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Mutation in ankyrin repeats of the mouse *Notch2* gene induces early embryonic lethality

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SUMMARY

***Notch* family genes encode transmembrane proteins involved in cell-fate determination. Using gene targeting procedures, we disrupted the mouse *Notch2* gene by replacing all but one of the ankyrin repeat sequences in the cytoplasmic domain with the *E. coli* β-galactosidase gene. The mutant *Notch2* gene encodes a 380 kDa Notch2-β-gal fusion protein with β-galactosidase activity. *Notch2* homozygous mutant mice die prior to embryonic day 11.5, whereas heterozygotes show no apparent abnormalities and are fully viable. Analysis of *Notch2* expression patterns, revealed by X-gal staining, demonstrated that the *Notch2* gene is expressed in a wide variety of tissues including neuroepithelia, somites, optic vesicles, otic vesicles, and branchial arches, but not heart. Histological studies, including in situ nick end labeling procedures, showed earlier onset and higher incidence of apoptosis in homozygous mutant mice than in heterozygotes or wild type mice. Dying cells were particularly evident in neural tissues, where they were seen as early as embryonic day 9.5 in *Notch2*-deficient mice. Cells from *Notch2* mutant mice attach and grow normally in culture, demonstrating that *Notch2* deficiency does not interfere with cell proliferation and that expression of the Notch2-β-gal fusion protein is not toxic *per se*. In contrast to *Notch1*-deficient mice, *Notch2* mutant mice did not show disorganized somitogenesis, nor did they fail to properly regulate the expression of neurogenic genes such as *Hes-5* or *Mash1*. In situ hybridization studies show no indication of altered *Notch1* expression patterns in *Notch2* mutant mice. The results indicate that *Notch2* plays an essential role in postimplantation development in mice, probably in some aspect of cell specification and/or differentiation, and that the ankyrin repeats are indispensable for its function.**

Key words: Notch2, Targeting, β-galactosidase, Gene expression, Cell death, Mouse

INTRODUCTION

Notch was initially described as a gene involved in determining cell fate in the neurogenic region of the developing *Drosophila* embryo. Subsequent studies in *Drosophila* have demonstrated that Notch is involved in determination of various types of cell lineages. The biological activity of Notch appears to be to maintain precursor cells in an undifferentiated state until they are competent to respond to compelling inductive cues (for review see Artavanis-Tsakonas et al., 1995; Robey, 1997; Lendahl, 1998; Weinmaster, 1998).

Mammals are different from *Drosophila* in having multiple Notch homologues, *Notch1*, *Notch2*, *Notch3* (Ellisen et al., 1991; Weinmaster et al., 1991, 1992; Franco del amo et al., 1992, 1993; Reaume et al., 1992; Kopan and Weintraub, 1993; Lardelli and Lendahl, 1993; Lardelli et al., 1994) and a distantly related *Notch4* (Robbins et al., 1992; Uyttendaele et al., 1996). All *Notch* genes encode large cell-surface receptors with several conserved domains (see Fig. 1A), including extracellular tandemly repeated copies of an epidermal growth factor (EGF)-like motif involved in ligand binding, repeats of another cystein-rich motif (LNR) with unknown function in the extracellular domain and CDC10/ankyrin-like repeats in the cytoplasmic domain. It has been reported that two proteins involved in mediating signal transduction, Suppressor of Hairless (Su(H)) and Deltex, bind to the ankyrin repeats, suggesting an important role of the latter in intracellular Notch signaling (for review see Artavanis-Tsakonas et al., 1995; Robey, 1997; Lendahl, 1998; Weinmaster, 1998). Su(H) (also known as RBP-Jκ and CBF-1) has also been reported to bind a novel short sequence termed RAM located between the transmembrane domain and the ankyrin repeats (Tamura et al., 1995; Honjo, 1996).

As in *Drosophila*, mammalian Notch proteins appear to play an important role in preventing cell differentiation in a wide variety of cell lineages. When the cytoplasmic domain of *Notch1* from mouse, human and *Xenopus* is overexpressed or ectopically

expressed it seems to act constitutively in preventing differentiation of myogenic or neurogenic cells in culture (Kopan et al., 1994; Nye et al., 1994; Lindsell et al., 1995), causing hypertrophy of neural and muscle tissue in vivo (Coffman et al., 1993), inducing human T-lymphoma (Ellisen et al., 1991) and mammary tumor development in mice (Robbins et al., 1992), and perturbing retinal development in *Xenopus* (Dorsky et al., 1995).

Studies on the expression of mammalian *Notch* genes showed complementary and combinatorial patterns during development, suggesting distinct functions for different members of the family (Weinmaster et al., 1992; Higuchi et al., 1995; Swiatek et al., 1994; Williams et al., 1995). Except for disorganized somitogenesis, targeted *Notch1*-deficient mice follow apparently normal development through the first 9 embryonic days, then die in utero (Swiatek et al., 1994, Conlon et al., 1995), suggesting that *Notch1* plays an important developmental role in various tissues. No such information has been reported for *Notch2* and *Notch3*. To study the developmental function(s) and expression patterns of *Notch2* in mice, we generated a mutation by replacing 5 of the 6 ankyrin repeats and part of the downstream sequence of the *Notch2* gene with the *E. coli* β-galactosidase gene. Here we show that *Notch2* mutant mice develop normally up to embryonic day (E) 9.5 and die prior to E11.5 with widely distributed, massive cell death. The onset of widespread cell death starts as early as E9.5 and is due to *Notch2* deficiency, not to expression of the introduced β-galactosidase gene. These results demonstrate that *Notch2* expression is indispensable for postimplantation development in mice.

MATERIALS AND METHODS

Construction of *Notch2* targeting vector

A 3.7 kb *Nco*I-*Eco*RI fragment coding for *trp-lacZ*, a 1.0 kb *Eco*RI fragment containing the polyA addition signal derived from SV40 DNA, and a 1.3 kb *Eco*RI-*Bam*HI fragment containing both the phosphoglycerate kinase gene promoter (pgk promoter) and the neomycin phosphotransferase gene (*neo*) (Yagi et al., 1993) were used for construction of the *Notch2* targeting vector (Fig. 1D). The *Nco*I site in the 3.7 kb fragment and the *Bam*HI site of the 1.3 kb fragment were converted into blunt ends with mung bean nuclease and T4 DNA polymerase, respectively.

A phage clone λNT2-2 carrying mouse genomic *Notch2* sequences (Fig. 1C) was isolated from a λEMBL3 Balb/c genomic DNA library. A *Xho*I-*Acc*I fragment of 1.3 kb and a 2.0 kb *Xho*I-*Sal*I fragment were isolated from λNT2-2, and the *Acc*I site and *Xho*I site of the 2.0 kb fragment were converted into blunt ends with mung bean nuclease and T4 DNA polymerase, respectively. The 4.0 kb *Eco*RI fragment which extends from the 3′ end of *Notch2* DNA into λNT2-2 was isolated from a λZAP *Eco*RI partial genomic library made from Balb/c genomic DNA.

To generate pYH, DNA fragments were cloned between the *Sal*I and *Xho*I sites of Bluescript (Stratagene) in the following order: the 1.3 kb *Xho*I-*Acc*I *Notch2* genomic fragment, *trp-lacZ*, a polyA signal sequence, pgk promoter-neo and the *Xho*I-*Sal*I *Notch2* fragment. To lengthen the 5′ *Notch2* sequence in the targeting vector, the XLI Blue bacteria harboring the plasmid pYH were infected by λNT2-2 in a liquid culture. Resulting phage were screened with a ^{32}P-labelled *neo* gene to isolate λLNNT2-2 (Fig. 1D). A *Sal*I digest of λLNNT2-2 DNA was ligated with pMCIDT-A, a vector encoding fragment A of diphtheria toxin (Yagi et al., 1993), linearized with *Sal*I. The final targeting vector was linearized with *Not*I before electroporation into embryonic stem cells. All enzymes used for the construction of the targeting vector were purchased from New England Biolabs.

DNA transfection of ES cells

Culture of an embryonic stem cell line, R1 (Nagy, 1993) with feeder cells and electroporation were carried out as described by Wurst and Joyner (1991). Electroporated ES cells (1×10^6) were inoculated into 6 cm diameter dishes. Two days later, the medium was replaced with selection medium containing 0.4 mg/ml G418. After 8-10 days, G418 resistant transformants were isolated and maintained in the same medium containing a 5 times lower concentration of G418. Chromosome counts on transfected ES cell clones were performed as described by Doyle et al. (1996).

Genotyping ES cell lines and mice by Southern blot analysis and PCR

DNA from G418-resistant transformants, tail biopsy samples, yolk sacs from embryos at E9.5-14.5 and whole embryos at E8.5, was prepared with lysis buffer [10 mM Tris-HCl (pH7.5), 100 mM NaCl, 10 mM EDTA, 0.1% SDS, 40 mg/ml proteinase K]. Isolated DNA (20 μg each) was digested with *Eco*RI, electrophoresed on 1% agarose gels and subjected to Southern blot analysis. PCR was carried out for 30 cycles at 94°C for 30 seconds and 70°C for 2.5 minutes and the product electrophoresed on a 1.5% agarose gel. PCR primers for the targeted allele were from the *neo* gene (5′-GTGCCCTGAATGAACTGCAG-3′) and a *Notch2*-reverse genomic sequence downstream from the *Xho*I site (5′-GAGACATACGTGTGAGGAGACTCGA-3′). The third primer for the wild-type was a *Notch2*-forward sequence (5′-TTTGCCAACC-GGGACATCAC-3′) derived from a genomic region upstream of the *Sma*I site.

Mice

Blastocysts were recovered from pregnant C57BL/6 females, injected with transfected ES cells and then re-implanted into pseudopregnant C57BL/6 mice. Chimeric mice were mated with C57BL/6. Germ line transmission was recognized by the agouti coat color of the progeny.

Western blot analysis

Embryos were homogenized in sample buffer containing 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS and 62.5 mM Tris-HCl (pH 6.8). Protein samples were separated on SDS-polyacrylamide (5%) gels and blotted onto nitrocellulose membranes. Notch2-β-galactosidase fusion protein was detected using anti-β-galactosidase monoclonal antibody (DSHB, Iowa) and alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma).

β-galactosidase assay

Whole embryos at the indicated days of gestation were fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS) for 2 hours at 4°C and washed with PBS. They were stained in X-gal reaction solution containing 84 mM sodium phosphate (pH 7.5), 8.4 mM KCl, 1 mM $MgCl_2$, 3 mM $K_4[Fe(CN)_6]$, 3 mM $K_3[Fe(CN)_6]$, 0.1% Triton X-100 and 1 mM X-gal for 2 hours at 37°C. Cell suspensions prepared from embryos and cultures by trypsinization were fixed in 1% glutaraldehyde for 10 minutes at room temperature, then washed and incubated with X-gal as described above. Reactions were stopped by adding 10 μl of 0.25 M EDTA. More than 1000 cells were counted to estimate the population size of β-galactosidase-positive cells.

Histology

For histological study, whole embryos fixed as described above were embedded in paraffin after staining with X-gal. Sections were cut at 6 μm thickness and stained with hematoxylin-eosin or nuclear fast red. In some samples, apoptotic cells were detected by an in situ nick end labeling (ISNEL) technique described previously (Tanaka et al., 1995), which was a modification of the TUNEL procedure of Gavrieli et al. (1992).

In situ hybridization

Whole-mount in situ hybridization was carried out according to

published procedures (Wilkinson and Nieto, 1993). The hybridization probes used included the 5′ region of *Notch1* and *Notch2* cDNA (Higuchi et al., 1995), a *Sac*I-*Eco*RV (825 bp) fragment of the *lacZ* gene, and full length cDNAs for *HES-1,-3,-5, MASH1, neurogenin-1* and *Pax1*.

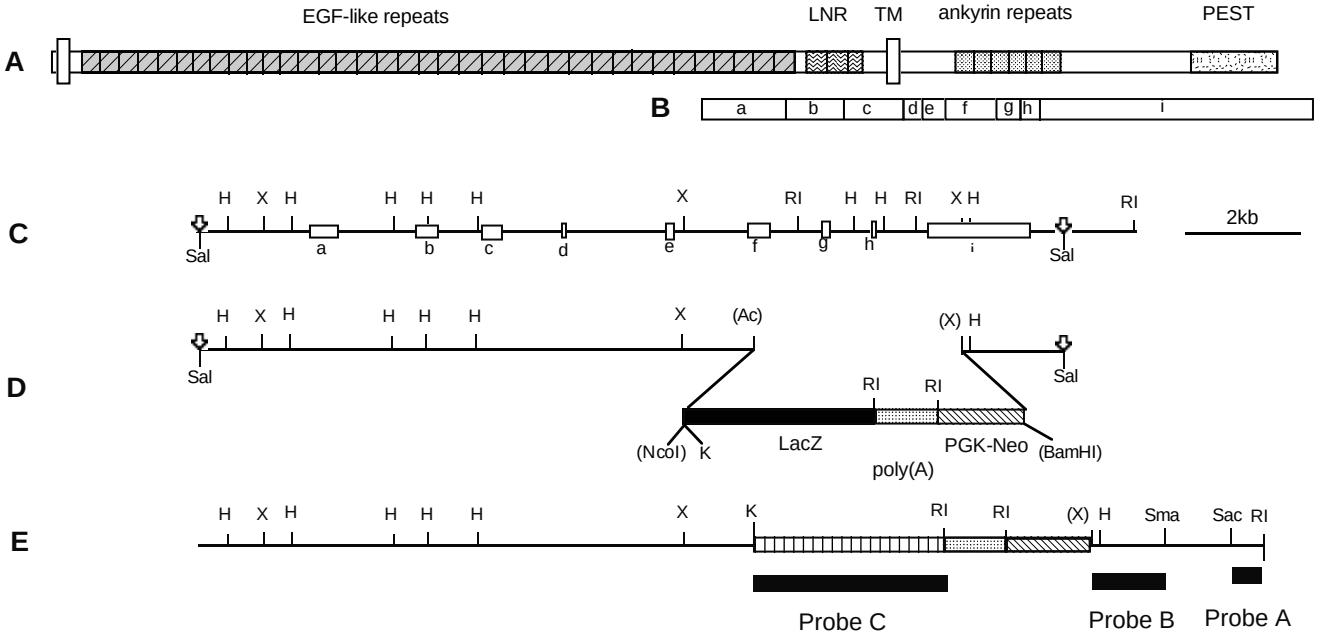
Primary cell culture

Embryos at E9.5 were incubated in 50 μl trypsin-EDTA solution (Gibco BRL) for 30 minutes at 37°C, 10 volumes of culture medium consisting of 90% Dulbecco's modified Eagle medium (Nissui, Co. Tokyo) and 10% fetal bovine serum (JRH Bioscience, KS) were added to stop the digestion and the cells were dissociated by pipetting. Each cell suspension was divided into 5 aliquots, inoculated into a 96-well culture dish and incubated at 37°C in a 5% CO_2 incubator. After 2 hours, when cells had attached, the inoculation medium was replaced with 300 μl culture medium. Subsequently the medium was changed every day. Cell number was counted with a hemocytometer after dispersing with trypsin-EDTA solution at the indicated days.

RESULTS

Genomic disruption of *Notch2* by gene targeting

Functional analyses of *Drosophila Notch* and the vertebrate *Notch1* gene suggest that the ankyrin repeat region in the cytoplasmic domain plays an important role in Notch signaling. Although the entire genomic organization of the *Notch2* gene is not known, it does possess an ankyrin repeat region (Fig. 1A). To construct a vector that would allow us to determine whether destruction of the repeats would result in abolition of Notch2 signaling, we inserted a genomic sequence comprising the distal 9 exons of the *Notch2* gene into a recombinant λphage clone (Fig. 1B). We replaced a genomic fragment encoding the amino acid sequence from the second (within exon f) to beyond the sixth (within exon i) ankyrin repeat of *Notch2*, such that a Notch2-β-galactosidase fusion protein could be generated (Fig. 1D). This allows not only



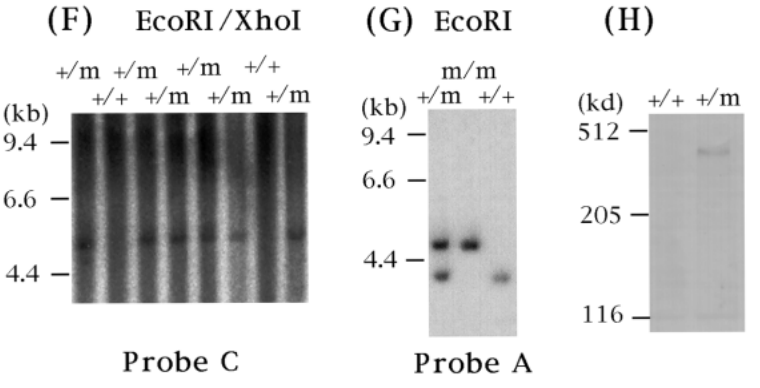


Fig. 1. Disruption of the *Notch2* gene. (A) Schematic diagram of the Notch2 protein. (B) Nine *Notch2* exons (a-i) are accomodated in recombinant phage, λNT2-2. Exons are aligned to correspond to the Notch protein shown in A. (C) Genomic organization of the *Notch2* gene. Exons are shown as open boxes and restriction sites by vertical lines. Open arrowheads indicate the 5′ and 3′ end of λNT2-2. (D) Structure of the *Notch2* targeting vector. (E) Predicted structure of the disrupted *Notch2* gene. Restriction endonucleases sites are indicated as follows: RI, *Eco*RI; X, *Xho*I; H, *Hin*dIII; K, *Kpn*I; Sac; *Sac*I; Sma, *Sma*I; Sal, *Sal*I. (X), (Ac), (NcoI) and (BamHI) indicate destroyed *Xho*I, *Acc*I, *Nco*I and *Bam*HI sites, respectively. Three probes, A (0.5 kb *Sac*I-*Eco*RI), B (1.4 kb *Xho*I-*Sma*I) and C (3.0 kb *Kpn*I-*Eco*RI) used for Southern blot analysis are also shown. (F) Southern blot analysis of offspring obtained from crossing a *Notch2*$^{+/m}$ male with a wild-type C57BL/6 female. Genomic DNA was extracted from tails, digested with *Eco*RI and *Xho*I, electrophoresed, blotted and then hybridized with ^{32}P-labeled Probe C as indicated. (G) Southern blot of +/+, +/m and m/m E9.5 embryos derived from crossing heterozygous *Notch2* mutant mice (*Notch2*$^{+/m}$ × *Notch2*$^{+/m}$). Yolk sac DNA was digested with *Eco*RI and then hybridized with Probe A. (H) Western blot analysis of the Notch2-β-galactosidase fusion protein. *Notch2*$^{+/+}$ and $^{+/m}$ embryos were obtained at E10.5. Genotypes of embryos were determined by Southern blot analysis of respective yolk sac DNAs. Proteins from the embryos were subjected to western blot analysis with anti-β-galactosidase antibody. Molecular weight of the fusion protein was calculated from molecular weight markers (sea urchin dynein heavy chain, myosin heavy chain and *E. coli* β-galactosidase).

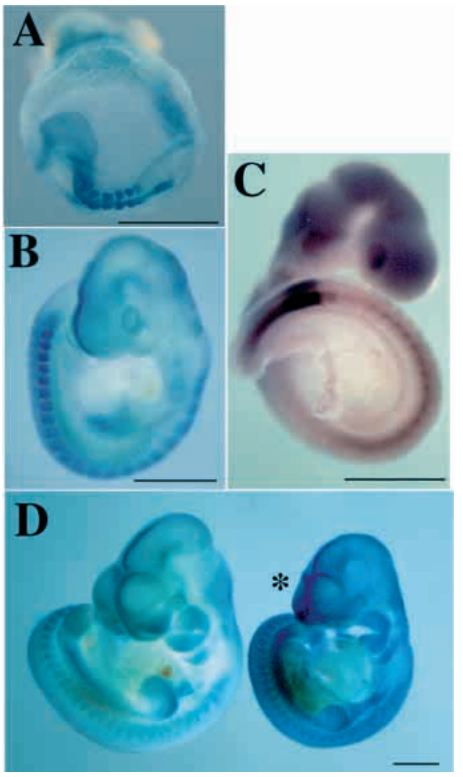


Fig. 2. Expression of the Notch2-lacZ fusion protein and the *Notch2* gene. *Notch2*$^{m/m,}$ *Notch*2$^{+/m}$ and *Notch2*$^{+/+}$ embryos were isolated from heterozygous crosses. After fixation, embryos were incubated in X-gal reaction solution for detection of the fusion protein or analyzed by in situ hybridization for *Notch2* mRNA as described in Materials and Methods. (A) Lateral view of a *Notch2*$^{+/m}$ embryo at E8.5 stained with X-gal. The fusion protein was strongly detected in somites, allantois, ventral-most neural groove and primitive streak. (B) A *Notch2*$^{+/m}$ embryo at E9.5 stained with X-gal. The fusion protein was strongly expressed in somites, and in the roof plate and floor plate of brain and spinal cord. Fusion protein expression also occurred in optic vesicles, otic vesicles and whole brain but not in the heart. Developmental retardation was not observed in *Notch2*$^{m/m}$ embryos until after E9.5. (C) Whole-mount in situ hybridization of *Notch2* RNA in a *Notch2*$^{+/m}$ embryo at E9.5, using a *Notch2*-specific riboprobe. Note that strong expression was observed in presomitic mesoderm but not in newly formed somites. Compare with the fusion protein expression pattern in B. (D) Comparison of *Notch2*$^{+/m}$ (left) and *Notch2*$^{m/m}$ (right) embryos stained with X-gal at E10.5. Fusion protein expression was pronounced in the midbrain roof plate, and was also observed in branchial arches, limb buds and somites. Developmental retardation is apparent in the *Notch2*$^{m/m}$ embryo, especially in the telencephalon (asterisk). Scale bar, indicate 1 mm.

evaluation of the function of the ankyrin repeats in Notch signaling but also permits examination of the expression pattern of *Notch2* during mouse development by assessing β-galactosidase activity. We screened nearly 250 G418-resistant embryonic stem (ES) cell clones for disruption of the *Notch2* gene by Southern blot analysis**,** using a 0.5 kb *Sac*I-*Eco*RI genomic *Notch2* fragment downstream of the inserted sequence as a probe (Probe A in Fig. 1E). Targeted disruption

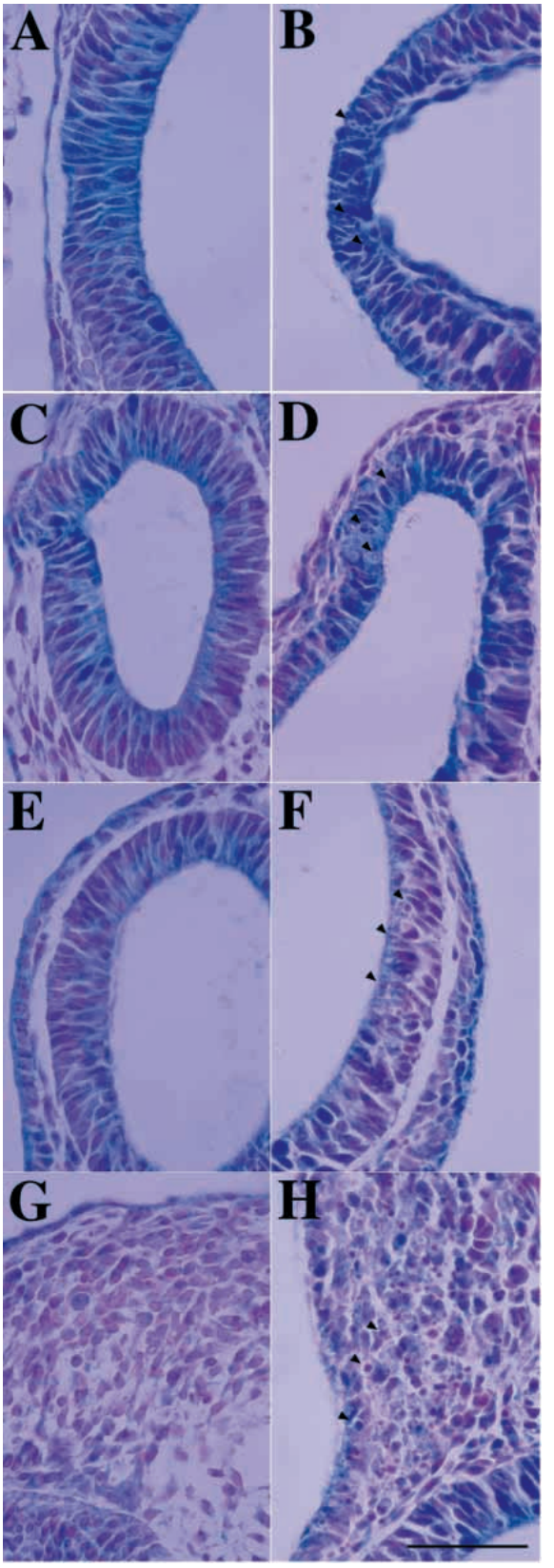


Fig. 3. Histological comparison of *Notch2*$^{+/m}$ and *Notch*2$^{m/m}$ embryos at E9.5. (A,B) Telencephalon. (C,D) Otic vesicles. (E,F) Optic vesicles. (G,H) Cranial ganglia. Pycnotic cells (arrowheads) were readily observable in neuroepithelia of the telencephalon, otic vesicles and optic vesicles, and in cranial ganglia of E9.5 *Notch2*$^{m/m}$ embryos (B,D,F,H), but were rarely seen in *Notch2*$^{+/m}$ embryos (A,C,E,G). Scale bar, 50 μm.

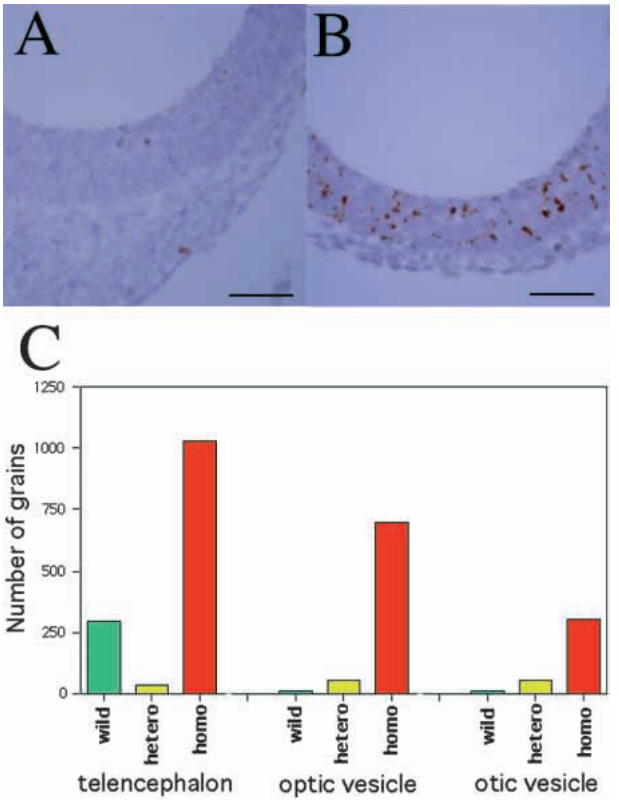


Fig. 4. Apoptotic cell death was enhanced in *Notch2*$^{m/m}$ embryos compared to *Notch2*$^{+/m}$ and *Notch2*$^{+/+}$embryos at E9.5. (A,B) ISNEL staining of telencephalic region of (A) *Notch2*$^{+/m}$ and (B) *Notch2*$^{m/m}$ embryos. Bars indicate 50 μm. (C) ISNEL-positive grain counts in sections of telencephalon, optic vesicles and otic vesicles of *Notch2*$^{+/+}$, *Notch2*$^{+/m}$ and *Notch2*$^{m/m}$ embryos at E9.5. Numbers represent cumulative grain counts on ten serial paraffin sections totalling 60 μm thickness.

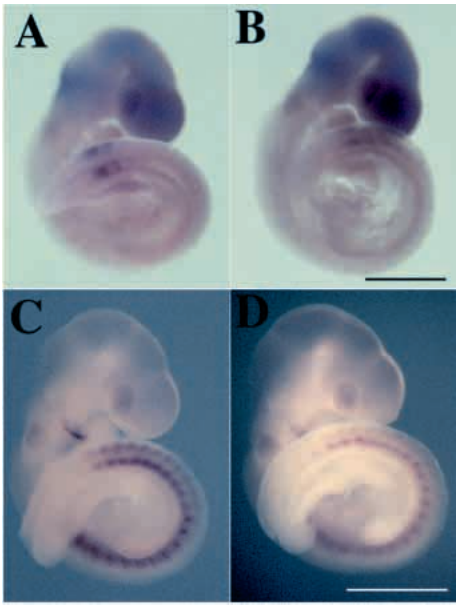


Fig. 5. Whole-mount *Notch1* and *Pax-1* expression profiles in somites. *Notch2*$^{+/+}$ (A,C) and *Notch2*$^{m/m}$ (B,D) embryos at E9.5 were analysed by whole-mount in situ hybridization with probes for *Notch1* (A,B) or *Pax1* (C,D). There is no significant difference in the expression levels of either gene when wild type and mutant embryos are compared. Scale bars indicate 1 mm.

should produce 4.6 kb and 4.0 kb *Eco*RI fragments, corresponding to mutant and wild-type *Notch2* alleles, respectively. We found 5 ES cell clones producing 4.6 kb and 4.0 kb *Eco*RI fragments in almost equal amounts, indicating that these clones were heterozygous for the disrupted *Notch2* gene (*Notch2*$^{+/m}$). Another probe, a 1.4 kb *Xho*I-*Sma*I *Notch2* gene fragment located elsewhere downstream in the targeting vector (Probe B), produced identical hybridization profiles in *Eco*RI digests of DNA from these clones (data not shown). Two clones with normal diploid karyotypes were used to produce chimeric mice as described in Materials and Methods. Chimeras from one clone transmitted the disrupted *Notch2* gene to their offspring.

Southern blot analysis of tail DNA from 8 offspring at 4 weeks post partum, obtained by crossing a *Notch2*$^{+/m}$ male and a *Notch2*$^{+/+}$ female, revealed that 6 were *Notch2*$^{+/m}$ and 2 were *Notch2*$^{+/+}$ (Fig. 1F). Yolk sac DNAs from E9.5 embryos obtained by intercrossing the heterozygotes showed three types of Southern blot profiles corresponding to *Notch2*$^{+/+}$, $^{+/m}$ and $^{m/m}$ genotypes (Fig. 1G). These results demonstrate that a mouse line has been established that carries a properly target-disrupted *Notch2* allele.

Western blot analysis of E10.5 whole embryo homogenates using a monoclonal antibody against β-galactosidase revealed an approximately 380 kDa protein in *Notch2*$^{+/m}$ embryos but not in wild type (Fig. 1H). This is slightly larger than the expected size of 317 kDa (201 kDa Notch2 amino acid sequence and 116 kDa β-galactosidase), probably because of glycosylation, commonly found with transmembrane proteins. We also carried out RT-PCR analysis which detected a spliced transcript hybridizable with both *Notch2* and *lacZ* probes in *Notch2*$^{+/m}$ mice but not in *Notch*$^{+/+}$ mice (data not shown).

Expression of *Notch2* during early embryogenesis

Expression of *Notch* family genes during early mouse development has been studied previously by in situ hybridization (Weinmaster et al., 1992; Reaume et al., 1992; Lardelli and Lendahl, 1993; Higuchi et al., 1995; Swiatek et al., 1994; Williams et al., 1995). In order to determine whether expression of the mutant gene mirrors that of the wild type, we examined *Notch2* expression patterns in whole-mount *Notch2*$^{+/m}$ and $^{m/m}$ embryos both by in situ hybridization and by assessing β-galactosidase activity**.** In E8.5 embryos, strong *Notch2* expression was observed in the ventral-most cells of the neural groove, somites, primitive streak and allantois (Fig. 2A). At E8.5-9.0, in addition to floor plate expression, the strongest signals were seen in the most recently formed somites, then gradually lessened toward the more rostral, older ones (data not shown, c.f. Fig. 2B). By E9.5 *Notch2* expression was observed in a variety of tissues (Fig. 2B). Prominent expression was observed not only in the younger somites, but also in the floor plate, roof plate and neuroepithelium of the brain, and in neuroepithelia of the optic and otic vesicles, whereas the heart did not show any signal at all. At E10.5 the expression patterns remain essentially the same, except that the signals in the roof plate of the midbrain and in the branchial arches become more pronounced (Fig. 2D). X-gal staining of

Notch2$^{m/m}$ embryos was much heavier than that of *Notch2*$^{+/m}$ embryos, reflecting the copy number of the mutated gene. For the most part, these expression patterns of the *Notch2* fusion proteins are consistent with previous in situ hybridization data (Swiatek et al., 1994; Williams et al., 1995). However, a remarkable difference in the results from these two methods is seen when somites and presomitic mesoderm are compared. As shown in Fig. 2C, in situ hybridization with a *Notch2* probe revealed that *Notch2* transcripts are restricted to the presomitic region. In contrast, the fusion protein is present in the somites but not in presomitic mesoderm (Fig. 2B). Additional in situ hybridization studies using a *lacZ* probe to visualize transcripts for the fusion protein in *Notch2*$^{+/m}$ mice demonstrated a localization pattern identical with that of the *Notch2* transcripts described above. Differences in the expression patterns revealed by these two methods could be due either to a time lag between transcription and translation, or to a difference in perdurability between the transcripts and the Notch2-β-galacotosidase fusion protein. Such a difference could result from the absence in the fusion protein of the Notch2 C-terminal region including the PEST sequence. Similar observations have been reported in mice in which the *lunatic fringe* gene has been disrupted by a *lacZ* insertion (Zhang and Gridley, 1998).

Embryonic lethality caused by homozygous *Notch2* mutation

Southern blot analysis of DNAs from 242 progeny (3-4 weeks post partum) derived from intercrosses of *Notch2*$^{+/m}$ mice revealed no mice homozygous for the *Notch2* mutation (*Notch2*$^{m/m}$) (Table 1), suggesting that homozygous disruption of *Notch2* leads to embryonic lethality. Up to E10.5, *Notch2*$^{m/m}$ embryos were found with the expected Mendelian segregation profile of approximately 1:2:1 (Table 1), whereas after E12.5 no homozygous mutant embryos were detected. At E11.5 only one homozygous mutant embryo was found among 33 embryos examined. These data indicate that *Notch2*$^{m/m}$ mice die at or around E11.5. Thus the *Notch2* gene must play a vital role during early postimplantation development in mice, and the ankyrin repeats must be essential for *Notch2* function.

Widespread apoptotic cell death in *Notch2*$^{m/m}$ mice at E9.5

All *Notch2*$^{m/m}$ embryos at E9.5 appeared normal compared with wild-type and *Notch2*$^{+/m}$ littermates whereas by E10.5 *Notch2*$^{m/m}$ embryos clearly were developmentally retarded (Fig. 2D). Histological examination of E10.5 *Notch2*$^{m/m}$ embryos revealed vast numbers of pycnotic cells widely distributed throughout the embryo. They were seen in the telencephalon, lateral plates of the spinal cord, otic vesicles, optic vesicles, cranial ganglia, neural crest and somites (data not shown). In an effort to determine when and where pycnotic cells first appear in *Notch2*$^{m/m}$ embryos, histological studies were carried out at E9.5. Pycnotic cells were observed in tissues where programmed cell death normally occurs during development (Glücksmann, 1951; Oppenheim, 1991; Conlon et al., 1995) such as brain neuroepithelia, otic vesicles, optic vesicles, cranial ganglia and neural crest. However, in spite of the normal size and appearance of E9.5 *Notch2*$^{m/m}$ embryos, pycnotic cells in these regions were already much more numerous and more widely distributed than in Notch2$^{+/m}$ embryos (Fig. 3). In order to determine whether cells were dying by apoptosis at E9.5, and if so to quantify it in each genotype, we carried out in situ nick end labeling (ISNEL) detection of DNA fragmentation on sections of an optic vesicle, an otic vesicle and the telencephalic region (Fig. 4A-C). For each region examined, there were 6-10 times more ISNEL-positive grains associated with *Notch2*$^{m/m}$ tissues than with *Notch2*$^{+/+}$ or *Notch2*$^{+/m}$. These observations demonstrate that a widespread onset of apoptosis occurs as early as E9.5 in *Notch2*$^{m/m}$ embryos, prior to significant developmental retardation. Widespread apoptosis has also been reported in *Notch1* mutant mice at E9.5, but only after developmental retardation has become apparent (Swiatek et al., 1994; Conlon et al., 1995).

Because disorganized somitogenesis has been reported in *Notch1* mutant mice, we examined the morphology of somites in *Notch2*$^{m/m}$ embryos from E9.5 to E10.5 in combination with the expression pattern of Pax-1, a marker for sclerotome differentiation (Ebensperger et al., 1995). We found no abnormality in early somitogenesis in *Notch2*$^{m/m}$ embryos (Fig. 5C,D), but a significant number of pycnotic cells was observed in already formed somites at E10.5 (data not shown). These results indicate that *Notch2* signaling via ankyrin repeats does not play an essential role in somitogenesis but is needed for survival of somite derivatives. To investigate the possibility that a deficiency in *Notch2* might affect the expression of *Notch1* in such a way that normal somitogenesis in *Notch2* mutants might be ensured, we performed in situ hybridization on E9.5 *Notch2*$^{m/m}$ and wild-type embryos using a *Notch1* specific riboprobe. As seen in Fig. 5, *Notch1* expression patterns and levels are nearly identical in the two genotypes and show no indication of compensatory regulation in the mutants.

Table1. Genotypes of progeny of F_1 intercrosses of *Notch2* heterozygous mice

Stage (dpc)	Number of litters	Total mice or embryos	Genotypes		
			+/+	+/m	m/m
8.5	3	22	5	11	6
9.5	5	34	9	17	8
10.5	8	52	6	36	10
11.5	4	33	16	16	1
12.5	5	34	13	21	0
14.5	3	18	6	12	0
Adult	–	242	88	154	0

Expression of neurogenic genes in *Notch2*$^{m/m}$ mice

Transfection experiments with cultured cells have shown that Notch1 signaling activates *Hes* genes, subsequently suppressing neurogenic gene expression and inhibiting the cells from differentiating into neural derivatives (Jarriault et al., 1995). *Hes-5* downregulation and *Mash1* upregulation was also observed in Northern blot and in situ hybridization analyses of mutant mice lacking either *Notch1* or *RBP-Jκ* (de la Pompa et al., 1997), indicating that *Hes-5* and *Mash1* are under the control of *Notch1* and *RBP-Jκ*. Because *Notch2* is also expressed in the developing mouse brain, we examined the expression of *Hes-1, Hes-3, Hes-5, Mash1* and *neurogenin* by in situ hybridization on E9.5 embryos to determine whether Notch2 also modulates the expression of these genes. No significant differences in expression were detected between

Notch2$^{m/m}$ and *Notch2*$^{+/+}$ embryos for any of these genes, either in distribution or intensity (Fig. 6). These observations suggest that the role of Notch2 signaling in neurogenesis is different from that of Notch1.

Retention of proliferating ability of *Notch2*$^{m/m}$ cells in culture

To study further the enhanced cell death caused by *Notch2* mutation, we examined the growth in vitro of cells obtained from embryos before and after the onset of massive pycnosis. Cells from *Notch2*$^{m/m}$ embryos at E9.5 proliferated at virtually identical rates as cells from *Notch*$2^{+/m}$ and *Notch*$2^{+/+}$ embryos (Fig. 7A). We also tested *Notch2*$^{m/m}$ cells from embryos at E10.5 when pycnosis in vivo was more extensive. These cells grew at the same rate as cells from E9.5 embryos (data not shown). It is likely that such cultures consist of the progeny of cells that were still healthy in vivo when isolated, not those that were already pycnotic. Even so, the results indicate that embryonic cells proliferate independently of their Notch2 genotypes and that cells from Notch2$^{m/m}$ embryos exhibiting massive pycnosis can survive and grow in culture even beyond the stage when embryonic lethality occurs in vivo.

To determine whether embryonic cells in vitro express *Notch2* and, if so, whether the presence of bacterial β-galactosidase is cytotoxic, cells from E9.5 embryos were cultured for 2 days, fixed, and stained with X-Gal. Approximately 30% of *Notch2*$^{+/m}$ cells and 50% of *Notch2*$^{m/m}$ cells were positive, both in the inocula and after 2 days of culture (Fig. 7B). The different values obtained for the two genotypes reflect sensitivity of the assay in relation to gene dosage – heterozygous cells are a paler blue and some low level positives would be scored as negative. These results indicate that embryonic lethality in homozygous *Notch2* mutant mice is not due to cytotoxicity of the Notch2-β-galactosidase fusion protein and that the mutant cell population can survive and proliferate in vitro in the absence of a functional *Notch2* ankyrin repeat domain.

DISCUSSION

We have used gene targeting procedures to generate *Notch2* mutant mice in which the region encoding the distal 5 ankyrin repeats and part of the following downstream sequence are replaced by the *E. coli lacZ* gene. The mutation allowed us to study the function of Notch2 and the significance of the ankyrin repeats in mouse development as well as Notch2 expression profiles. *Notch2*$^{m/m}$ mice showed developmental retardation, widespread cell death, and embryonic lethality before E11.5 after developing apparently normally up to the 25-26 somite stage. The widespread cell death observed in *Notch2*$^{m/m}$ embryos is unlikely to be related to high expression levels of the *Notch2*-β-galactosidase fusion protein in these embryos, since cells isolated from E9.5 *Notch2*$^{m/m}$, *Notch2*$^{+/m}$ and *Notch2*$^{+/+}$ embryos survive and proliferate in culture at similar rates whether or not they express the mutant protein. Thus, the abnormalities revealed by *Notch2*$^{m/m}$ mice most likely are due to a lack of functional Notch2 signaling.

In spite of the normal external appearance of E9.5 *Notch2*$^{m/m}$ embryos, the onset of a histological abnormality, pycnosis, starts prominently in some *Notch2*-expressing tissues, particularly the neuroepithelium of the telencephalon, cranial ganglia cells, and optic and otic vesicles. These tissues show pycnotic features in *Notch2*$^{+/m}$ and *Notch2*$^{+/+}$ embryos also, but the frequency is much lower than in *Notch2*$^{m/m}$ embryos. In subsequent development of *Notch2*$^{m/m}$ embryos, pycnosis progressed extensively in these regions and also spread to additional tissues where the *Notch2* gene was found to be normally expressed, such as spinal cord, somites and hind gut epithelium. However, there appears to be a relatively poor correlation between the level of *Notch2* expression and the frequency of pycnotic cells in any given tissue. For instance, the floor plate shows strong expression of *Notch2*, but nonetheless appears normal at E10.5.

Survival and growth in vitro of *Notch2*$^{m/m}$ cells compared with Notch$2^{+/+}$ cells seems to be in sharp contrast to the high incidence of pycnosis observed in vivo. Up to 50% of the cultivatable cells express *Notch2* but are neither killed by it nor are dependent on it for survival. There are three possible explanations. First, growth factors contained in the culture medium may rescue prospective pycnotic cells. Second, culture procedures may selectively favor cell types for which *Notch2* deficiency is not lethal. Third, intracellular signals derived from the unaltered extracellular domain of the mutant *Notch2* protein may be involved in the observed cell death, and trypsinization may disrupt such a signaling pathway. The first possibility seems the most likely because, in chimeras between *Notch2*$^{m/m}$ and wild type, mutant cells appear normal at E11.5 even in the telencephalon and otic and optic vesicles (Y. Kadokawa et al., unpublished data). Thus, we speculate that widespread cell death is not a direct consequence of the *Notch2* mutation, but results from a defect in production of an unidentified pleiotrophic factor in homozygous mutant embryos, or from a systemic deficiency, perhaps in hematopoiesis (Bigas et al., 1998).

The cytoplasmic regions of Notch family proteins possess several copies of well-conserved ankyrin repeats, the importance of which in mammalian Notch function has been documented using cultured cells (Kopan et al., 1994; Jarriault et al., 1995). The ankyrin repeat domain has been thought to bind to RBP-Jκ and Deltex proteins, both of which have been implicated in developmental regulation of gene expression via Notch (for review, see Artavanis-Tsakonas et al., 1995; Robey, 1997; Lendahl, 1998; Weinmaster, 1998). Physical interaction between RBP-Jκ and the intracellular Notch domain has been reported to occur in mouse Notch family members, but with the RAM sequence, not with the ankyrin repeats (Tamura et al., 1995; Kato et al., 1996). One function of RBP-Jκ may be to assist the binding of factors to the ankyrin repeats, thus facilitating Notch signaling and subsequent regulation of gene expression. We have shown that Notch2 protein lacking 5 of its 6 ankyrin repeats fails to support normal development in mice, suggesting that Notch signaling is required for embryonic development and that the ankyrin repeat region is indispensable for that function.

Notch2$^{m/m}$ phenotypes are similar to those reported in *Notch1* mutant mice except that in *Notch1* mutants, developmental retardation is obvious at E9.5 and is accompanied by disorganized somitogenesis (Swiatek et al., 1994, Conlon et al., 1995). In the *Notch2* mutants studied here, retardation is not apparent until E10.5, and somitogenesis

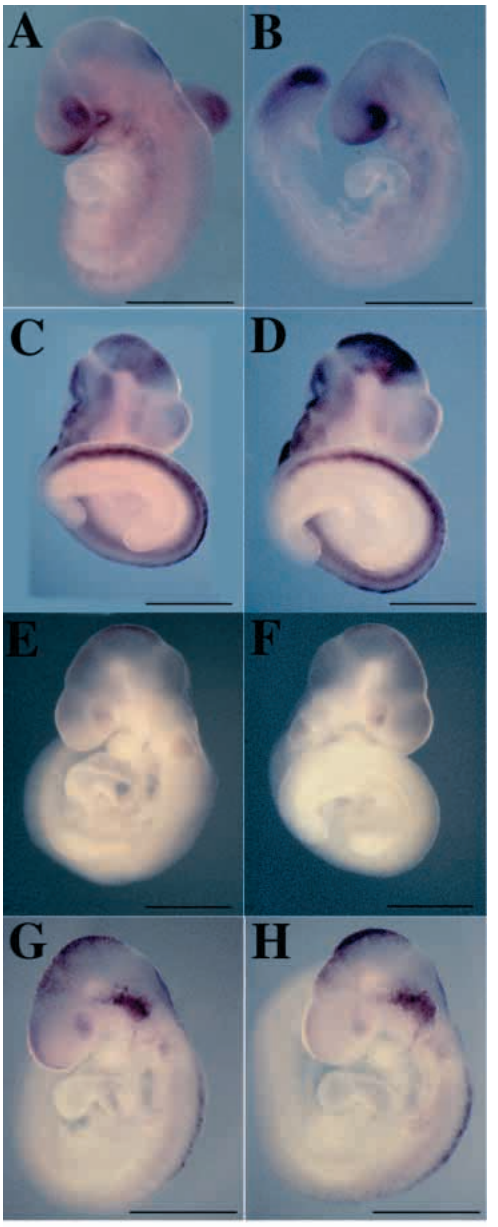


Fig. 6. Neurogenic gene expression patterns in *Notch2*$^{+/m}$ and *Notch2*$^{m/m}$ embryos at E9.5. *Hes-1* (A,B), *Hes-5* (C,D), *Mash1* (E,F) and *neurogenin* (G,H) probes were used for in situ hybridization in *Notch2*$^{+/m}$ (A,C,E,G) and *Notch2*$^{m/m}$ (B,D,F,H) embryos. There are no consistent differences in the patterns or expression levels of the examined genes between *Notch2*$^{+/m}$ and *Notch2*$^{m/m}$ embryos. Scale bars, 1 mm.

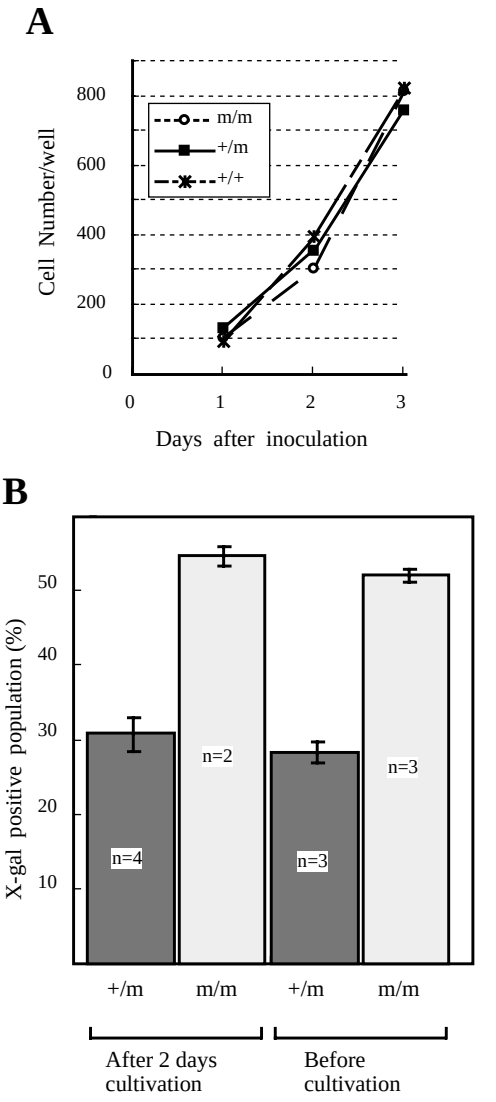


Fig. 7. Cell growth in vitro relative to *Notch2* genotype and β-galactosidase expression. (A) *Notch2*$^{m/m}$ (*n*=2), *Notch2*$^{+/m}$ (*n*=3) and wild-type (*n*=2) embryos at E9.5 were isolated from a heterozygous cross, dissociated, and cells were inoculated into culture medium. Cell counting was begun after 24 hours of cultivation as described in Materials and Methods. (B) Embryos of *Notch2*$^{+/m}$ and *Notch2*$^{m/m}$ at E9.5 were dissociated in culture medium, fixed with glutaraldehyde and stained with X-gal. In one experiment cells from E9.5 embryos were cultivated in vitro for 2 days prior to X-gal staining. Dark and gray bars with standard errors indicated by brackets represent *Notch2*$^{+/m}$ and *Notch2*$^{m/m}$ genotypes, respectively.

prior to that time appears normal. *Notch1* mutants develop apparently normally through the first 9 embryonic days, up to the 14-16 somite stage, after which prominent pycnosis was found in the neuroepithelium of the central nervous system, otic vesicles and in neural crest-derived cells such as cranial sensory ganglia and cephalic mesenchyme. A similar progression is reported here for *Notch2* mutants, but offset to a slightly later time frame. Both classes of mutants die prior to E11.5. Transcription of *Notch1*, *2* and *3* genes has been reported to begin by gastrulation, E7.0 (Williams et al., 1995), well before the stages at which the mutant phenotypes of *Notch1* and *Notch2* first become apparent. Our studies utilizing β-galactosidase staining demonstrate that the fusion protein is present and active by E8.5 (Fig. 2A). Since the size of the fusion protein on western blots indicates that the mutant *Notch2* mRNA is correctly translated at E8.5 (data not shown), it is likely that Notch2 mRNA in wild-type embryos is correctly translated then as well. Thus, there is a significant delay between the time at which the first Notch gene products are present and the time at which their functions become

essential for cell survival. It is instructive to consider in this context the phenotype caused by a null mutation of *RBP-Jκ*, which may be a common signaling mediator for all members of the Notch family (Kato et al., 1996). RBP-Jκ mutant mice showed developmental retardation at E8.5, earlier than was seen with either *Notch1* or *Notch2* mutants (Oka et al., 1995). These results imply that functional overlaps may exist among Notch family genes in early development. In contrast, several tissues express both *Notch1* and *Notch2*, yet exhibit defects at E9.5 if either gene is defective, suggesting that functional overlapping does not always occur. The otic and optic vesicles are two examples. Such tissues could be mosaics of *Notch1*- or *Notch2*-dependent cells, or tissue-specific differences may exist in the signaling pathways that serve different *Notch* family members.

Since the *Notch2* mutant protein retains the EGF-repeats, the LNR region, and the RBP-Jk binding domain, the mutant might act in a dominant negative fashion for other Notch family proteins by capturing Notch signaling molecules as reported in *Drosophila* (Rebay et al., 1993). However, Rebay et al., observed the dominant negative phenotype only when a mutant Notch was overexpressed, thus overriding wild-type Notch. On the other hand, the *Notch2* mutant gene is under the control of the intrinsic promoter, producing the mutant Notch2 at a relatively low level, and therefore, it seems unlikely that the mutant protein overrides the wild-type products of other Notch members. Furthermore, no abnormalities were apparent in *Notch2*$^{+/m}$ mice. Taken together, it seems likely that the Notch2 mutant protein does not function as a dominant negative mutant. This notion is supported by several other observations. In contrast to *Notch1* mutant mice, disorganized somitogenesis was not observed in *Notch2*$^{m/m}$ mice at E9.5. Although expression of neurogenic genes such as *Mash1*and *Hes-5* has been reported to be perturbed by *Notch1* or *RBP-Jκ* mutation (de la Pompa et al., 1997), this is not observed in *Notch2* mutants. Furthermore, chimeric embryos that are mosaics of *Notch2*$^{m/m}$ and wild-type cells continue apparently normal development beyond E11.5 (Kadokawa et al., unpublished data). No abnormalities have been observed in somites or other tissues where *Notch1*, *Notch2* and *Notch3* are co-expressed even though *Notch2*$^{m/m}$ cells contribute significantly to these tissues.

The results presented here indicate that the Notch2 mutant protein does not function as a dominant negative mutant against Notch1 or Notch3, and strongly suggest that the abnormalities observed in *Notch2*$^{m/m}$ mice are due specifically to Notch2 deficiency. We conclude that the ankyrin repeat region of Notch2 plays an essential, but as yet undefined role in the embryonic development of several tissue types.

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