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Citation	Mammalian Genome, 18(11), 779-786 https://doi.org/10.1007/s00335-007-9062-0
Issue Date	2007-11
Doc URL	https://hdl.handle.net/2115/33866
Rights	The original publication is available at www.springerlink.com
Type	journal article
File Information	thidMS(revised).pdf



A deletion mutation of the protein tyrosine phosphatase kappa (*Ptprk*) gene is responsible for the T-helper immunodeficiency (*thid*) in the LEC rat

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Abstract

Bone marrow (BM)-derived T-cell progenitors differentiate into CD4 or CD8 single positive (SP) cells in the thymus. We have previously reported that a single autosomal mutation, *thid*, causes a defect in maturation of CD4 SP thymocytes and an abnormality of peripheral helper-T cells in the LEC rat. In this study, we attempted to identify a gene responsible for the *thid* mutation. We first performed genetic linkage analysis and mapped *thid* locus between *Myb* and *D1Rat392* on Chr 1. In this region, we found a ~380 kb deletion from intron 3 of the *Ptpnk* gene, which encodes a receptor-like protein tyrosine phosphatase type κ (RPTP κ), to intron 1 of *RGD1560849* predicted gene in the LEC rat genome. Reconstitution with syngenic BM cells transduced *Ptpnk* but not *RGD1560849* predicted gene rescued development of CD4 SP cells in the LEC rat thymus. It is confirmed by this result that the *Ptpnk* gene is responsible for the *thid* mutation in the LEC rat. Our results further suggest that RPTP κ plays a critical role in the development of CD4 SP cells in the thymus.

Introduction

The thymus is a major site of the development of T lymphocytes. Bone-marrow (BM)-derived T cell progenitors enter the thymus, differentiate into mature T cells through sequential stages defined by expression of T cell receptors (TCRs), expression of accessory molecules on the

cell surface, and antigen-induced selection (Singer and Bosselut 2004). The most immature T cells in thymus express neither the TCR nor the coreceptors, CD4 and CD8 (double negative, or DN). The DN thymocytes are found in subcapsular zone and outer cortical region of the thymus, and migrate to the inner cortex (Lind et al. 2001; Porrit et al. 2003). During this step, DN thymocytes differentiate into CD4 and CD8 double positive (DP) cells. The DP cells are selected by the extent of signaling depending on the interactions between TCR and self peptide-bound MHC molecules, leading to either deletion (negative selection) or development of MHC-restricted CD4 or CD8 single positive (SP) cells (positive selection) (Ladi et al. 2006). The self MHC on the cortical thymus epithelial cells mediates positive selection (Bousso et al., 2002; Witt et al. 2005). The CD4/CD8 lineage commitment is regulated by the strength or duration of TCR-MHC binding, and the activities of signaling pathway control the expression of CD4/CD8 molecules (He et al. 2005; Laky et al. 2006; Liu and Bosselut 2004; Sun et al. 2005). SP cells move to the medulla, and are negatively selected for removal of thymocytes bearing TCRs reactive to self-antigens (Siggs et al. 2006). Negative selection is supported by the medullary thymic epithelial cells that present tissue-specific antigens on MHC molecules (Gallegos and Bevan 2004).

We have previously reported that a mutant strain of rats, LEC, exhibits a defect in T-cell maturation from DP to CD4 SP but not to CD8 SP cells in the thymus (Agui et al. 1990,

1991b). Although the thymocytes from LEC rats contain less than 1% of CD4 SP cells, some CD4⁺ T cells appear in the peripheral lymphoid organs. However, these peripheral CD4⁺ cells are not functional as helper T cells: that is, the secretion of IL-2 after treatment with mitogenic lectin is impaired (Sakai et al. 1990). Moreover, LEC rats do not produce antibodies against T-cell-dependent antigen, sheep red blood cells (Agui et al. 1990). These defects in the LEC rat are caused by a single autosomal recessive locus designated as *thid* (T-helper immunodeficiency) (Yamada et al. 1991). In this study, we attempted to identify a gene responsible for the *thid* mutation by positional cloning and succeeded to show that a deletion mutation of the *Ptprk* gene, encoding a receptor-type protein tyrosine phosphatase type κ (RPTP κ), is responsible for the *thid* in the LEC rat.

Materials and Methods

Animals. LEC/Ncu, BN/SsN and (BN x LEC)F₁ x LEC backcrossed progenies were maintained at animal breeding rooms in the Center for Experimental Animal Science, Graduate School of Medical Sciences, Nagoya City University, and Graduate School of Veterinary Medicine, Hokkaido University. Animal breeding rooms were kept at 23 ± 2°C and 50 ± 10% relative humidity with a 12-hour light-dark cycle. Research was conducted according to the Guidelines for the Care and Use of Laboratory Animals of both the Graduate

School of Medical Sciences, Nagoya City University and the Graduate School of Veterinary Medicine, Hokkaido University. The experimental protocols were approved by the Institutional Animal Care and Use Committee of both the Graduate School of Medical Sciences, Nagoya City University and the Graduate School of Veterinary Medicine, Hokkaido University.

Genome mapping. A total of 197 (BN x LEC) F_1 x LEC backcrossed progenies were used for PCR-based SSLP analysis. The sequences of all microsatellite primers were based on the Rat Genome Database <<http://rgd.mcw.edu/>> (RGSC 3.4, Dec 2004). We found a 12 bp-length polymorphism in the coding region of *Myb* gene between the LEC and BN rats (data not shown). The sequences of the primers for detecting this polymorphism were shown in Table 1. The *thid* genotype was estimated from the phenotype determined by the ratio of CD4⁺ T cells in mesenteric lymph node cells with flow cytometry as described previously (Jung et al. 2001). Briefly, backcrossed progenies showing the normal ratio (40-50%) of CD4⁺ cells in mesenteric lymph node cells were classified as *thid*/+ genotype, whereas backcrossed progenies showing the small ratio (10-20%) of CD4⁺ cells were classified as *thid*/*thid* genotype. Backcrossed progenies were clearly segregated into two groups with 1:1 ratio as reported previously (Yamada et al. 1991; Wei et al. 1997; Jung et al. 2001). Linkage analysis was performed by Map Manager QTXb20 software (Manly et al. 2001).

Identification a gene responsible for the thid and sequencing of genomic DNA.

The sequence around the *Ptprk* gene in the rat genome was searched in the rat genome browser of Ensembl <<http://www.ensembl.org/index.html>> (Release 43, Feb 2007) and NCBI <<http://www.ncbi.nlm.nih.gov/>> by BLAST software (Ver. 2.2.15, Oct 2006) with the sequence of mouse genome based on the information of the homologous synteny. Obtained sequence was used for the construction of PCR primers for genes locating in the *thid* region. The sequences of PCR primers were shown in Table 1. Sequencing of PCR products was performed with an ABI 377 genetic analyzer (Applied Biosystems, Foster City, CA).

Flow cytometry. Mesenteric lymph node cells and thymocytes (1×10^6 cells) stained with fluorescein isothiocyanate (FITC)-conjugated anti-rat CD4 (Calbiochem, San Diego, CA), and phycoerythrin (PE)-conjugated anti-rat CD8 (Calbiochem) were analyzed with an EPICS XL ADC flow cytometer (Beckman Coulter, Fullerton, CA).

Lentiviral transduction. The rat *Ptprk* and *RGD1560849* predicted gene cDNAs were amplified by RT-PCR with primers shown in Table 1 using total RNA from the BN rat thymus as template. The amplified cDNAs were sequenced (accession number AB297790 and XR_008922 for *Ptprk* and *RGD1560849* predicted genes, respectively) and cloned downstream of CMV promoter of pLenti6/V5 lentiviral vector (Invitrogen, Carlsbad, CA). Recombinant lentivirus was generated by using ViraPower lentivirus expression system

(Invitrogen).

Generation of *BM-reconstituted rats*. Recipient female LEC rats at 6-8 weeks of age were treated with 6 Gy X-irradiation, which was lethal dose for LEC rats (Hayashi et al., 1994). BM cells (1×10^6 cells) from donor male LEC rats (8 weeks old) were infected with recombinant lentivirus, and transplanted to recipient LEC rats through tail vein on the next day. At 5-6 weeks after BM-reconstitution, thymocytes were examined with flow cytometry.

Results

Genetic linkage analysis of the *thid* locus. It has been reported that the *thid* locus is mapped between markers *DIMgh17* and *DIMgh3* on Chr 1 (Wei et al. 1997). To identify the precise position of the *thid* locus, we performed genetic linkage analysis using markers closer to the *thid* locus than the previous markers. We produced 197 (BN x LEC) F_1 x LEC backcrossed progenies. The ratio of peripheral CD4⁺ T cells in the mesenteric lymph nodes was examined by flow cytometry in all progenies to classify *thid/thid* or *thid/+* phenotype (Fig. 1A). Genotyping with markers on Chr 1 was also performed in all progenies (Fig. 1B) and a linkage panel was generated (Fig. 1C). We found that the *thid* locus locates between markers *Myb* and *D1Rat392* at interval of 2 cM. Based on the rat genomic sequence data (RGSC 3.4, Dec 2004) between *Myb* and *D1Rat392* and the homologous region of the

mouse genome in terms of the synteny, it was indicated that there are six genes and two predicted gene in the *thid* locus. These genes were positioned as follows: *Myb* (16.5 Mb)-*Hbs1l* (16.7 Mb)-*LOC683474* (*Aldh8a1*) (16.75 Mb)-*RGD1560849*-predicted (17.2 Mb)-*Ptprk* (17.8 Mb)-*RGD1560849*-predicted (18.1 Mb)-*Lama2* (18.4 Mb)-*Arhgap18* (19.0 Mb)- *L3mbtl3* (19.5 Mb)-*D1Rat392* (19.6 Mb).

Identification of the *thid* mutation. We examined expression of all genes locating in the *thid* region in the BM and thymus and compared it between BN control and LEC rats (Fig. 2). Among genes locating in the critical region, the *Ptprk* and *RGD1560849* predicted genes were found to be expressed differently between BN and LEC rats. Thus, the *Ptprk* gene was expressed in both BM and thymus of BN rats but not of LEC rats, while the *RGD1560849* predicted gene was expressed in the thymus of BN rats but not of LEC rats. Therefore, we next analyzed the genomic structure of *Ptprk* and *RGD1560849* predicted genes in the LEC rat. Since complete sequence data of the region around the rat *Ptprk* was not registered in the rat genome database, we searched sequence data in the trace archive of the rat genome by comparing with the sequence of the mouse genome using BLAST. Using these sequence data, we designed several PCR primers and performed genomic PCR. Among them, the data for A, B, C, and D shown in Fig. 3A were informative. Both A and B sites locate at

~3 kb downstream from the donor site of exon 1 of *RGDI560849* predicted gene, which locates at next downstream of the *Ptprk* gene. The sites C and D locate at ~17 kb downstream from the donor site of exon 3 of the *Ptprk* gene (Fig. 3A). The sites A and D could be amplified, when genomic DNA of LEC rats was used as a template as well as BN control rats. We used LEA and WKAH rats as additional controls, because the LEA rat has been established from the Long-Evans closed colony simultaneously with the LEC rat and is the most genetically closed strain to the LEC rat. The WKAH rat was used as a non-relevant control, because we and others have been used it as a control strain for the LEC rat in previous reports (Agui et al. 1990; Yamada et al. 1991; Sakai et al. 1993; Hayashi et al. 1994; Wei et al. 1997). In contrast, the sites B and C could not be amplified in the LEC rat only (Fig. 3B). These results suggest that the region expanding from the intron 3 of *Ptprk* gene to the intron 1 of *RGDI560849* predicted gene is largely deleted in the LEC rat genome. Since the genomic region between B and C could be amplified using the forward primer of B and the reverse primer of C in the LEC rat, we sequenced the PCR product to confirm the break point of the *thid* mutation. The PCR product of the LEC rat contained the sequence of parts of both the *Ptprk* intron 3 and *RGDI560849* predicted intron 1 as expected (Fig. 3C). Since the *Ptprk* and *RGDI560849* predicted genes are not completely assembled in rat genome database at present, the precise size of deleted region in the *thid* locus is unknown. However, the size is

estimated to be ~380 kb in consideration of the homologous region of mouse genome.

Ptprk expression rescues T-helper immunodeficiency phenotype in LEC rats. Our results indicate that two genes, *Ptprk* and *RGD1560849* predicted genes, are defective in the LEC rat. *Ptprk* encodes a receptor protein tyrosine phosphatase κ (Jiang et al. 1993), and *RGD1560849* predicted gene encodes a hypothetical protein similar to *E430004N04Rik* of the mouse, of which function is unknown. We performed BM-reconstitution with the syngenic BM cells transduced *Ptprk* or *RGD1560849* predicted gene by lentiviral gene expression system in X-irradiated recipient LEC rats, since it has been reported that BM cells but not thymic epithelial cells are defective due to the *thid* mutation (Agui et al. 1990a). When *Ptprk* or *RGD1560849* predicted gene was transduced into LEC-derived BM cells, BM cells sufficiently expressed each gene (Fig. 4A). Next, these BM cells were reconstituted into X-irradiated recipient LEC rats. After 5-6 weeks, recipient rats were sacrificed and expression of these genes in the thymus and spleen was examined. As shown in Fig. 4B, each gene was sufficiently expressed, indicating that the reconstitution with exogenous gene-transduced BM cells succeeded. Then, thymocyte subsets were examined by flow cytometry (Fig. 4C). When *RGD1560849* predicted gene-transduced donor BM cells were reconstituted, CD4 SP cells in the thymus was as few as LEC rats. In contrast, when *Ptprk*-transduced donor BM cells were reconstituted, CD4 SP cells apparently appeared as consisting of 3.7% of the total thymocytes,

which correspond to a half value of normal rat CD4 SP cells. These results indicate that *Ptprk* is a gene responsible for the defect in the development of CD4 SP cells in the LEC rat thymus, and further suggest that *Ptprk* expression in BM cells is a prerequisite for the development of CD4 SP cell lineage in the thymus.

Discussion In the present study, we have identified a deletion mutation of the *Ptprk* and *RGDI560849* predicted genes in the *thid* locus of the LEC rat. Reconstitution with *Ptprk*- but not *RGDI560849* predicted gene-transduced BM cells rescued development of CD4 SP cells in the thymus. Therefore, we conclude that a deletion mutation of the *Ptprk* gene is responsible for T-helper immunodeficiency in the LEC rat.

Ptprk gene encodes a receptor-like protein tyrosine phosphatase type κ (RPTP κ) protein. RPTP κ contains a meprin/A5 antigen/RPTP μ (MAM) domain, a immunoglobulin-like (Ig) domain, and four fibronectin type III (FN) repeats in extracellular region, and two protein tyrosine phosphatase domains in cytoplasmic region (Jiang et al. 1993). The *Ptprk* gene in the LEC rat loses exons 4 through 31. It is unknown whether the truncated mRNA or protein of *Ptprk* is produced. However, it seems unlikely, since the *Ptprk* mRNA is anticipated not to possess the polyadenylation signal. Furthermore, even if we assume that the truncated mRNA or protein would be present, it loses important domains such

as Ig, FN, and tyrosine phosphatase. Thus, it considers that the LEC rat possesses a null mutation of the *Ptp^{rk}* gene. The extracellular region of RPTP κ plays a critical role in homophilic binding, which leads to cell-cell adhesion mediated by RPTP κ (Sap et al. 1994; Zontag et al. 1995). RPTP κ is up-regulated by transforming growth factor (TGF)- β , associated with epidermal growth factor (EGF) receptor, and reduces phosphorylation of EGF receptor in a mammary epithelial cell line, MCF10A. Consequently, cell proliferation is reduced when RPTP κ is activated (Wang et al. 2005). In contrast, down-regulation of RPTP κ modulates Src and FAK phosphorylation, and prevents TGF- β -mediated cell adhesion and migration (Wang et al. 2005). In addition, it is suggested that RPTP κ plays a role in tumor suppression. Previous reports indicate that human Chr 6q, which contains *PTPRK* gene, has been deleted frequently in various tumors (Cooney et al. 1996, Theile et al. 1996). Indeed, loss of heterozygosity of 6q22-23, in which *PTPRK* locates, was found in primary central nervous system lymphomas (Nakamura et al. 2003). Moreover, *PTPRK* mRNA was down-regulated in some melanoma cells (McArdle et al. 2001). It is reported that *PTPRK* mRNA is expressed in various tissues such as spleen, prostate, ovary, brain, kidney, liver, and epithelial cell line (Jiang et al. 1993; Shen et al. 1999; Yang et al. 1997). In contrast, a low expression of *PTPRK* mRNA was observed in human thymus, and was not seen in peripheral blood leukocytes in the previous report (Yang et al. 1997). To date, the expression and the

biological function of RPTP κ in the immune cells were unknown. The present study is the first report describing the function of RPTP κ in the immune system. RPTP κ may play a role in the regulation of adhesion, proliferation and migration of T-precursor cells in the thymus. *Ptprk*-deficient mice were generated by gene trap (Shen et al. 1999; Skarnes et al. 1995). However, they were viable, fertile, and absent of overt phenotypes. Abnormality of the immune system was not examined. There are four homologous proteins, RPTP κ , RPTP λ , RPTP μ , and RPTP ρ in the protein tyrosine phosphatase family that contains MAM, Ig, and FN extracellular domains in humans and rodents (Alonso et al. 2004). Therefore, it is suggested that other members compensate the biological functions when one is deficient. Indeed, homozygous mice that the *Ptprm* is gene-trapped are viable and appear to be normal like *Ptprk*-disrupted mice (Koop et al. 2003). However, our results indicate first time that a deficiency of a member of the protein tyrosine phosphatase family, *Ptprk*, is not compensated by other three homologs, showing a deficient phenotype with respect to the development of CD4 SP cells in the thymus.

After we submitted this paper, a similar report was published elsewhere (Kose et al. 2007). Although they reached *Ptprk* gene as responsible for the *thid* mutation, their report is incomplete. Thus, they did not show the deleted region in the LEC rat genome nor verify their result by the rescue experiment.

In summary, we show that the T-helper immunodeficiency mutation in the LEC rat is attributed to a deletion mutation of the *Ptpnk* gene, encoding a receptor-like protein tyrosine phosphatase type κ . It was verified by the data that BM-reconstitution with *Ptpnk*-transduced BM cells could rescue the T-helper immunodeficiency phenotype in LEC rats. Thus, the present study proposes a crucial role of the RPTP κ in the positive selection and/or maintenance of CD4 SP cells in the thymus.

Table 1 Primers used for PCR.

Genes	Primers	Length of PCR products (bp)
RT-PCR		
<i>Ptprk</i>	F: 5'-ACAAATGTCAGCCTCAAGATGAT-3' R: 5'-ATCAACGCCTTCATAGTCAGGTA-3'	429
<i>RGD1560849</i>	F: 5'-GTAAGGAAT CAACAGAGCCA-3' R: 5'-TCTCGGAATGATGCACTAGA-3'	724
<i>Aldh8a1</i>	F: 5'-AGCACATTTTGGAGAAAGAATCA-3' R: 5'-CTCCCTCTCTTCCTATTCCAGAG-3'	344
<i>Arhgap18</i>	F: 5'-AGAACA AATGAAAACGAAGACCA-3' R: 5'-ATATAGGGCAGTGAGTTCGATCA-3'	324
<i>Hbs1l</i>	F: 5'-TTACCTTCTGAGGAAAATGGACA-3' R: 5'-AACATCCATTGTTACTCCCCTTT-3'	450
<i>L3mbt13</i>	F: 5'-GAATTCCGAGCTTTGGAAGTTAT-3' R: 5'-CTTGGAGTCAGCCTTCAGAGATA-3'	438
<i>Lama2</i>	F: 5'-ACTGATAGAAGTATCGCGTCTGC-3' R: 5'-AGGTATGGAAGGTTGTCCAAAAT-3'	438
<i>RGD1560695</i>	F: 5'-CGTACTTCTACCTGGGTTGTG AA-3' R: 5'-AATGACCTTTATGGTGGATTGTG-3'	419
<i>Ptprk</i> (full length)	F: 5' -GCTGGCTGTCTGGATTTGCGCGCGA T-3' R: 5' -GGAACAGGTGCAACAGCTGCTGGC T-3'	4,464
<i>RGD1560849</i> (full length)	F: 5'-CGGTGACAAATTGACATCTGGAAGC-3' R: 5'-AGAGTTCTGTAGGCACATCAGTGCT-3'	1,996
Genomic PCR		
<i>Myb</i>	F: 5' -TGCTATCAAGAACCACTGGA-3' R: 5' -GGATATTCGCCGTTGACTGA-3'	186 (BN) 174 (LEC)
Region A	F: 5' -AGCTCCTGCTGACCTGAAGA-3' R: 5' -GCAGAAATCAGATGGTCCCA-3'	258
Region B	F: 5' -TAGCAATGCATCCTGCAGAT-3' R: 5' -GCTTATTGACATGCAGCCCT-3'	388
Region C	F: 5' -ACCTCAGAGAGAACAGTTCC-3' R: 5' -CTGTGACTTTGGCTGAGAGC-3'	297
Region D	F: 5' -TGGCCACAGTATAGGGTCAT-3' R: 5' -CGACTGTGACCAGAGCAACT-3'	406

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Figure legends

Fig. 1 Genetic mapping of the *thid* locus. (A) FACS analysis of mesenteric lymph node cells. Backcrossed progenies possessing normal ratio of CD4⁺ cells (40-50%) were classified as *thid*/+ genotype, while less ratio of CD4⁺ cells (10-20%) were as *thid*/*thid* genotype. Figure is shown representative of each genotype. (B) Genotyping panel generated from 197 (BN x LEC)F₁ x LEC backcrossed progenies. Open and filled squares indicate BN/LEC heterozygous and LEC/LEC homozygous genotypes, respectively. The values under the squares indicate the numbers of progenies. (C) Linkage map generated from the data of genotyping panel. The values indicate the genetic distance (cM) ± standard error between the two loci.

Fig. 2 Expression of genes locating in the *thid* locus. RT-PCR was performed with cDNAs prepared from the thymus and BM tissues of BN and LEC rats. Primes for each gene were shown in Table 1.

Fig. 3 Identification of the *thid* mutation. (A) Genomic structure around the *thid* locus on rat Chr 1. A deleted region between region A in intron 1 of *RGDI560849* predicted gene and

region D in intron 3 of the *Ptprk* gene is indicated as a filled column. (B) PCR amplification of regions A through D indicated in (A). Genomic DNAs from WKAH, LEA, BN, and LEC rats were used to amplify regions A through D. B-C indicates the PCR products of the region between B and C. (C) DNA sequencing of PCR products. The sequence of BN rat was derived from PCR product of region C in panel (B). The sequence of LEC rat was derived from PCR product of region B-C in panel (B).

Fig. 4 Rescue experiment by transplantation of BM cells transduced *Ptprk* or *RGD1560849* predicted gene. (A) Expression of *Ptprk* and *RGD1560849* predicted genes in LEC rat BM cells, which had been transduced these genes by the lentiviral vector. Lane 1; *RGD1560849* predicted gene-transduced BM cells, lane 2; *Ptprk* gene-transduced BM cells. (B) Expression of *Ptprk* and *RGD1560849* predicted genes in the thymus and spleen of LEC rats transplanted with *Ptprk* or *RGD1560849* predicted gene-transduced BM cells. *Ptprk*⁺ and *RGD1560849*⁺ indicate the data from recipient LEC rats transplanted with BM cells, which had been transduced respective genes. Note that the *Ptprk* gene was expressed in both thymus and spleen, whereas *RGD1560849* predicted gene was expressed in the thymus but not in the spleen. (C) Flow cytometry of thymocytes from LEC rats transplanted with *Ptprk* or *RGD1560849* predicted gene-transduced BM cells. BN and LEC indicate the data from

untreated rats.

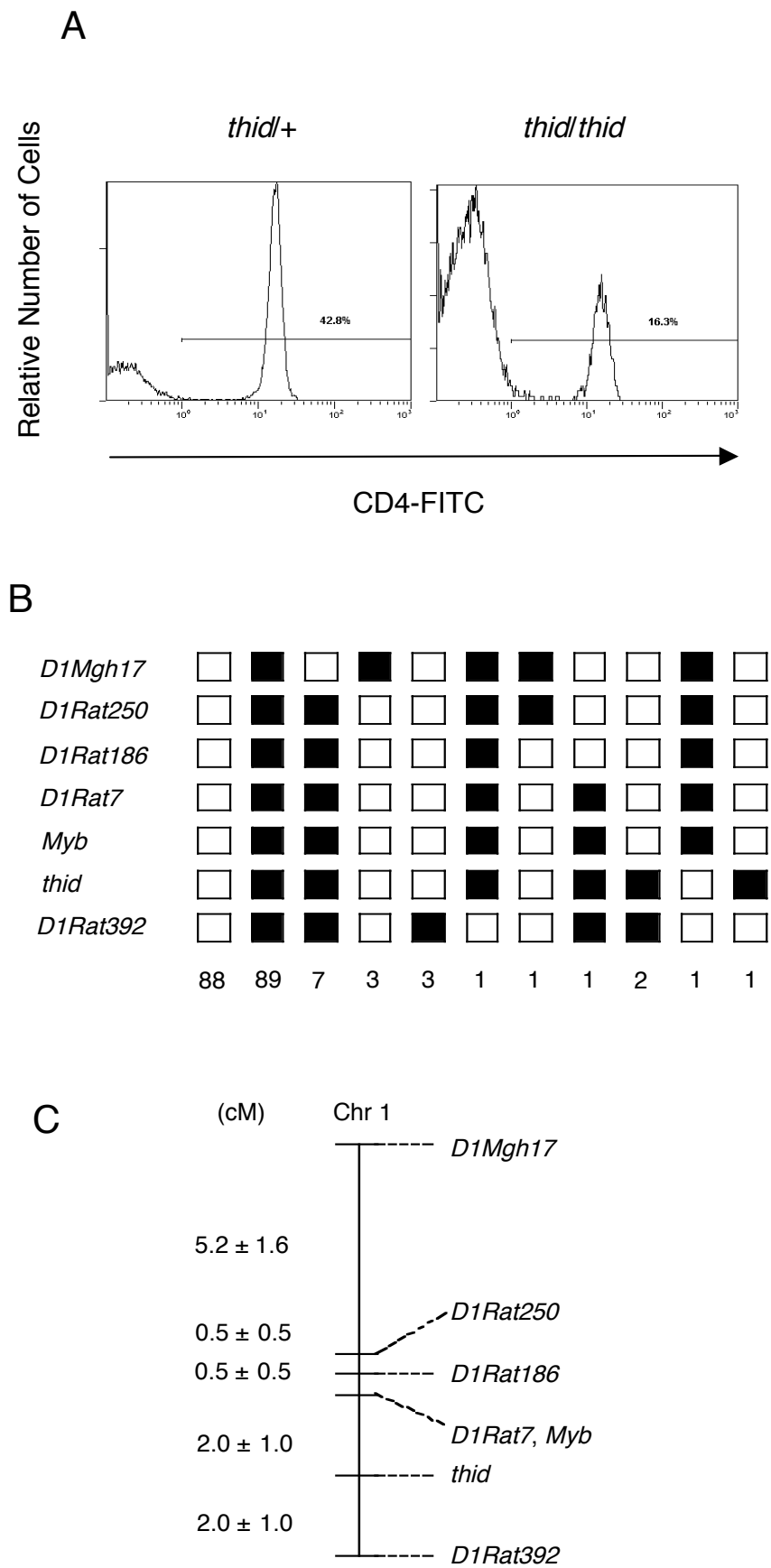


Fig. 1

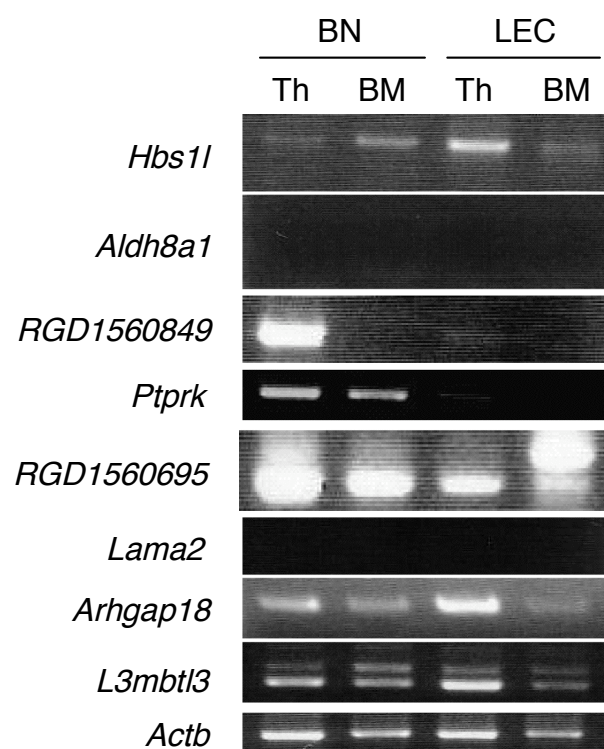


Fig. 2

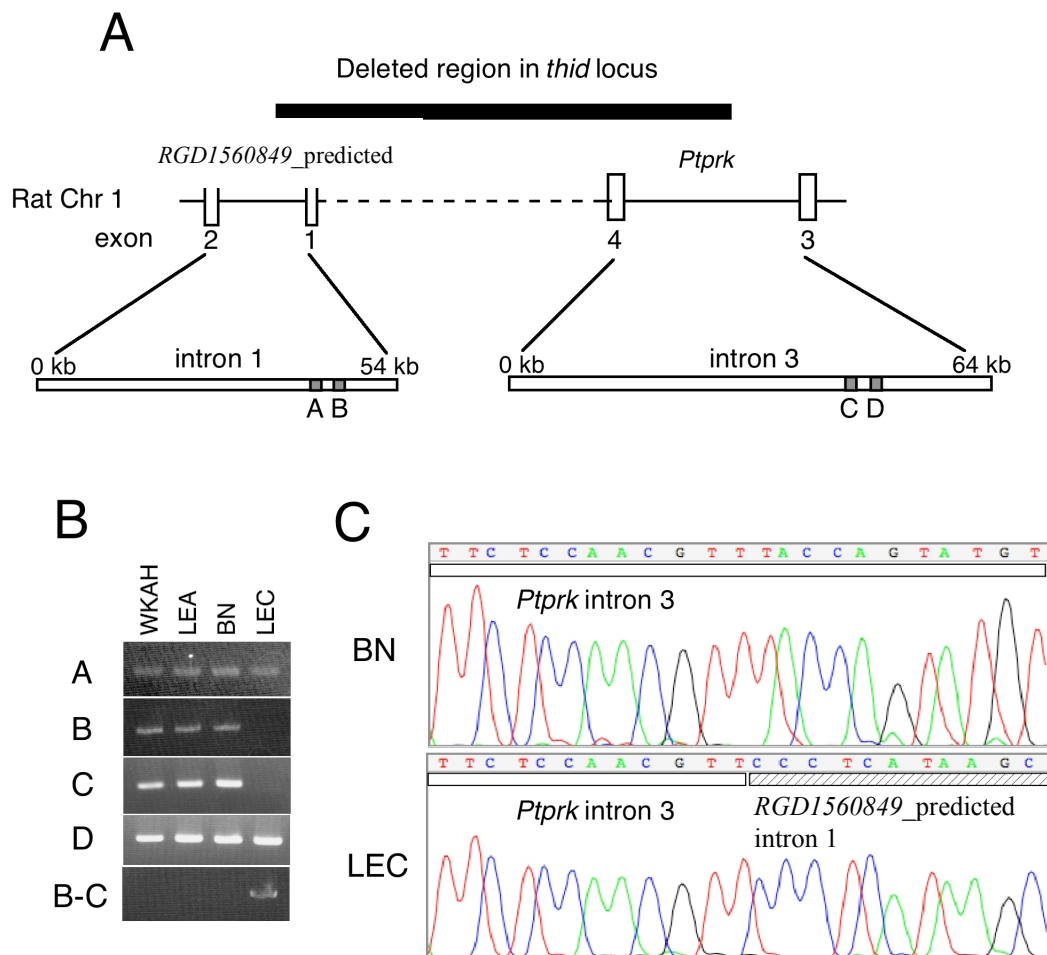


Fig. 3

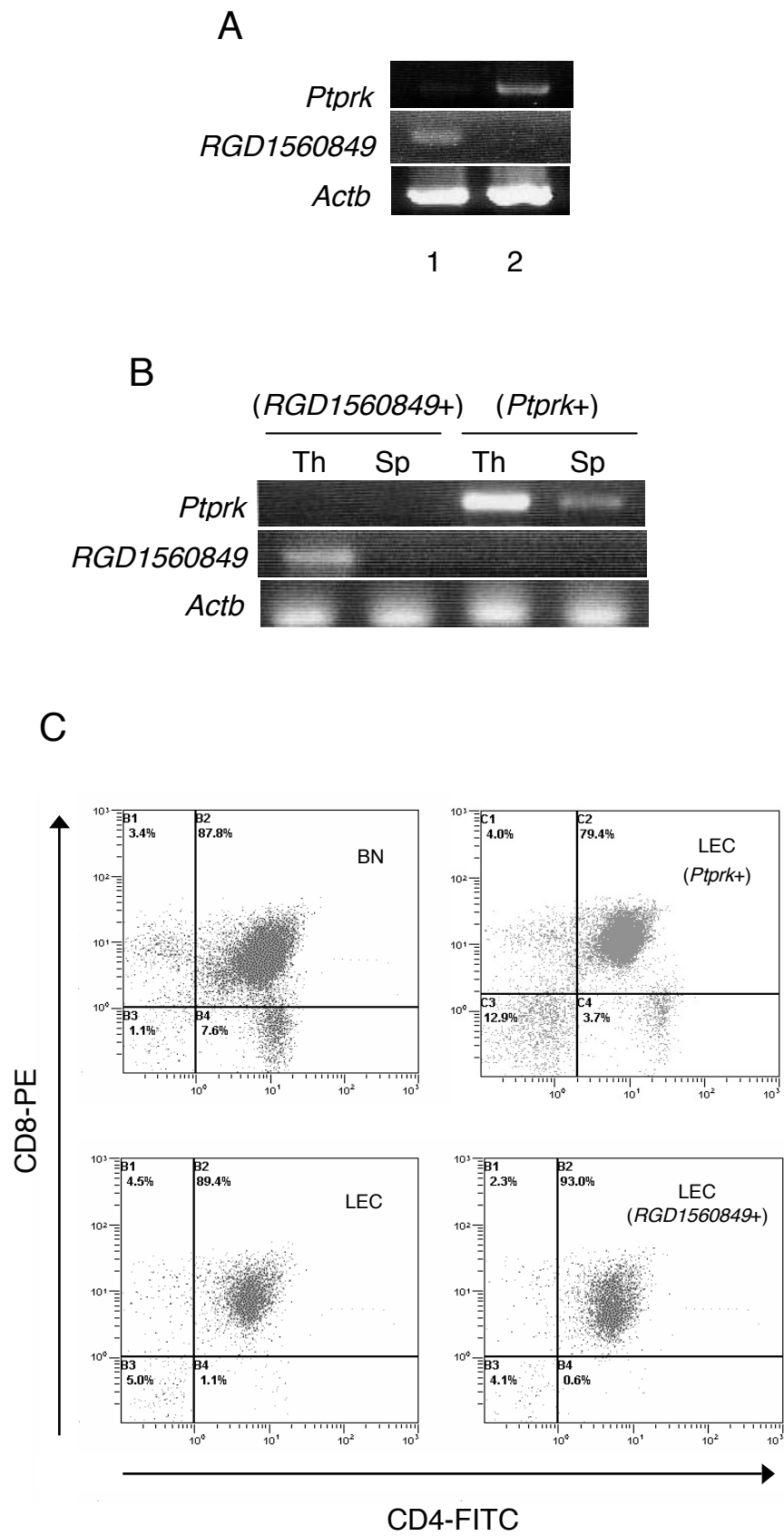


Fig. 4