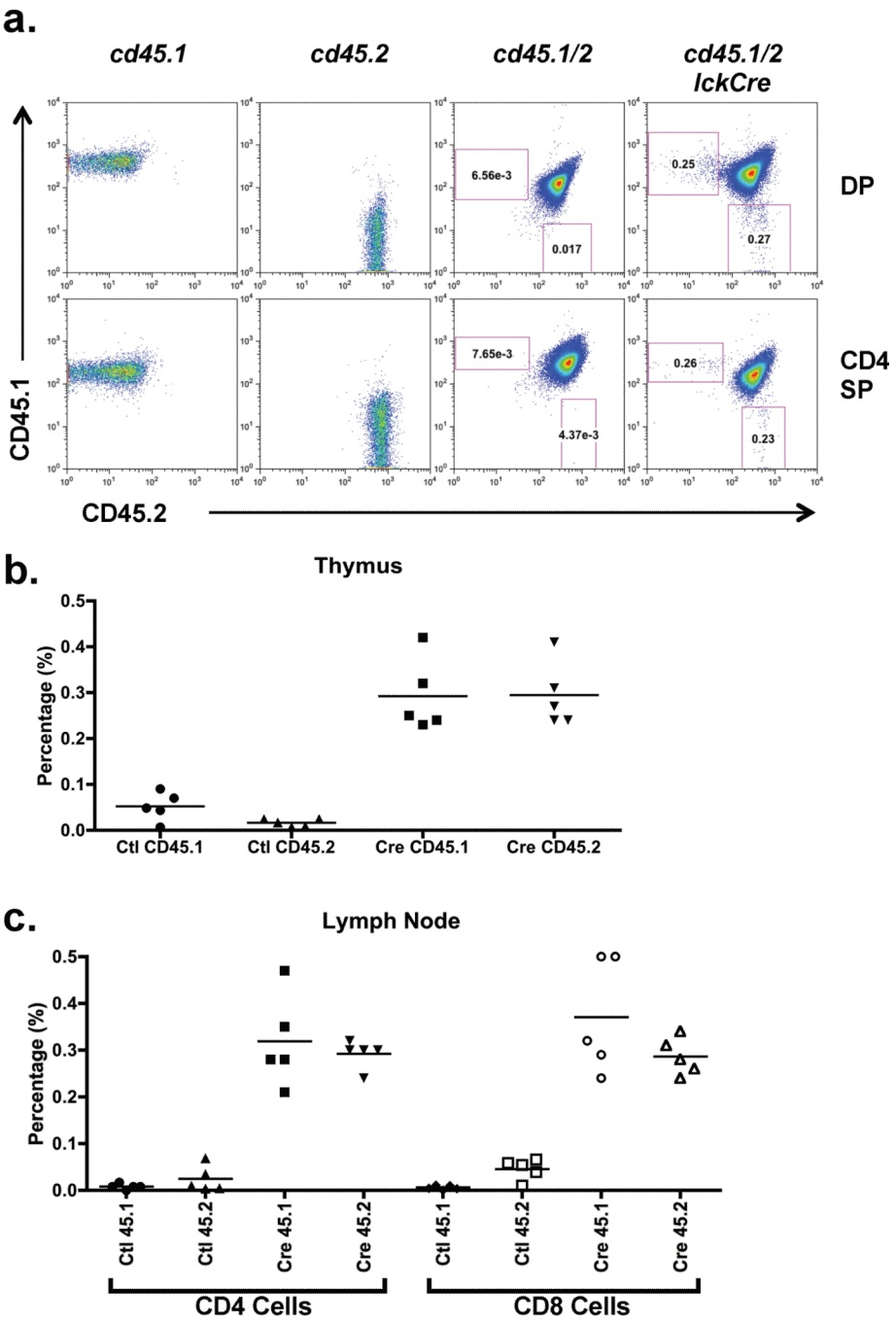

Fig. 2.

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 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).

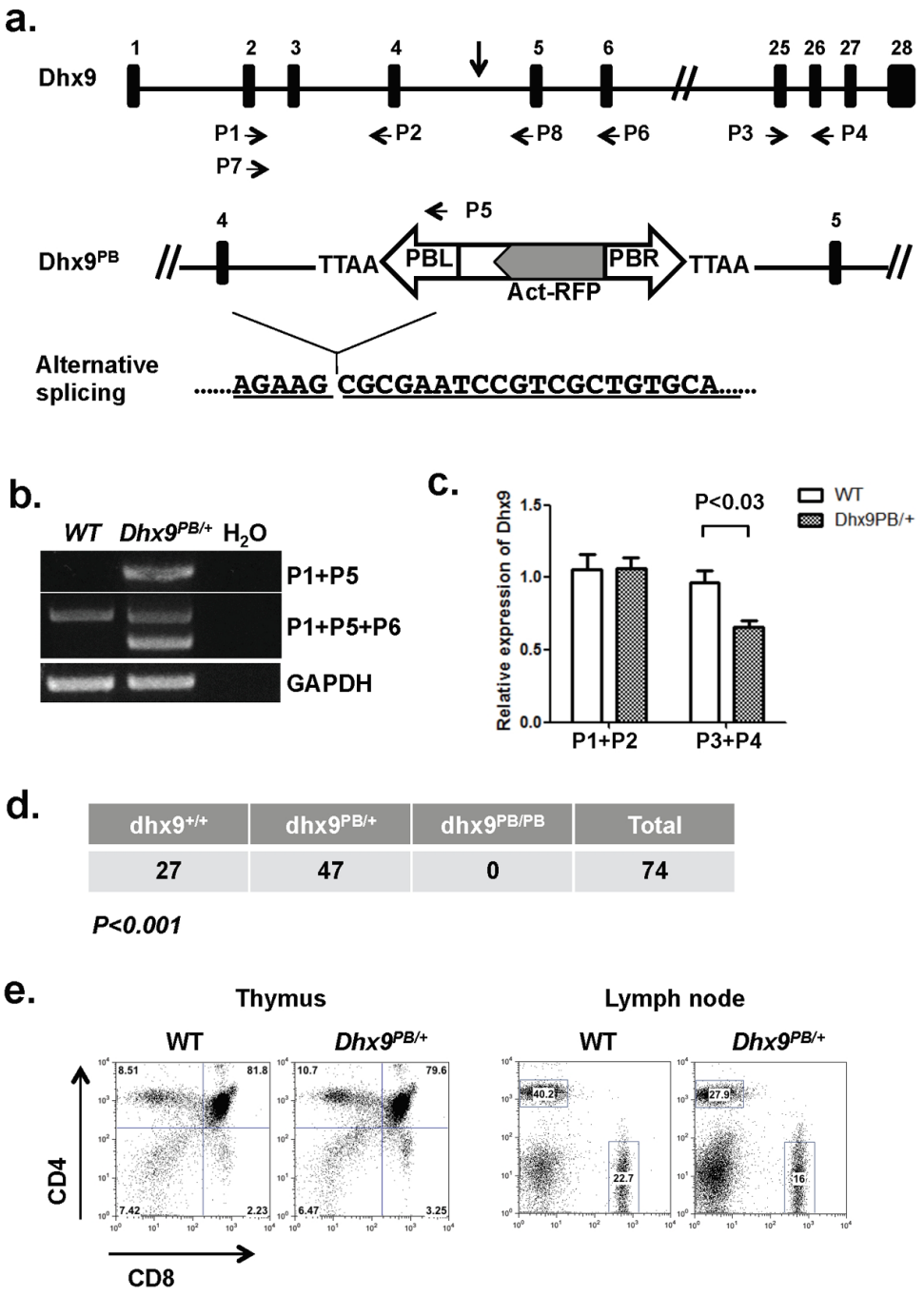

Fig. 3.

Description of the Dhx9^{PB} mutation. (a) Mouse Dhx9 gene contains 28 exons on chromosome 1(ENSMUSG00000042699, NCBI m37). The *piggyBac* Act[RFP] insertion in the Dhx9^{PB} 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 slice site from the Dhx9^{PB} allele is shown on the bottom. (b) RT-PCR detection of an alternative splice product from the Dhx9^{PB} allele. PCR reaction with total thymocyte RNA from wild type or Dhx9^{PB} heterozygous mice were performed with either two primers (P1 + P5) or three primers (P1 + P5 + P6). GAPDH was

included as a loading control. (c) Realtime 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 $Dhx9^{PB}$ heterozygous mice. (d) Genotyping results were obtained from offspring of $Dhx9^{PB/+}$ 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 $Dhx9^{PB/+}$ mice. Plots are representative of three pairs.

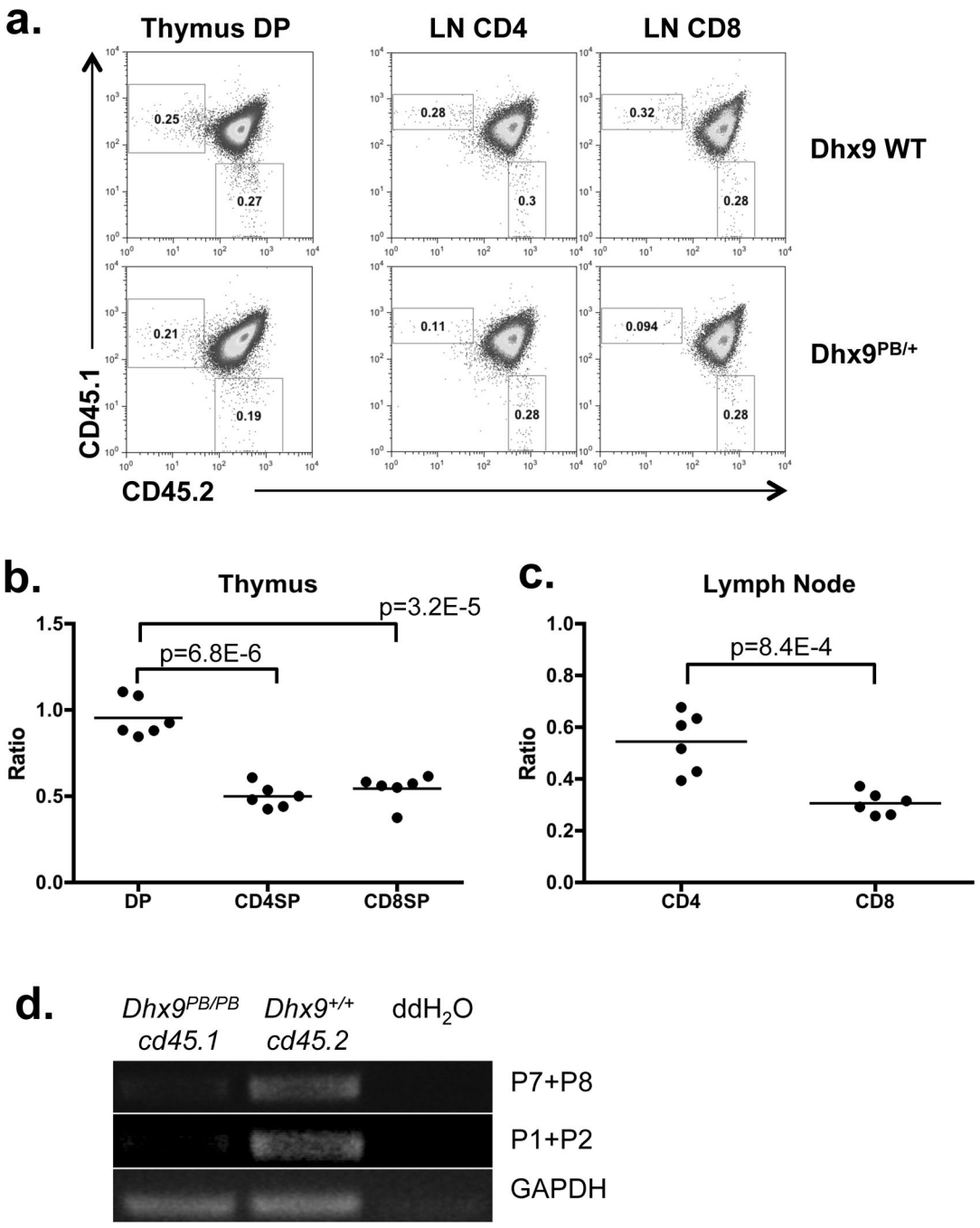

Fig. 4.

Mosaic analysis of Dhx9 during T lymphocyte development. (a) FACS analysis of Dhx9 wild type and mutant clones from Dhx9$^{PB/+}$ 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 to CD45.2 single positive cells in mosaic thymi. Relative percentage of CD45 single positive cells were calculated using the same gating criteria in Fig. 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 post sorting FACS analysis. Result is representative of two independent sorts.