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Normal Immune System Development in Mice Lacking Deltex-1 RING finger domain.

Running title : Deltex-1 deficiency in the immune system

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ABSTRACT

The Notch signalling pathway controls several cell fate decisions during lymphocyte development, from T-cell lineage commitment to peripheral differentiation of B and T lymphocytes. Deltex-1 is a RING finger ubiquitin ligase, conserved from *Drosophila* to humans, which has been proposed to be a regulator of Notch signalling. Its pattern of lymphoid expression as well as gain-of-function experiments suggest that Deltex-1 regulates both B-cell lineage and splenic marginal zone B cell commitment. *Deltex-1* was also found to be highly expressed in germinal center B cells. To investigate the physiological function of Deltex-1, we generated a mouse strain lacking the Deltex-1 RING finger domain, which is essential for its ubiquitin ligase activity. *Deltex-1*^{ΔΔ} mice were viable and fertile. A detailed histological analysis did not reveal any defect in major organs. T- and B-cell development was normal, as were humoral responses against T-dependent and T-independent antigens. These data indicate that the Deltex-1 ubiquitin ligase activity is dispensable for mouse development and immune function. Possible compensatory mechanisms, in particular from for a fourth *deltex* gene identified during the course of this study are discussed.

INTRODUCTION

Notch proteins are evolutionarily conserved transmembrane receptors which control cellular differentiation processes in nearly every organ (reviewed in references (2, 19)). Mammals have four homologues of the *Drosophila Notch* gene. Upon interaction with one of their ligands, Notch receptors undergo a cascade of proteolytic cleavages, which ultimately releases the Notch Intra-Cellular Domain (NICD) from the membrane (reviewed in reference (40)). NICD subsequently translocates to the nucleus and forms a complex with RBP-J, which upregulates transcription of various targets. RBP-J is thus the central mediator of Notch signal transduction. However, a RBP-J-independent pathway has also been described (37) (reviewed in reference (22)), but its relevance *in vivo* remains to be addressed in Mammals.

Notch-1 is essential for lymphoid development (reviewed in reference (35)). Notch-1 activation in the thymus (13) commits common lymphocyte precursors to the T-cell lineage and prevents them from adopting a B-cell fate (34, 36, 45). In agreement with this function, NICD can repress the activity of E2A (30, 32), a transcription factor required for early B-cell ontogeny. Notch/RBP-J pathway also regulates later steps of T cell differentiation such as VDJ β rearrangement, β -selection (47), $\alpha\beta/\gamma\delta$ (44) and TH1/TH2 (1) lineage decisions. Gain-of-function experiments suggested that Notch-1 might similarly influence CD4⁺/CD8⁺ lineage decision (38), but conditional inactivation of RBP-J did not alter the CD4/CD8 ratio (44). At last, new data point to a potential function of the Notch pathway in the regulation of peripheral T cell activation (6) and the development of regulatory T cells (reviewed in reference (27)).

A role for the Notch pathway in B-cell development was recently uncovered. Conditional deletion of either *RBP-J* (43) or *Notch-2* (39) in the B-cell lineage results in the selective loss of

splenic marginal zone B-cells. Moreover, RBP-J deletion leads to a concomitant increase in the number of follicular B-cells, while mice deficient in *Mint* (18), a suppressor of the Notch/RBP-J pathway, display the reciprocal phenotype. It was thus suggested that Notch-2/RBP-J instruct splenic transitional B cells to adopt a marginal zone B cell versus follicular B cell fate. Notch-2 has also been proposed to regulate B1/B2 lineage decision (46), but conflicting data have been reported (43).

E3 ubiquitin ligases are key regulators of Notch signalling, which control the trafficking and the stability of Notch receptors and ligands (20). For example, Itchy/Suppressor of deltex and c-Cbl are thought to drive the endocytosis of a membrane-anchored form of Notch to the lysosomal compartment (16, 26), while a ubiquitin ligase complex containing SEL-10 targets nuclear NICD to the proteasome (11, 31, 48).

Deltex is another ubiquitin ligase that binds Notch and modulates its signalling, but its precise function remains unclear. Deltex was initially identified in *Drosophila* as a positive regulator of the Notch pathway : *deltex* loss-of-function mutants display a phenotype similar to that caused by weak *Notch* alleles (10, 49), whereas *deltex* overexpression partially mimics *Notch* gain-of-function (23). However, the overexpression of *Deltex-1*, one of the three mammalian homologues of *Drosophila deltex* gene (17, 33), can either enhance (24) or antagonize (41) Notch/RBP-J signalling, depending on the cellular context. In addition, Deltex is thought to mediate RBP-J-independent Notch signals (32, 37).

Deltex proteins share three functional domains (17). Domain I mediates physical interaction with Notch ankyrin repeats (23). Domain II consists of prolin-rich sequences, which may serve as a docking site for an unknown WW- or SH3-protein (24). Domain III contains a highly conserved RING finger domain which mediates homo- and hetero-dimerization (25) and confers ubiquitin ligase activity *in vitro* (42).

Deltex-1 is expressed in a wide array of tissues, particularly in the central nervous system, testis and endothelial cells (24, 28). *Deltex-1* has been suggested to play a role in neurogenesis (17), myogenesis (32), oligodendrocyte maturation and myelination (4, 14). *Deltex-1* also displays a dynamic pattern of expression in thymocytes (15, 29) (5) and peripheral T cells (29) and may thus play a role in T cell development. On the basis of its ability to inhibit E2A *in vitro* (32), *Deltex-1* was initially thought to mediate Notch signal during T-cell commitment. However, Izon *et al.* reported opposite effects of *Deltex-1* on E2A and showed that enforced expression of *Deltex-1* in hematopoietic stem cells results in a phenotype that mirrors that caused by *Notch-1* inactivation (15). The reason for this discrepancy is not clear, but the latter results suggest that *Deltex-1* may actually antagonize Notch signalling in common lymphocyte precursors to promote B-cell fate. *Deltex-1* could be important for later steps of B cell differentiation as well, since it is highly expressed in mouse marginal zone B cells (39) and human germinal center B cells (12).

To investigate the physiological function of *Deltex-1*, we generated a mouse strain lacking the C-terminal half of *Deltex-1*, which contains the RING finger domain. To our surprise, *Deltex-1*^{ΔΔ} mice were viable and fertile and displayed normal lymphocyte differentiation and immune function. Possible compensatory mechanisms are discussed.

Material and Methods

Construction of targeting vector.

A mouse 129/SvJ genomic library (λ FixII; Stratagene) was screened with a 1 kb probe located in the 3' untranslated region of DTX-1. A 15 kb phage encompassing exons 4 to 10 (see Fig. 1) was selected. A 4.3 kb fragment upstream of exon 4 was amplified with Pfu-Turbo (Stratagene) using primers GAGCCACGTGCTCCTGTTTG (forward) and GGCCTGGAACCCAACCTATC (reverse). A 3.6 kb fragment downstream of the poly(A) signal was amplified using primers CCAGGAGAATGAGGAAGACC (forward) and λ Fix5' (reverse) (see Fig. 1). Fragments were inserted using restriction sites added in the primers in the *Sall* and *XhoI* cloning sites flanking the neomycin resistance gene (neo^R) of a modified pLNTK vector (Bertocci, 2002).

Generation of gene-targeted mice.

E14.1 embryonic stem cells (ES) were transfected as described (Torres, 1997). G418- and ganciclovir-resistant clones were screened by PCR (35 cycles with the Long Expand PCR system (Roche)), using the following external (E) and internal (I) primer sets : 5'(E) forward ACAAGTTCCCAAGTCTTGCAGGAGC and 5'(I) reverse GCTGGACGTAAACTCCTCTTCAGAC ; 3'(I) forward GTCTGAAGAGGAGTTTACGTCCAGC and 3'(E) reverse CTCACCCATGGGTTTACACTTAGCC. Homologous recombination was confirmed by Southern blot analysis of DNA from ES clones and thymus of gene-targeted mice, using probe S (Fig. 1). Three recombinant clones (Deltex-1 Δ^+) were obtained among 384 clones and were injected into BALB/c or C57BL/6 blastocysts to generate chimeric mice for germline

transmission of the mutant allele. Deltex-1^{Δ/Δ} mice of mixed genetic background were analyzed after 6 weeks of age. Genotyping of mice was performed by PCR, with simultaneous amplification of wild-type (400 bp) and mutant allele (180 bp), using the primers CAGAGTGTTCTGCAGGAATCGATGC (forward), GGGATCCATAAGTGTGGACTCTATCGG (reverse) and GCTGGACGTAACTCCTCTTCAGAC (reverse in neo^R).

Analysis of *Deltex-1* expression in gene-targeted mice.

Total RNA was extracted with Trizol (Invitrogen). 5 μg of poly A⁺ mRNA isolated from total RNA with Micro Fast Track 2.0 (Invitrogen) were analyzed by northern blotting. The blots were probed with probe 5' (contained in exon 3) and 3' (encompassing exons 5 to 8) (see Fig. 1). Probe 5' was amplified by RT-PCR with the primers CTAGGACAGACATTGCCTAC (forward) and GATGCATGGATTGTAGGTCGATG (reverse). Probe 3' is a 500 bp digestion product by BamHI and EcoRI of a RT-PCR product cloned into pCR2.1-TOPO (Invitrogen) (primers : CTAGGACAGACATTGCCTAC (forward) and GCTGTGTCCCTGTCTTCTC (reverse)). Signals were normalized to β-actin.

Histology.

Organs were fixed in 4% PFA for 48 hours and embedded in paraffin blocks. From the blocks, 5 μm-thick sections were stained with hematoxylin, eosin and saffron (HES).

Flow cytometry analyses.

Single-cell suspensions from spleen, Peyer's patches, thymus, bone marrow and peritoneal cavity were analyzed using a FACStar apparatus and CellQuest software (BD Biosciences) after staining with the following reagents : goat anti-mouse IgM-FITC, from Southern Biotechnology Associated; anti-CD16/CD32 (clone 2.4G2), anti-B220-PE (clone RA3-6B2), anti-CD19-bio (clone 1D3), anti-CD21-FITC (clone 7G6), anti-CD23-PE (clone B3B4), anti-CD5-FITC (53-7-3), anti-CD4-PE (clone RM 4-5), anti-CD8-FITC (clone 53-6.7), anti-CD25-FITC (7D4) and Streptavidin-CyChrome from BD Biosciences ; peanut agglutinin (PNA)-FITC from Vector.

Immunizations and determination of immunoglobulin titers.

Deltex-1^{ΔΔ} and littermate control mice were immunized by intraperitoneal injection of 100 μg of TNP-Ficoll or 100 μg of alum-precipitated TNP-KLH (Biosearch Technologies). TNP-KLH-immunized mice were boosted 21 days later. Serum samples were collected before immunization and at day 7 after TNP-Ficoll immunization or at day 14 and 28 after TNP-KLH immunizations. Plates were incubated overnight at 4°C with TNP-BSA capturing antigen (Biosearch Technologies) (50μg/ml) and saturated with PBS-BSA 1% (1 hour at 37°C). Serial dilutions of serum samples were added to the wells for 2 hour at room temperature, washed and incubated with HRP-conjugated goat-anti-mouse isotypes at 1/500 (Southern Biotechnology Associated). After revelation with ABTS substrate, the optical density at 405nm was recorded. Serum from a pool of immunized wild-type mice served as a standard control between plates.

Basal serum immunoglobulin levels were quantified by ELISA using goat anti-mouse Ig (H+L) and SBA Clonotyping System/HRP from Southern Biotechnology Associated.

To study somatic hypermutation, mice were immunized with phenyl-oxazolone and splenic cells were treated as described in (8).

Analysis of gene expression through semi-quantitative RT-PCR.

Splenic marginal zone B cells (MZB) ($CD19^+CD21^{hi}CD23^{-/lo}$) and follicular B cells (FoB) ($CD19^+CD21^{int}CD23^{hi}$) were sorted with a FACSVantage SE apparatus (BD Biosciences). Total RNA was extracted from $2 \cdot 10^5$ cells using the RNeasy Kit (Qiagen) and cDNA was synthesized using the ProSTAR First-Strand RT-PCR kit (Stratagene). RT-PCR were performed with the Advantage 2 polymerase (BD Biosciences) using the following set of primers : *Deltex-1* forward GGTGGCCATGTACTCCATG and *Deltex-1* reverse TTGGCCATGGCCTCAGAAAC ; *Deltex-2* forward CAATGCTACCTGCCAGATAG and *Deltex-2* reverse AAGAAGCTGACCTGAAGCTG ; *Deltex-4* forward TTGTTACCTTCCAGACAGCGAG and *Deltex-4* reverse CCTTGACTACCCAGAAGTGAAG. Semi-quantitative PCR was performed on serial dilutions of the templates. Reaction products were separated by electrophoresis, transferred onto Hybond N+ membrane (Amersham) and hybridized with internal ^{32}P -labeled oligonucleotides. Quantitation was obtained using Storm 840 Phosphoimager (Molecular Dynamics).

RESULTS

Generation of a mouse strain lacking the Deltex-1 ring finger domain (Deltex-1^{ΔΔ} mice).

We engineered a gene-targeting vector that replaced exons 4 to 10 of *Deltex-1* with a neomycin resistance cassette (neo^R) (Fig. 1 A). These exons code for the C-terminal half of Deltex-1 (G 321 to A 626) which contains one of the proline-rich sequences, the RING finger domain and a motif highly conserved across all Deltex proteins (17). Exon 10 also contains the 3' untranslated region of *Deltex-1* with the polyadenylation signal.

Homologous recombination was confirmed by PCR on both sides of the construct (data not shown) and by Southern blot analysis of DNA from the three ES clones chosen for injection and from thymus DNA of the resulting heterozygous (Deltex-1^{+Δ}) and homozygous (Deltex-1^{ΔΔ}) mice (Fig. 1 B). RT-PCR and northern blot confirmed the expected deletion, while showing the appearance of three new RNA products in Deltex-1^{+Δ} and Deltex-1^{ΔΔ} mice (Fig. 1 D and data not shown). The highest (marked "a") and the lowest (marked "c") forms hybridized with a neo^R probe and disappeared after removal of the neo^R cassette in B cells by mating Deltex-1^{ΔΔ} mice with CD19-CRE mice (Fig. 1 E). Given its large size, the highest form probably corresponds to a partially spliced pre-mRNA stabilized by the poly-adenylation signal brought by the neo^R cassette. Sequencing of a RT-PCR product suggested that the lowest truncated form is created by splicing of the donor site of the third exon to a cryptic acceptor site in neo^R, which creates an in frame stop codon seven amino acids farther (data not shown). The intermediate truncated product (marked "b") is still present after removal of the neo^R cassette (Fig. 1 E). Both truncated products b and c are at least three to five times less abundant than the wild-type *Deltex-1* mRNA (Fig. 1 D

and 1 E). However, due to the lack of an antibody against Deltex-1 N-terminus, we cannot evaluate whether a truncated protein devoid of RING finger domain is expressed. As a conservative estimate, we therefore qualify our mutant mice as being deleted for the ring finger domain of Deltex-1 (Deltex-1^{ΔΔ}).

Gross phenotypic and histologic analysis of Deltex-1^{ΔΔ} mice.

Homozygous Deltex-1^{ΔΔ} mice are viable and fertile and show no apparent defects. Since *Deltex-1* is expressed in a wide array of organs (17, 24, 28), we performed a detailed histological analysis of four adult Deltex-1^{ΔΔ} mice. Given the mixed genetic background, wild-type littermates were used as a control. No abnormalities were detected in brain, spinal cord, eye, liver, kidney, urinary bladder, pancreas, salivary glands, lung, testis, ovary, uterus, mammary gland, skin, aorta and bone marrow (data not shown). Analysis of spleen and mesenteric lymph node did not reveal any differences between wild-type and Deltex-1^{ΔΔ} mice : primary follicles as well as germinal centers were present in normal numbers (Fig. 2).

Normal lymphocyte development of lymphocytes in Deltex-1^{ΔΔ} mice.

Deltex-1 overexpression in mouse hematopoietic stem cells inhibits T cell development while inducing ectopic B cell development in the thymus (15), which suggests that Deltex-1 antagonizes Notch-1 signal to promote B cell development. Thus, we first examined lymphocyte differentiation in the thymus and bone marrow of Deltex-1^{ΔΔ} mice. The distribution of bone marrow B cell subpopulations (pro-B/pre-B B220^{lo}IgM⁻ ; immature B220^{lo}IgM⁺ ; mature

B220^{hi}IgM⁺) found in mutant mice is indistinguishable from that in wild-type mice (Fig. 3 A). Similarly, as shown in figure 3 B, the proportions of thymic T cell subpopulations (CD4⁻CD8⁻ ; CD4⁺CD8⁺ ; CD4⁺CD8⁻ and CD4⁻CD8⁺) are not altered in mutant mice and the numbers of ectopic B cells in the thymus remain unchanged (data not shown).

We next checked T cell populations in the spleen, since the Notch pathway is known to influence peripheral T cell development and activation (1, 6, 27). In particular, *Deltex-1* was recently shown to be constitutively expressed in human CD4⁺CD25⁺ regulatory T cells and downregulated after activation of these cells with anti-CD3 (29). However, wild-type and mutant mice display similar numbers of CD4⁺ and CD8⁺ T cells in the spleen, and splenic CD4⁺CD25⁺ population was not affected in *Deltex-1*^{Δ/Δ} mice (Fig. 3 B).

Deltex-1 was also suggested to play a role in late B cell differentiation (12, 39). First, *Deltex-1* is highly expressed in mouse marginal zone B cells, in a Notch-2 dependent fashion (39). We therefore examined splenic B cell subsets. Three populations can be distinguished according to their relative expression of CD21 and CD23 : transitional B cells 1 (CD19⁺CD21⁻CD23⁻), marginal zone B cells (MZB : CD19⁺CD21^{hi}CD23^{lo}) and follicular B cells (FoB, comprising transitional B cells 2 and long-lived recirculating mature B cells : CD19⁺CD21^{int}CD23^{hi}) (21). In contrast with the selective loss of marginal zone B cells observed in *Notch-2*^{-/-} (39) or *RBP-J*^{-/-} mice (43), all three populations are present in the spleen of *Deltex-1*^{Δ/Δ} mice in normal percentages (Fig. 3 A). Contrary to what was observed in *Notch-2*^{-/-} mice (39), CD21 level was not decreased at the surface of splenic B cells (data not shown). As *Deltex-1* had been reported to modulate E2A activity (15, 17, 32), a transcription factor essential for early and late B cell development, we checked E2A activity *in vitro* using a reporter assay (15). *Deltex-1*^{Δ/Δ} and wild-type splenic B cells stimulated with lipo-polysaccharide (LPS) have similar

level of E2A activity (data not shown). Secondly, we have reported that Deltex-1 is highly expressed in sheep and human germinal center B cells, as well as in their malignant counterpart (12). We thus investigated whether chronic germinal center formation occurred normally in Peyer's patches of mutant mice by staining cells with peanut agglutinin (PNA) which selectively binds centroblasts and centrocytes. The B220⁺PNA^{hi} germinal center B cell population is comparable between wild-type and Deltex-1^{ΔΔ} mice. Thirdly, Notch-2 has also been proposed to play a role in peritoneal B1 cell development (46), potentially through a RBP-J independent pathway (43). We could not observe any difference in the size of the B1 cell population between wild-type and mutant mice (Fig. 3 A).

Together, all these data show that the Deltex-1 RING finger domain is dispensable for both early and late lymphocyte development in mice.

Deltex-1^{ΔΔ} mice have normal humoral immune responses.

In order to look for a role of Deltex-1 in the terminal differentiation of B lymphocytes, we checked the ability of Deltex-1^{ΔΔ} mice to mount humoral responses. First, we found that Deltex-1^{ΔΔ} splenic B lymphocytes proliferated in response to LPS stimulation and were able to undergo class-switch recombination *in vitro* upon stimulation with LPS and IL4 (data not shown). Comparison of the serum concentrations of immunoglobulin classes did not reveal any significant differences between naive Deltex-1^{ΔΔ} and wild-type mice (Fig. 4 A). We then compared humoral responses *in vivo* of mutant and wild-type mice by challenging them with a T-independent type 2 antigen, TNP-Ficoll, and a T-dependent antigen, TNP-KLH. Titers of IgM and IgG3 were similar in both groups of mice after immunization with TNP-Ficoll (Fig. 4 B). Similarly, mutant and

wild-type mice displayed comparable IgM, IgG1, IgG2a, IgG2b and IgG3 anti-TNP antibody titers after primary and secondary immunizations with TNP-KLH (Fig. 4 C).

Deltex-1 was found to be strongly expressed in hypermutating lymphocytes of sheep ileal Peyer's patches and human germinal center centroblasts (12). We therefore investigated whether somatic hypermutation occurs normally in *Deltex-1*^{ΔΔ} mice. To this end, we immunized mice with the hapten phenyl-oxazolone which elicits a well-characterized antibody response and sequenced the rearranged VκOx1 gene segments of B220⁺PNA^{hi} splenic B cells. *Deltex-1*^{ΔΔ} mice showed a mutation rate equivalent to that of wild-type mice (data not shown).

Together, these data show that *Deltex-1*^{ΔΔ} mice mount normal T-dependent and T-independent type 2 humoral responses *in vivo*.

KIAA0937 is a fourth mammalian Deltex protein (Deltex-4).

The absence of an obvious phenotype in mice lacking the *Deltex-1* RING finger domain prompted us to study the expression profile of other *Deltex* family members that could have a redundant function. Three mouse *Deltex* genes were initially described (17). While *Deltex-2* protein sequence is quite similar to that of *Deltex-1*, *Deltex-3* is far more divergent since it lacks the domain I and does not bind Notch proteins *in vitro* (17). Thus, only *Deltex-2* is likely to compensate for the absence of *Deltex-1*.

A chicken *Deltex* gene was cloned (*cDTX2*) and shown to be the orthologue of human KIAA0937 (9). KIAA0937 was therefore called *Deltex-2*. Sequence comparison actually demonstrates that KIAA0937 (and its mouse orthologue (NM_172442)) is a genuine fourth

mammalian *Deltex* gene, which encodes a protein even closer to Deltex-1 (Fig. 5 A and 5 B). Therefore, we propose to name this gene *Deltex-4*.

We first determined the relative levels of expression of *Deltex-1*, *Deltex-2* and *Deltex-4* in different adult organs by northern blot. All three *Deltex* genes have very different, though partially overlapping, expression patterns (Fig. 6 A). A search of EST sequences revealed that human *DELTEX-4* (XM_166213) is expressed in many fetal, adult and cancerous tissues, particularly in the brain (35 EST), heart (17 EST), colon (11 EST), stomach (8 EST) and lung (7 EST). Mouse EST for *Deltex-4* can be found in the brain (38 EST), eye (9 EST) and thymus (4 EST) as well as other organs. We then determined the relative levels of *Deltex-1*, *Deltex-2* and *Deltex-4* in marginal zone B cells and follicular B cells by semi-quantitative RT-PCR. As previously described, *Deltex-1* is far more expressed (9 fold more) in marginal zone B cells (Fig. 6 B). Interestingly, *Deltex-4* is hardly detectable in spleen by northern blot. However, this gene shows a similar bias of expression in favor of marginal zone B cells (9 fold more, Fig. 6 B). On the contrary, *Deltex-2* is hardly detectable in any splenic B cell subset (Fig. 6 B), suggesting that T cells are major contributors to its splenic expression level. We next investigated whether *Deltex-1* inactivation induces an upregulation of *Deltex-2* or *Deltex-4* transcript that could compensate for its absence. *Deltex-2* and *Deltex-4* transcript levels remain unchanged in the brain, testis and spleen of *Deltex-1*^{ΔΔ} mice, compared to wild-type mice (Fig. 6 C).

DISCUSSION

In the course of a cDNA subtraction, we identified Deltex-1, a modulator of Notch signalling pathway, as being highly expressed in human and sheep germinal center B cells (12). Meanwhile, *Deltex-1* was shown to be highly expressed in mouse marginal zone B cells (39) and overexpression studies suggested that Deltex-1 promotes B cell lineage commitment (15). Moreover, its expression profile indicated a potential role for Deltex-1 in T cell differentiation (5, 29, 32) and several other developmental processes (4, 14, 17, 24, 28, 41).

In this study, we generated a mouse strain defective for the *Deltex-1* gene. We chose to delete the C-terminal RING finger domain responsible for the ubiquitin ligase activity of Deltex-1 (42). Since residual expression of the mRNA coding for the N-terminal half of the protein was observed in the mutant mice obtained, we refer to these strains as deleted for the RING finger domain (Deltex-1^{ΔΔ}). Our results demonstrate that this domain is dispensable for mouse development and normal immune system functions. First, Deltex-1^{ΔΔ} mice have normal lymphoid development in the thymus and bone marrow and peripheral B- and T-cell subpopulations are present in expected proportions. In particular, neither marginal zone B cells nor germinal center B cells are affected by this mutation. Secondly, Deltex-1^{ΔΔ} mice mount efficient T-independent type 2 and T-dependent humoral immune responses, which suggests that the Deltex-1 RING finger domain is also dispensable for terminal B cell differentiation and function. Considering the potential role of Deltex-1 in lymphopoiesis and embryonic development, these results are quite surprising. Two hypotheses can account for this lack of overt phenotype.

One explanation could be that the Deltex-1 RING finger domain is dispensable for Deltex-1 function *in vivo*. Prior experiments supported this hypothesis since Deltex domain I was able to rescue the *deltex* phenotype in *Drosophila* mutants on its own (23). However, the nature of the mutation has not been determined in these mutants, which are likely hypomorphic and may be specifically impaired for some function of Deltex relying on the sole domain I. Similarly, the overexpression of Deltex-1 domain I in cell lines has been shown to antagonize the transcriptional activity of the NICD/RBP-J complex (15). However, several articles report that Deltex proteins lacking a RING finger domain rather behave as a weak form of Deltex (23 , 25), or even as a dominant-negative form of Deltex *in vitro* (50) and *in vivo* (4, 7, 14). At last, it should be noted that the experiments investigating Deltex-1 effects on NICD overexpression may not be fully relevant and could result in non-physiological effects, such as competition with other factors for binding to Notch. Indeed, Deltex is rather likely to act downstream of full-length Notch and upstream of an activated NICD (25). Since Deltex-1 is a bona-fide ubiquitin ligase (42), one possibility is that Deltex-1 ubiquitinates a membrane-anchored form of Notch through its RING finger, to control its stability and/or its subcellular localization (40). Moreover, it should be noted that the level of the truncated *Deltex-1* transcript that we observe in *Deltex-1^{ΔΔ}* mice is quite low compared to *Deltex-1* transcript in wild-type mice. Preliminary results show that excision of the neo^R cassette in B lymphocytes by mating *Deltex-1^{ΔΔ}* mice with CD19-CRE mice results in a stronger decrease in the expression of the truncated mRNAs with still no alteration of early and late B cell development (our unpublished results). We therefore think that the lack of phenotype observed in *Deltex-1^{ΔΔ}* mice is unlikely to originate from an incomplete inactivation of this gene.

A second hypothesis could be that gene redundancy compensate for the absence of *Deltex-1*. *Deltex-1* indeed belongs to a multigenic family (17). We show here that this family consists of four different members, since we observed that KIAA0937, a previously described human orthologue of chicken *Deltex-2* (9), actually encodes a fourth *Deltex* gene, which should thus be called *Deltex-4*. Both *Deltex-2* and *Deltex-4* have a sequence highly similar to that of *Deltex-1*, but their expression patterns only partially overlaps that of *Deltex-1*. Moreover, neither *Deltex-2* nor *Deltex-4* transcripts are upregulated in *Deltex-1*^{ΔΔ} mice. Therefore, *Deltex-2* and *Deltex-4* are not likely to compensate for *Deltex-1* absence in all tissues. Another possibility is that *Deltex-1* deficiency is compensated for by an unrelated protein that displays functional convergence, as it might be the case with Neuralized and Mind bomb, two E3 ubiquitin ligases required for the endocytosis of a Notch ligand (reviewed in (20)).

If an imperfect compensation process is taking place, one might envision to uncover more subtle phenotypes associated with specific differentiation processes, possibly by mating *Deltex-1*^{ΔΔ} mice with strains heterozygous for a mutation in another component of the Notch pathway.

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FIGURE LEGENDS

FIG.1. Targeted disruption of mouse *Deltex-1* gene. (A) Schematic representation of Deltex-1 protein domains, with the deleted region including the RING finger domain underlined. (B) Schematic representation of wild-type and targeted *Deltex-1* locus. Dotted boxes represent non coding sequences within exons. Positions of *BamHI* sites (B) and of the probes used for Southern (S) and northern (5' and 3' cDNA probes) blot analysis are indicated. *PGK* p(A) signal, polyadenylation signal of phospho-glycerate kinase gene. TK, Herpes simplex thymidine kinase gene. Triangles flanking the neomycin resistance gene (neo^R) represent LoxP sites. (C) Southern blot analysis of *BamHI*-digested thymic DNA from wild-type mice (+/+) and from mice heterozygous (+/ Δ) and homozygous (Δ/Δ) for the targeted *Deltex-1* gene. (D) Northern blot analysis of *Deltex-1* expression in the spleen and brain from wild-type, heterozygous and homozygous mice, with a cDNA probe outside (probe 5') or inside (probe 3') the deletion. a, b and c mark the three truncated forms of *Deltex-1* transcript observed in mutant mice (see text for details). Blots were normalized with an *actin* probe.

FIG. 2. *Deltex-1* ^{Δ/Δ} mice have normal lymphoid organ structure. Sections from spleen (A and B), and mesenteric lymph node (C) from wild-type (left panel) and *Deltex-1* ^{Δ/Δ} mice (right panel) were stained with hematoxylin, eosin and saffron and photographed under a light microscope at 40x. (B) Higher magnification view of (A) showing a primary B cell follicle and adjacent marginal zone.

FIG. 3. Normal lymphoid development in Deltex-1^{Δ/Δ} mice. (A) B cell subpopulations in the bone marrow, spleen, peritoneal cavity and Peyer's patches were determined by fluorescence-activated cell sorting (FACS). (B) Thymic and splenic T-cell subpopulations were determined by FACS. Mean values and standard deviations for at least 5 animals of each genotype are indicated.

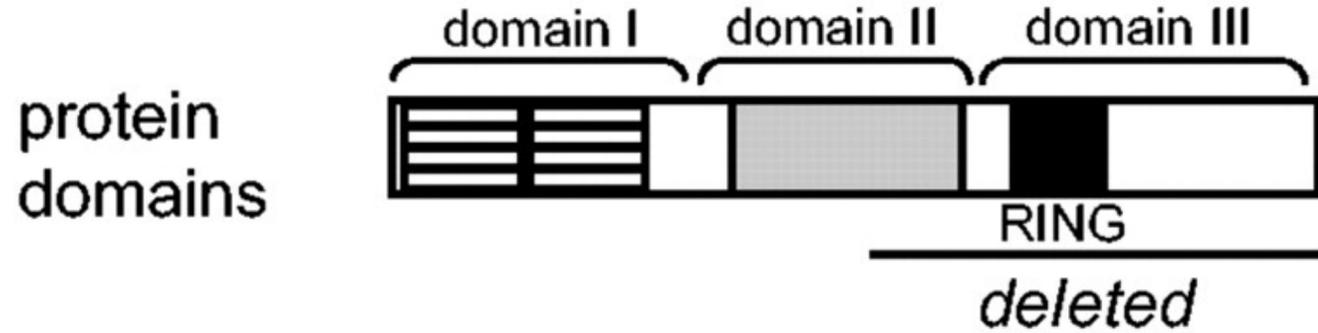
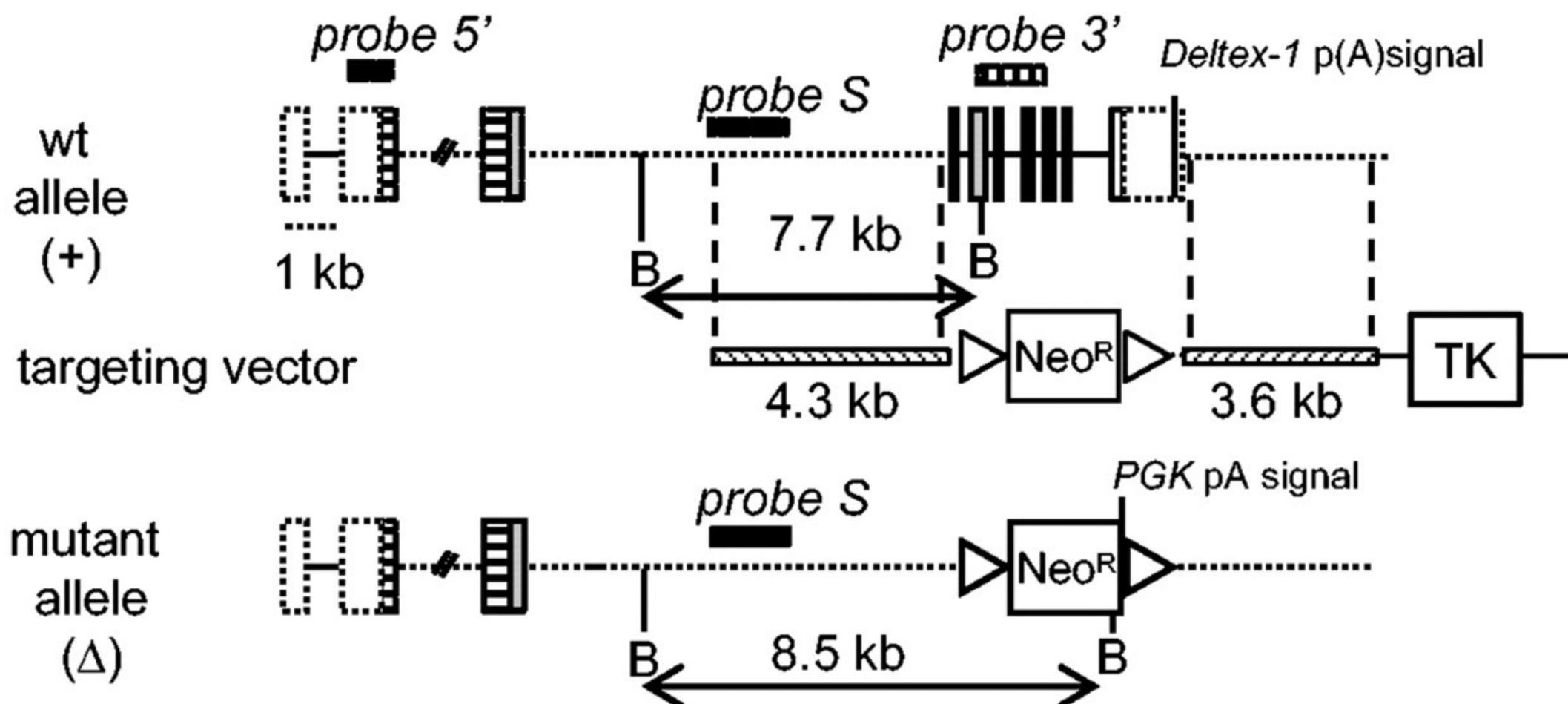
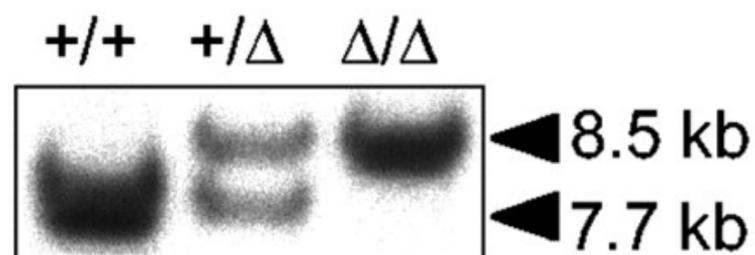
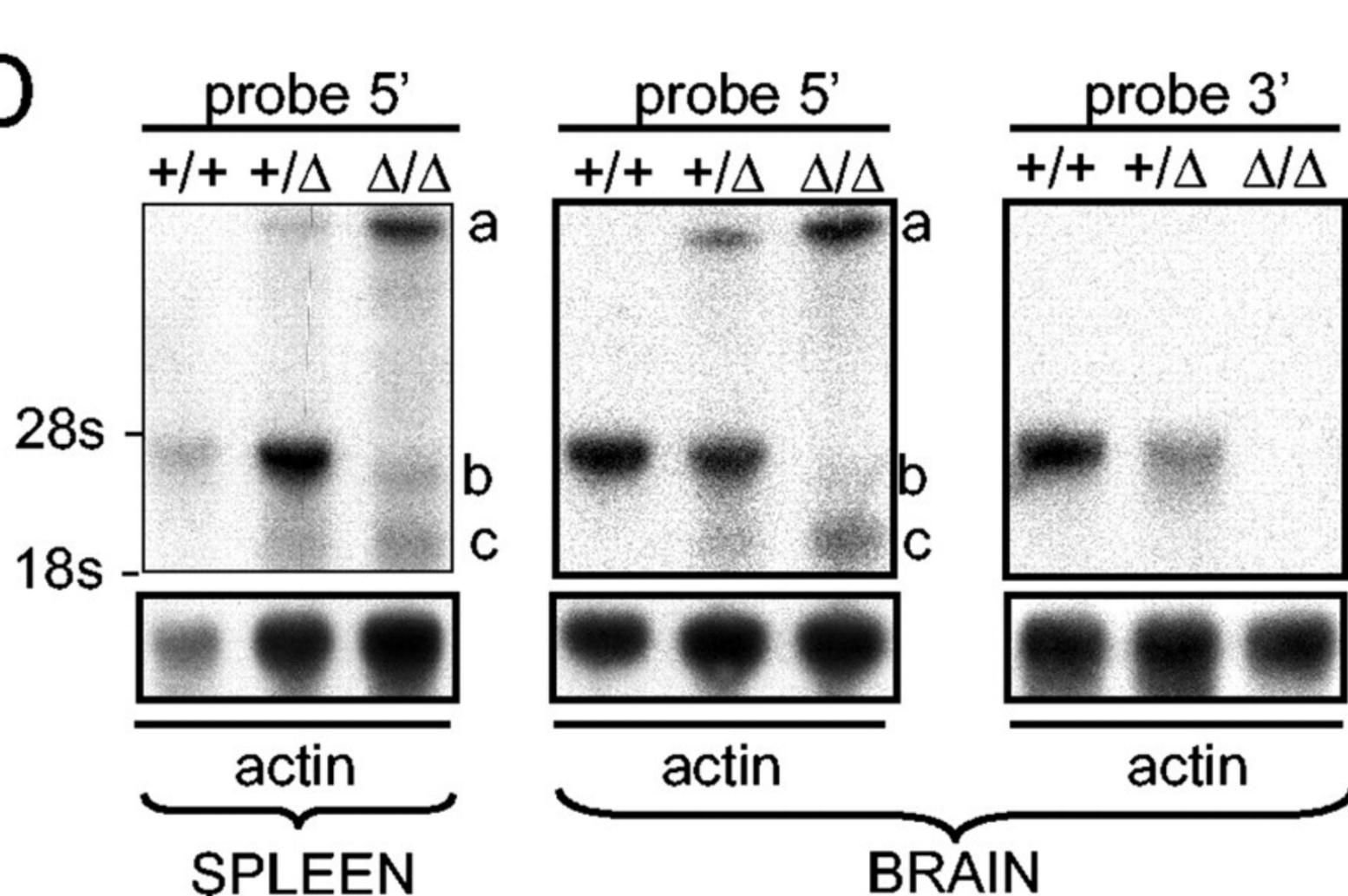
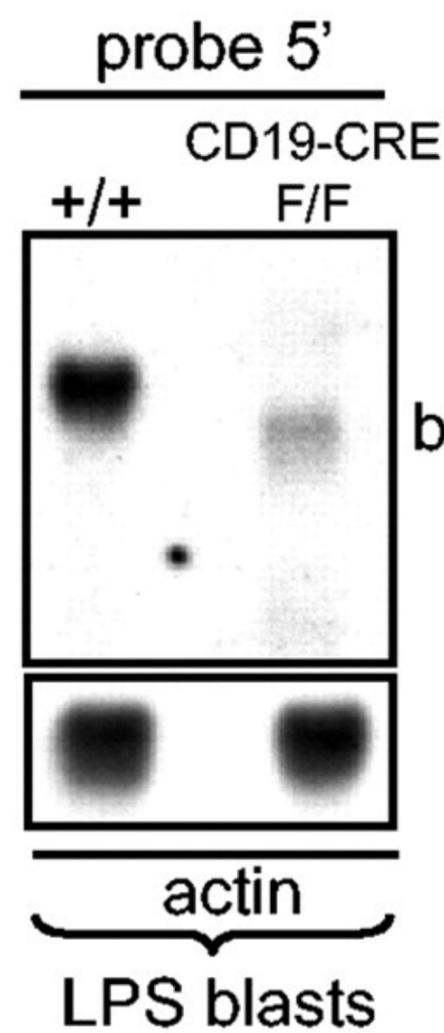
FIG. 4. Deltex-1^{Δ/Δ} have normal basal serum immunoglobulin levels and mount normal humoral responses. The plotted values represent the serum concentrations of each mouse relative to the average concentration of all wild-type mice. (A) The concentrations of the indicated serum immunoglobulin isotypes of 10 Deltex-1^{Δ/Δ} (filled circles) and 10 littermate (open circles) control mice were measured by ELISA. (B) T-independent type-2 response. 7 wild-type and 7 Deltex-1^{Δ/Δ} mice were immunized with TNP-Ficoll and sera were quantified for the presence of TNP-specific antibodies of IgM and IgG3 isotypes by ELISA. (C) T-dependent response. 8 wild-type and 8 Deltex-1^{Δ/Δ} mice were immunized with TNP-KLH and boosted at day 21, as marked by an arrow. Sera were quantified for the presence of TNP-specific antibodies of IgM, IgG1, IgG2a, IgG2b and IgG3 isotypes by ELISA.

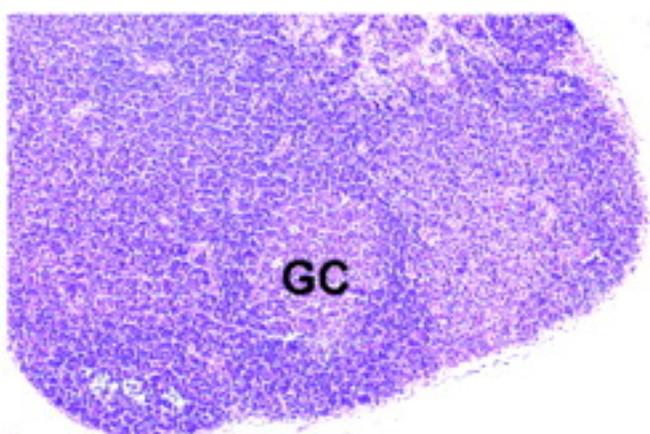
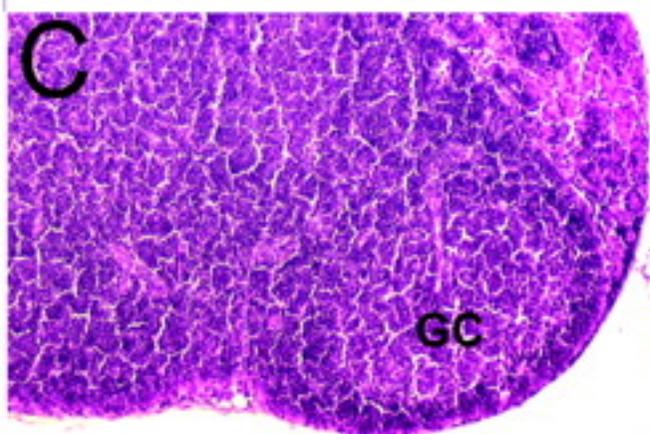
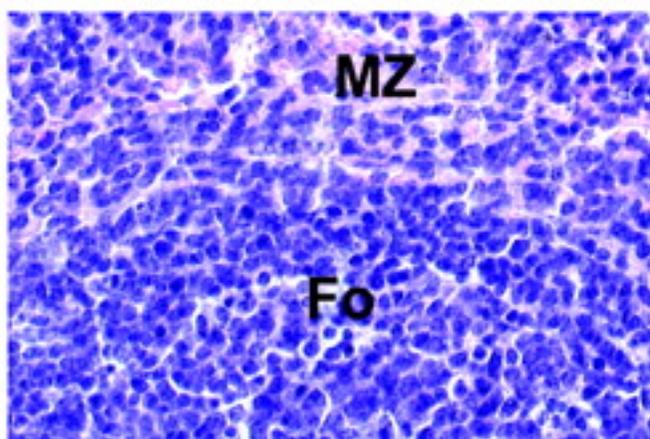
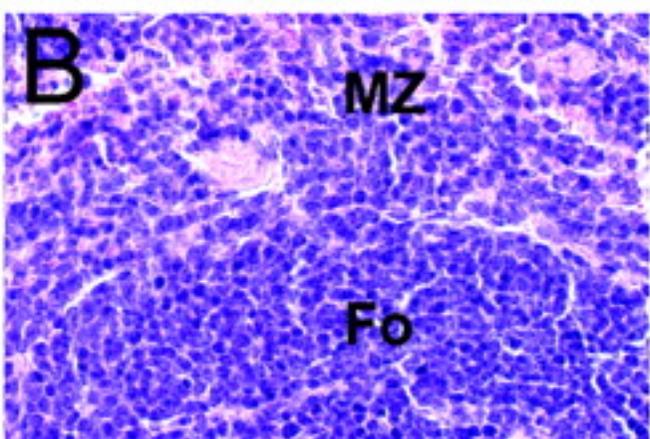
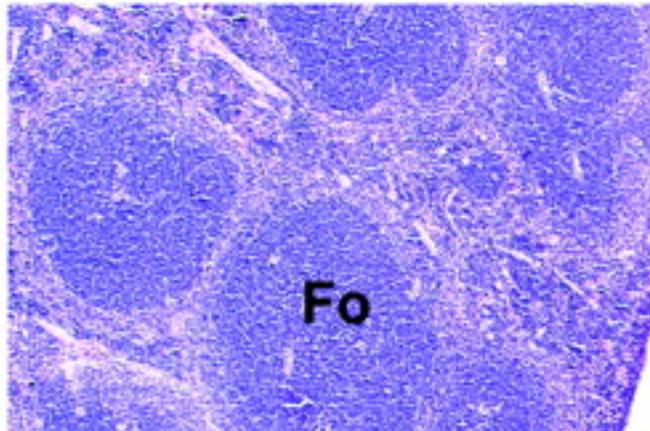
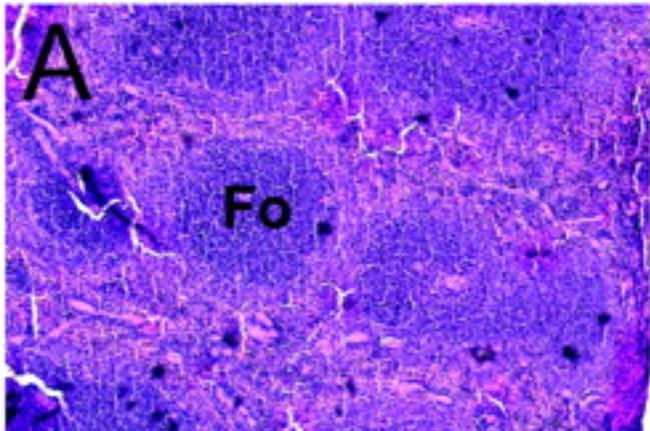
FIG. 5. Deltex-4 protein sequence and transcript expression profile. (A) Comparison of human and mouse Deltex-1 (accession NP_004407 and BAB18939) and Deltex-4 (XP_166213 and AAH58647) proteins performed using Multalin program (3). Conserved amino acids are indicated in bold letters. Shaded and open boxes represent proline rich sequences and RING finger motif, respectively. The dotted line indicates the region deleted in Deltex-1^{Δ/Δ} mice. (B) Two-by-two comparison of mouse Deltex-1 (BAB18939), Deltex-2 (BAB18940), Deltex-3 (BAB18942) and Deltex-4 (AAH58647) proteins performed using BLAST. The number of amino

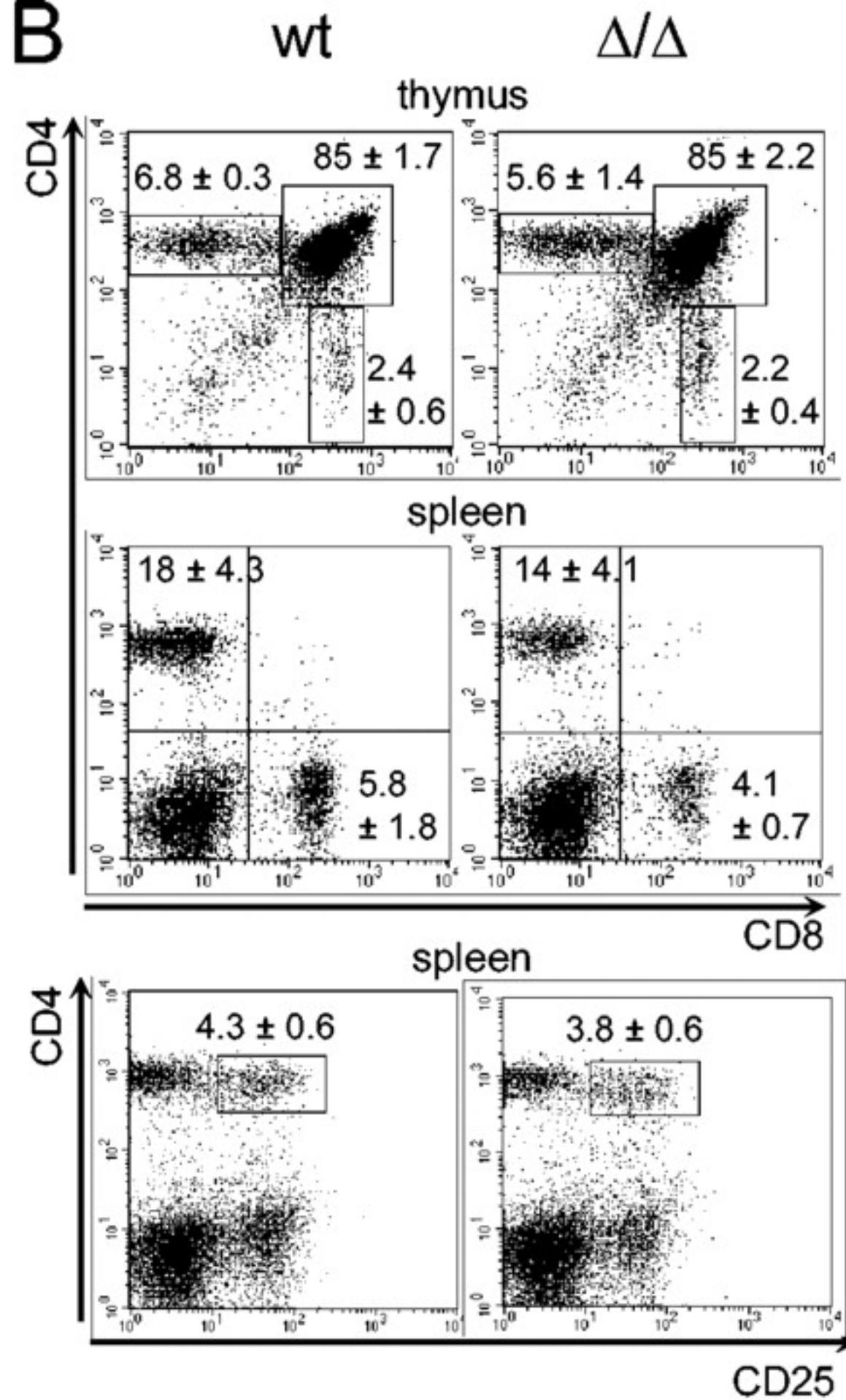
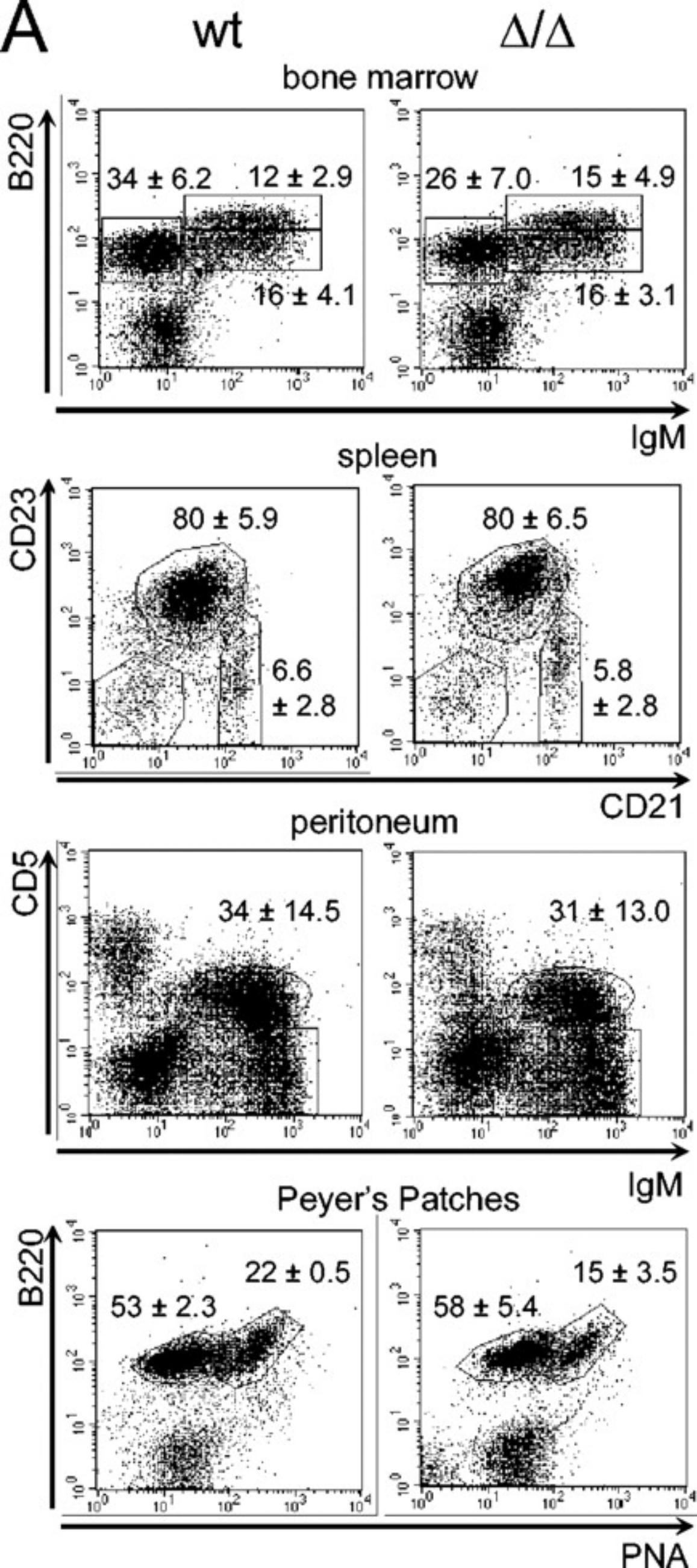
acids and the identity (left) and similarity (right) values are indicated for each pair of proteins.

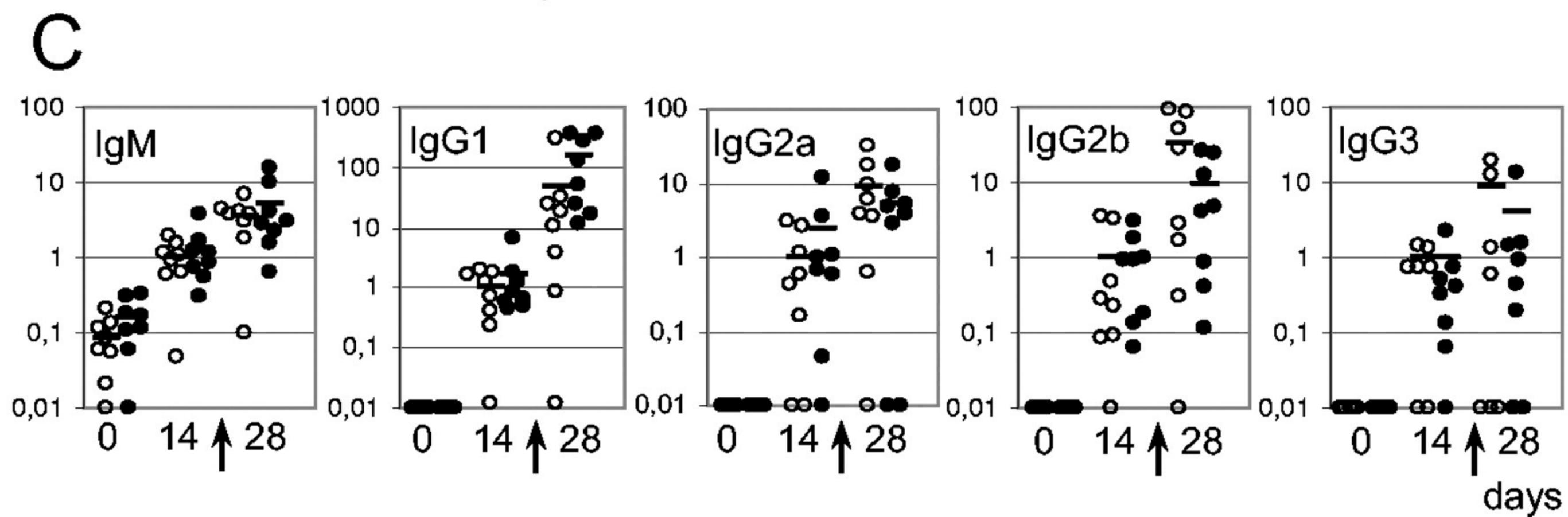
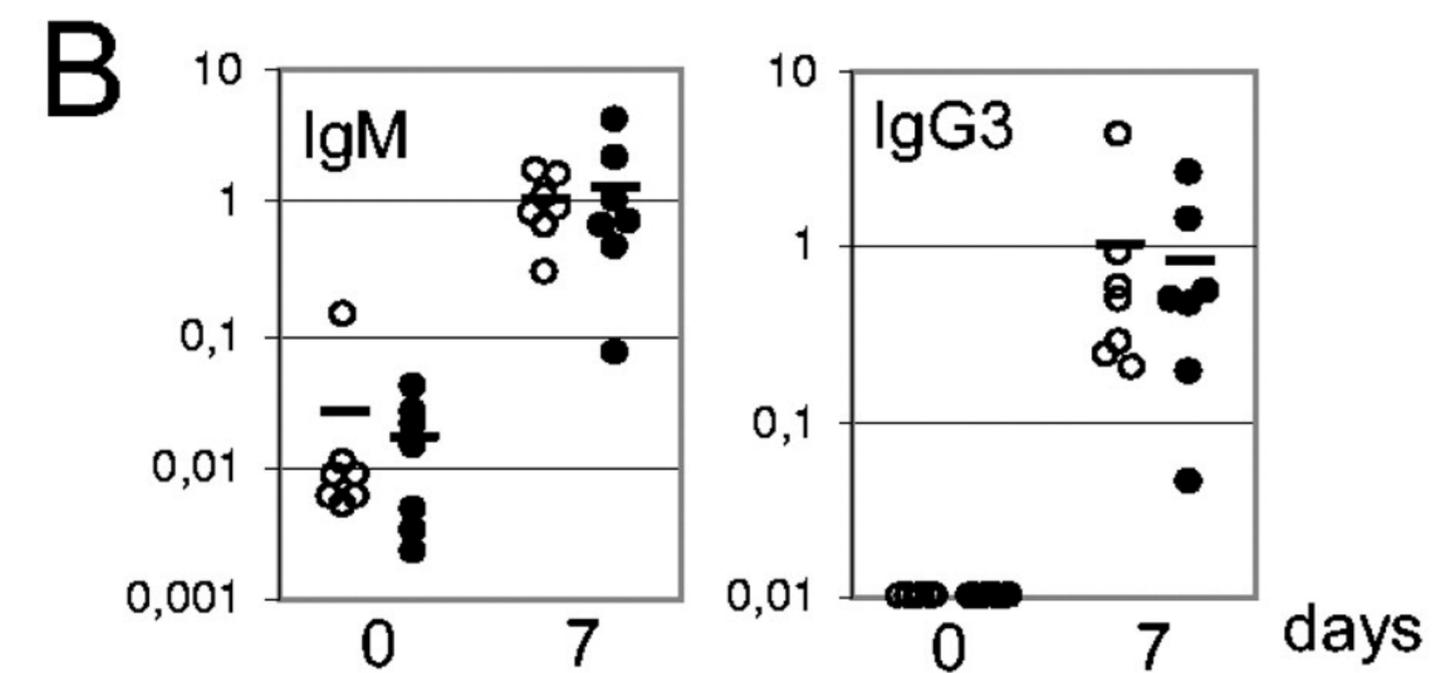
(C) Northern blot analysis of mouse *Deltex-1*, *Deltex-2* and *Deltex-4* expression. Spleen, brain, testis, mouse embryonic fibroblasts (MEF) and LPS-stimulated B cells (LPS-B) were prepared from *Deltex-1*^{+/ Δ} mice. Exposure time : 7 (*Deltex-1*), 5 (*Deltex-2*) and 6 (*Deltex-4*) days.

Normalization was performed using an *actin* probe. (D) Semi-quantitative RT-PCR analysis of *Deltex-1*, *Deltex-2* and *Deltex-4* expression in follicular B cells (FoB : CD19⁺CD21^{int}CD23^{hi}) and marginal zone B cells (MZ-B : CD19⁺CD21^{hi}CD23^{-/lo}). Serial dilutions of reverse transcription products were used. The number of cycles used in each reaction is indicated.

A**B****C****D****E**







A

hDTX_1 MSRPGHGGLMPVN-GLGFPPQNVARVVVWEWLNESHRRWRPYTATVCHHIEN--VLKEDAR-----GSVVLGQVDAQLVPIIIDLQSMHQFRQDTGTMR
 mDTX_1 MSRPQGVMVPVN-GLGFPPQNVARVVVWEWLNESHRRWRPYTATVCHHIEN--VLKEDAR-----GSVVLGQVDAQLVPIIIDLQSMHQFRQDTGTMR
 hDTX_4 -----MLLASAVVVWEWLNESHRRWRPYTATVCHHIEN--VVRAGPRAG-----GSVVLGQVDSRLAPYIIDLQSMHQFRQDTGTLR
 mDTX_4 -----MLLASAVVVWEWLNESHRRWRPYTATVCHHIEN--VVRAGPRAG-----GSVVLGQVDSRLAPYIIDLQSMHQFRQDTGTLR
 hDTX_2 -----MAMAPSPSLVQVYTPAAVAVWEWQDGLGTWHYPYSATVCSFIEQQFVQKQKQRFGLGSLAHSIPLQADPSLAPYIIDLPSWTQFRQDTGTMR
 mDTX_2 -----MAMAPSSSLPQVYPSHVVVAVWEWQDGLGIWHYPYSATVCSFIEQHFVQRQKQHFGLGSLAHSIPLQADPSLAPYIIDLPSWTQFRQDTGTMR
 Consensus VvWEWlnesh rWrPY VchhIE V r gsvvlGQvD L PYIIDLqSm QFRQDTGT R

101
 hDTX-1 FVRRNFYDPSSAPGKGIWWEWENDGGAWTAYDMDICITIONAYEKQHPWLDLSSLGFCYLIYFNMSQMNRRQTRRRRRLRRRLDLAYPLTVGSIPKSSQSW
 mDtx-1 FVRRNFYDPSSAPGKGIWWEWENDGGAWTAYDMDICITIONAYEKQHPWLDLSSLGFCYLIYFNMSQMNRRQTRRRRRLRRRLDLAYPLTVGSIPKSSQSW
 hDTX-4 FVRRNYDPSSAPGKGIWWEWENDNGSWTPYDMEVGITIQAYEKQHPWLDLSSIGFYSYIDFNMGQINRQTRQRRVRRRLDLIYPMVTGTLPKAQSW
 mDtx-4 FVRRNYDPSSAPGKGIWWEWENDNGSWTPYDMEVGITIQAYEKQHPWLDLSSIGFYSYIDFNMGQINRQTRQRRVRRRLDLIYPMVTGTLMPKQSW
 hDTX-2 AVRRHLFPQHSAPGRGVVWEWLDGGSWTAEASVCDYLEQQVARGNQLVDLAPLGYNTTVNYTHTQTNTKSSFCRSVRRQAGPPYPVTIIAPPGHT-
 SVRRHLFSQNSAPGQGIWWEWLDGGSWVAYEARICDYLEQQVARGIQVVDLAPLGYNTTVNYATLTQTNTKSSFCRSVRRQAGPPYPVTSDIAPVRQ-
 Consensus pVRRn ydpsSAPGkG VWEWenD G Wt Ydm itiq ayekqhpw DL s Gf Y i f m Q Nrqt r Rr RRrldl YP g pk qsw

201
 hDTX-1 FVG-----ASSGQPCSCQCCLLVNSTRAASNAILASQRRKAP-----PAPPLPPP-PPGGPPGALAVRPSATFTGAALWAAAPAAGPAEPAP-PPGAPP
 mDtx-1 FVG-----ASSGQPCSCQCCLLVNSTRAASNAILASQRRKAP-----PAPPLPPP-PPGGPPGALAVRPSATFAGAALWAAAPATGTEPAP-PPGVPP
 hDTX-4 FVSPGATSPFMSPCSCPQCCLVMSVKAIV-VNGSTGLQLPVTTRKMPGGVVKLPPLPG--SGAKPLDSTGTIRGPKLTAPSQVIRQASSMPTGTTM
 mDtx-4 FVSPGATSPFMSPCSCPQCCLVMSVKAIV-VHGGTGP---PAVRKHMALSGVGLKLPQPPG--PGAKPLDSTGTIRGPKLTAPSQVIRQVSNAPAGATV
 hDTX-2 -----GVACSCHQCLSGSRTGPVS---GRYRHSMTNLPAYPVPQHPHRTASVFGTHQAFAPYKPKSLSG-ARSAPRLNTTNWGAAPPVSLGS
 mDtx-2 -----GLICFCQCCLLHSGSGTGPVS---GRYRHSMTNLPAYPAPQ-APHRTTTVSGAHQAFAPYKPKSLSG-ARSAPRLNTTNWAAAPPVAGN
 Consensus pv pCaC QC lv s aa p p pg gA t G A Pg

301
 hDTX-1 RSPGAPGGARTPGQN----NLNRPGPQRTTSVSARASIPPGVPALPVKLNLTGTPVHPALAGMTGILLCAAGLPVCLTRAPKPI LHPPVSKSDVKPVPG
 mDtx-1 RSPSAPNGAPTQGN----NLNRPGPQRTTSVSARASIPPGVPALPVKLNLTGTPVHPALAGMTGILLCAAGLPVCLTRAPKPI LHPPVSKSDVKPVPG
 hDTX-4 GSPASPPGPNKTRVALATLNRLNLQRLAIAQSRVLIASGVPTVPVKNLNGSSPVNPALAGITGILMSAAGLPVCLTRPKLVLHPPVSKSEIKSIPG
 mDtx-4 GSPTSPPGPNKTRVALATLNRLNLQRLAIAQSRVLIASGVPTVPVKNLNGSSPVNPALAGITGILMSAAGLPVCLTRPKLVLHPPVSKSEIKSIPG
 hDTX-2 QPLYRSSLSHLGPQLLPPGSSSTSGAVSASLPSGPPSS--PGSVFATVPMQMPKPSRVQALAGMTSVLMSAIGLPVCLSRAPQPT--SPPASRLASKSHGS
 mDtx-2 QSLFHSSLSHLGPQLLPPGSSSTSGASASFPSPSSSSPGSAPTTVPVQMPKASRVQALAGMTSVLS-AIGLPVCLSRAPPT--GPPASRPASKSHSS
 Consensus sp p g l r qrs r i gvP pvknlng pV pALAG TgiL AaGLPVCLtR Pk lhpPvSks K pg

401
 hDTX-1 VPGVCRKTKKKHLKSK-NPEDVVRRYMOKVKNPPDEDCICMERLVTASGYEGVLRHKGVRPELVGRLGFCRHMVHLLICLVAMYSNGNKDGSLOCPTCK
 mDtx-1 VPGVCRKTKKKHLKSK-NPEDVVRRYMOKVKNPPDEDCICMERLVTASGYEGVLRNKSVRPELVGRLGFCRHMVHLLICLVAMYSNGNKDGSLOCPTCK
 hDTX-4 VSNTSRKTTKKQAKKKGK-TPEVLKYLQKVRHPPDEDCICMERLVTASGYEGVLRNKSVRPELVGRLGFCRHMVHLLICLVAMYSNGNKDGSLOCPTCK
 mDtx-4 VSNTSRKTTKKQAKKKGK-TPEVLKYLQKVRHPPDEDCICMERLVTASGYEGVLRNKSVRPELVGRLGFCRHMVHLLICLVAMYSNGNKDGSLOCPTCK
 hDTX-2 VKRLRKMSVKGATPKPEPEPEQVIRKYTEELKVPPDEDCICMEKLSAASGYSDVTDSDKAIGSLAVGHLTHCERHLLICLLAMYCNGNKDGSLOCPTCK
 mDtx-2 VKRLRKMSVKGATPKPEPEPEQVIRKYTEELKVPPDEDCICMEKLSAASGYSDVTDSDKAIGSLAVGHLTHCERHLLICLLAMYCNGNKDGSLOCPTCK
 Consensus V rkt Kk kk k PE V Y qkv pPdEDCtICMERL SGY g v p lVG L rCgH yH CLvAMY NGNKDGSLOCPTCK

501
 hDTX-1 AIYGEKGTQPPGKMEFHLIPHSLPGFPDQTIRIVYDIPTGIQGPENPNPGKFTARGFPRHCYLPNNEKGRKVLRLLI TAWERRLIFTIGTSNTTGES
 mDtx-1 AIYGEKGTQPPGKMEFHLIPHSLPGFPDQTIRIVYDIPTGIQGPENPNPGKFTARGFPRHCYLPNNEKGRKVLRLLI TAWERRLIFTIGTSNTTGES
 hDTX-4 TIYGKGTQPPGKMEYHLIPHSLPGHPDCKTIRIYISIPPGIQQPEHPNPGKFSARGFPRHCYLPDSEKGRKVLKLLVAVDRRLIFAIGTSSTTGES
 mDtx-4 TIYGKGTQPPGKMEYHLIPHSLPGHPDCKTIRIYISIPPGIQQPEHPNPGKFSARGFPRHCYLPDSEKGRKVLKLLVAVDRRLIFAIGTSSTTGES
 hDTX-2 TIYGEKGTQPPGKMEVLRFQMSLPGHEDCGTILIVYSIPHGIQGPENPNPGKFTARGFPRHCYLPDSEKGRKVLKLLVAVDRRLIFAIGTSSTTGET
 mDtx-2 TIYGEKGTQPPGKMEVLRFQMSLPGHEDCGTILIVYNIPHGIQGPENPNPGKFTARGFPRHCYLPDSEKGRKVLKLLVAVDRRLIFAIGTSSTTGET
 Consensus IYG KTGTQPPGKME hliPhSLPG D TirI Y IP GIQGPENpNPGK F ARGFPRHCYLP ekGRKVL LL AW RRLIF iGTSsTTGES

601
 hDTX-1 NNEKGRKVLRLLI TAWERRLIFTIGTSNTTGESDTVWNEIHHKTEFGSNTLGHGYPDASYLQNVLAELTAQGVSEAAAKA---
 mDtx-1 NNEKGRKVLRLLI TAWERRLIFTIGTSNTTGESDTVWNEIHHKTEFGSNTLGHGYPDASYLQNVLAELTAQGVSEAMAKA---
 hDTX-4 DSEKGRKVLKLLVAVDRRLIFAIGTSSTTGESDTVWNEVHHKTEFGSNTLGHGYPDANYLQNVLAELAAQGVSEDSSTAQEKD
 mDtx-4 DSEKGRKVLKLLVAVDRRLIFAIGTSSTTGESDTVWNEVHHKTEFGSNTLGHGYPDANYLQNVLAELAAQGVSEDSSTSHKED
 hDTX-2 DNAQGRKVLKLLVAVDRRLIFAIGTSSTTGETDTVWNEIHHKTEMDRNI TGHGYPDPNYLQNVLAELAAQGVTEDCLEQQ--
 mDtx-2 DSPQGRKVLKLLVAVDRRLIFAIGTSSTTGETDTVWNEIHHKTEMDRNV TGHGYPDPNYLQNVLAELAAQGVTEDCLEQQ--
 Consensus eKGRKVL LL AW RRLIF iGTSsTTGESDTV WNE HHKTEfgsNlTGHGYPDa YLdNVLAEL AqG sE

B