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Brain- and heart-specific *Patched-1* containing exon 12b is a dominant negative isoform and is expressed in medulloblastomas ☆

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Abstract

Mutations in the human tumor suppressor gene, *Patched-1*, are associated with nevoid basal cell carcinoma syndrome characterized by developmental abnormalities and tumorigenesis, such as basal cell carcinoma and medulloblastoma. During the investigation of complex alternative splicing in *Patched-1*, we identified an alternative exon, exon 12b, located between exon 12 and 13, both in humans and in mice. Since exon 12b has an in-frame stop codon, the mRNA isoform containing this exon (*Patched12b*) encodes a truncated patched-1 protein. RT-PCR and whole mount *in situ* hybridization revealed that mouse exon 12b was expressed in the brain and heart, particularly in the cerebellum, in both adults and embryos. We next performed a functional analysis of *Patched12b* using a GLI-responsive luciferase reporter. Luciferase activity was suppressed when transfected with a plasmid encoding *Patched-1*, but not with a plasmid for *Patched12b*. The suppressive activity of Patched-1 was relieved when cotransfected with a plasmid for *Patched12b*. This implies that the Patched12b protein has a dominant negative effect on Patched-1. Interestingly, *Patched12b* was found to be expressed in some of the medulloblastoma tissues and cell lines, indicating an important role in the pathogenesis of medulloblastoma as well as brain development.

Keywords: Alternative splicing; Medulloblastoma; Nevoid basal cell carcinoma syndrome; Patched-1

The *Patched-1* gene (*Ptc1*) controls cell growth and specification of the developing and postnatal tissues of many animals [1]. The nevoid basal cell carcinoma syndrome (NBCCS), also called Gorlin syndrome, is associated with mutations in a human *Ptc1* homolog, *PTCH* [2,3]. NBCCS is an autosomal dominant neurocutaneous disorder characterized by developmental malformations, such as syndactyly and spina bifida, and an increased incidence of a variety of tumors, including basal cell carcinoma (BCC) and medulloblastoma [4]. Mutations of *PTCH* are also detected in a small fraction of holoprosencephaly characterized by a failure of the complete separation of the forebrain into right and left halves [5]. Heterozygous loss of *PTCH* found in certain sporadic and familial cases of BCC and medulloblastoma indicates that *PTCH* is also a tumor suppressor gene [6–8]. Ptc1, a 12-pass transmembrane protein, is the ligand-binding component of the receptor complex for a secreted protein, Sonic hedgehog (Shh). In the absence of Shh binding, Ptc1 is thought to hold Smoothened (Smo), another component of the Shh receptor, in an inactive state and thus inhibit signaling to

☆ *Abbreviations:* AS, alternative splicing; BCC, basal cell carcinoma; EGFP, enhanced green fluorescent protein; NBCCS, nevoid basal cell carcinoma syndrome; NMD, nonsense-mediated mRNA decay; PTC, premature termination codon; Shh, Sonic hedgehog.

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downstream genes. Upon the binding of Shh, the inhibition of Smo is released and signaling is transduced, leading to the activation of target genes by the Gli family of transcription factors [1].

We and others have identified a number of *PTCH* mRNA isoforms generated by alternative splicing (AS) [9–11]. Among these isoforms, the one containing exon 12b conserved in both humans and mice (*PTCH12b* and *Ptc12b*, respectively) is particularly interesting since it is expressed in a brain- and heart-specific fashion, at least in human tissues [12]. Here, we show that mouse *Ptc12b* is also preferentially expressed in the brain and in the heart. Since this isoform has an in-frame stop codon, it encodes truncated Ptc1, which does not seem to have any functions. However, the functional analysis of this isoform demonstrated that it functions as a dominant negative isoform against Ptc1. Furthermore, *PTCH12b* was found to be expressed in some of the medulloblastoma tissues and cell lines, indicating an important role in the pathogenesis of medulloblastomas as well as brain development.

Materials and methods

Constructs. The plasmids for myc-PTCH and 8 × GLI-Luc were kindly provided by Dr. J. Ming and Dr. S. Ishii, respectively. Mouse *Ptc1* cDNA sequence, exon 12-12b-13, was amplified by RT-PCR. The primers used for the amplification were 5′-TTCTCCCTCCAGTACTGATG-3′ (exon 12 forward), 5′-CACCACAGCAGCCTTGGGAG-3′ (exon 13 reverse). The PCR product was subcloned into pGEM-T Easy (Promega) and used for*in situ* hybridization. pMyc-PTCH and pMyc-PTCH12b were described previously [12]. To produce pPTCH-EGFP and pPTCH12b-EGFP, *PTCH* sequences for exon 1a-exon23 and exon 1a-exon12b, respectively, were amplified by PCR using pMyc-PTCHM or pMyc-PTCH12b as a template and subcloned into pEGFP-N3 (Clontech). The primers used for the amplification were 5′-GGGGTACCGCTATGGGGAAGGCTA CTGG-3′ (exon 1a-2 forward), 5′-CGGGATCCGTTGGAGCTGCTT CCCCGGG-3′ (exon 23 reverse), and 5′-CGGGATCCCTCCTCG TAAGGAAACCTCATGTA-3′ (exon 12b reverse). Restriction enzyme recognition sequences (underlined) were added to facilitate subcloning.

RT-PCR. Total RNA was extracted using the RNeasy kit from Qiagen according to the manufacturer's recommendations. RT-PCR was performed as previously described using 5 μg of total RNA [11]. Primers used for RT-PCR were 5′-TGGCCCATGCATTCAGTGAAACA-3′ (mouse exon 11 forward), 5′-GAGGGTCATACTCTGTGCGGA-3′ (mouse exon 14 reverse), 5′-GTGTTGGTGTGGATGATGTTT-3′ (human exon 11 forward), and 5′-CGGGATCCTTGTAAAACAGCAGAAAAT-3′ (human exon 13 reverse).

Western blotting. Immunoblot analysis was performed as described previously [13]. In brief, 30 μg of the cell lysate was subjected to SDS–PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with anti-c-Myc mouse monoclonal antibody (Santa Cruz, 9E10) followed by horseradish peroxidase-conjugated anti-mouse immunoglobulins (DAKO) or with anti-GFP rabbit polyclonal antibody (Medical & Biological Laboratories, Japan) followed by horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz).

Luciferase assay. I-23 cells growing on six-well plates were cotransfected using Effectene reagent (Qiagen) with various combinations of plasmids as indicated in Fig. 4. The total amount of transfected DNA was adjusted to 3 μg with an empty plasmid, pcDNA3.0. Twenty-four hours after the transfection, cells were harvested and subjected to the luciferase assay with the reagents and protocols provided by Promega. Firefly luciferase activity was normalized by Renilla luciferase activity from a cotransfected pRL-SV40 (Promega).

In situ hybridization. The plasmids described above were linearized and digoxigenin-labeled cRNA probes were synthesized using T7 or SP6 RNA polymerase. E10.5 embryos on a C57BL/6J background were fixed in 4% paraformaldehyde in PBS, dehydrated in methanol, and stored at −20 °C. For hybridization, embryos were rehydrated in 0.1% Tween 20 in PBS (PBT) and incubated with proteinase K (10 μg/ml in PBT) for 15 min at 37 °C. Digestion was stopped by washing with 2 mg/ml glycine in PBT, and embryos were refixed in 4% paraformaldehyde and 0.25% glutaraldehyde in PBT, washed in PBT, and hybridized overnight at 65 °C with 2 μg/ml of digoxigenin-labeled RNA probes in hybridization solution (50% formamide, 5 × SSC, 2% blocking powder (Roche), 0.1% Tween 20, 0.5% CHAPS, 50 μg/ml yeast RNA, 5 mM EDTA, and 50 μg/ml heparin). Embryos were washed in hybridization solution and in 2 × SSC, 0.1% CHAPS at 65 °C, and incubated for 30 min with 20 μg/ml RNase A in 2 × SSC, 0.1% CHAPS at 37 °C. After washing, embryos were blocked for 3 h in 10% sheep serum, 1% BSA in PBT and incubated overnight at 4 °C with anti-digoxigenin antibody (Roche) (1:2000 diluted in 10% sheep serum, 1% BSA in PBT with 1.5 mg/ml mouse embryo powder). Embryos were washed 5 times in 1% BSA in PBT for 1 h each, 3 times in NTMT (100 mM NaCl, 100 mM Tris–HCl, pH 9.5, 50 mM $MgCl_2$, and 0.1% Tween 20) for 10 min each, and stained with NBT/BCIP stock solution (Roche) (1:50 diluted in NTMT) for about 2 h at room temperature.

Immunostaining and confocal microscopy. Immunostaining was performed essentially as described previously [14]. Briefly, HeLa cells were seeded on chamber slides (Nalge Nunc International) and were transfected with the constructs indicated in the figure legend. After 24 h, the slides were fixed with 4% paraformaldehyde, permeabilized, stained with anti-c-myc antibody (Santa Cruz, 9E10) followed with FITC-labeled anti-mouse immunoglobulins (DAKO), and observed with an Olympus microscope FV300. Nuclear localization was confirmed by Hoechst33342 staining. EGFP fusion proteins were observed as described previously [15].

Results

Tissue-specific regulation of Ptc12b expression in mice

Previously, we identified a *patched-1* isoform containing a novel exon, exon 12b, between exon 12 and exon 13 both in humans (*PTCH12b*) and in mice (*Ptc12b*) (GenBank Accession Nos. AB214500 and AB214501, respectively) [12]. Using RT-PCR and exon junction microarrays, *PTCH12b* was demonstrated to be expressed in a brain- and heart-specific fashion [12]. The nucleotide sequence of and adjacent to exon12b was relatively conserved in humans and in mice, especially around the 3′-end of the exon (Fig. 1A). Since premature termination codons (PTCs) were identified in both exons, they are expected to encode proteins truncated just after the sterol-sensing domain (Fig. 1B), whose function in PTCH remains elusive [16]. The splicing regulatory element, UGCAUG, reported to be phylogenetically and spatially conserved in introns that flank the brain-enriched alternative exons [17] was found in the intron regions upstream and downstream of exon 12b in both species (Fig. 1C), supporting the hypothesis that this element is a critical component for tissue-specific splicing events. We next investigated whether exon 12b was also preferentially

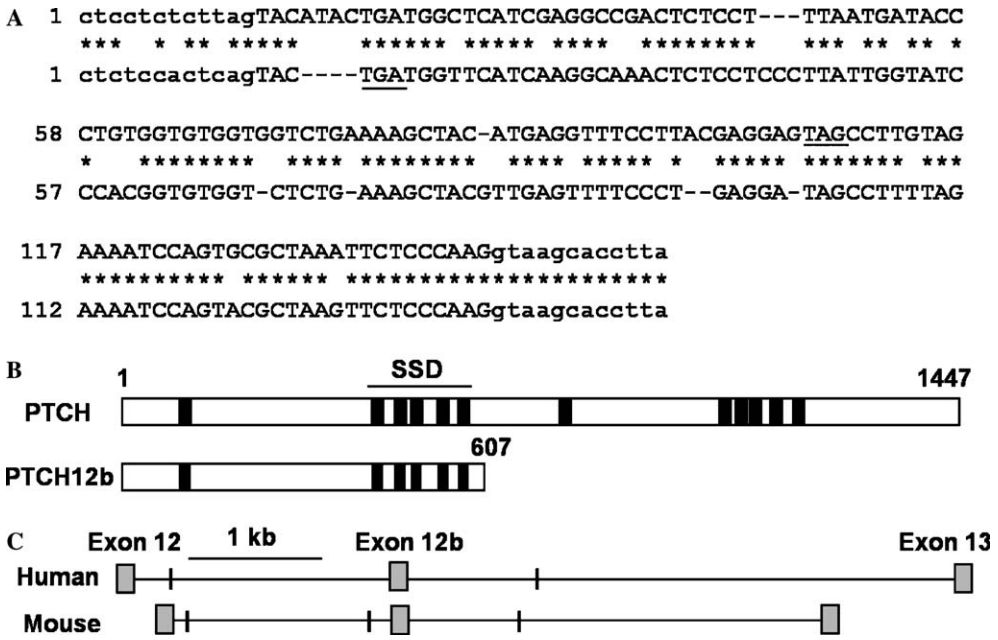

Fig. 1. Exon 12b and flanking splicing elements are conserved in humans and in mice. (A) Alignment of human (upper) and murine (lower) exon 12b and surrounding sequences. Upper- and lowercase letters indicate the exon and intron sequences, respectively. Nucleotides are numbered arbitrarily. Conserved nucleotides are marked by asterisks. In-frame stop codons are underlined. (B) PTCH protein isoforms. Numbers refer to amino acid positions relative to the first methionine of PTCH (NM_000264). Transmembrane domains are indicated by filled boxes. The region containing the 2nd to 6th transmembrane domains comprises the sterol-sensing domain (SSD). (C) Location of UGCAUG hexamers near exon 12b. The location of hexamers is indicated by small vertical thick lines.

expressed in the mouse brain and heart. In adult mice, *Ptc1* (12b−) was more or less expressed in various tissues. However, the *Ptc12b* isoform (12b+) was specifically expressed in the brain and in the heart, particularly in the cerebellum, but not in other tissues, such as the testis and the liver (Fig. 2A). To investigate the expression pattern in the mouse embryo, we performed whole mount *in situ* hybridization. *Ptc12b* was also expressed in the brain and in the heart (Fig. 2B), indicating some role yet to be identified in the development of these tissues. The specificity of the result was confirmed by the negative staining with the sense probe. Taken together, these results imply that the tissue-specific expression of this isoform is evolutionarily conserved.

PTCH12b is expressed in some medulloblastoma tissues and cell lines

Individuals with NBCCS are at high risk of medulloblastomas, which are primitive neuroectodermal tumors. Since medulloblastoma commonly arises in the cerebellum where *PTCH12b* is specifically expressed, we next addressed the question if this isoform is expressed in medulloblastoma cell lines and tissues. Out of 5 medulloblastoma cell lines analyzed, I-23 expressed very high level of *PTCH12b*. None of the 9 non-medulloblastoma cell lines expressed *PTCH12b*. Interestingly, it was also expressed in two out of two medulloblastoma tissues we examined, indicating that this isoform plays a role in the formation of medulloblastoma (Fig. 3A).

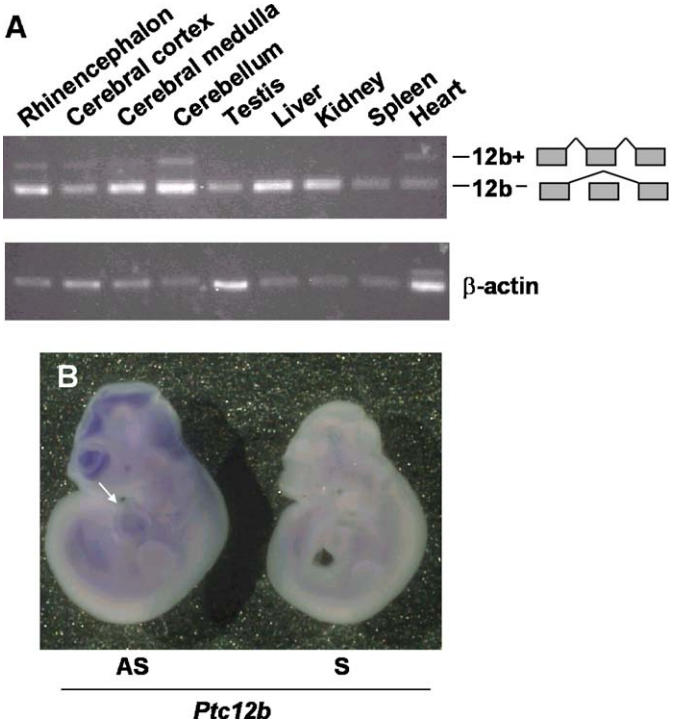

Fig. 2. Tissue-specific expression of *Ptc12b* in mice. (A) Total RNAs obtained from a panel of mouse tissues were subjected to RT-PCR with β-actin as an internal control. A forward primer for exon 11 and a reverse primer for exon 14 were synthesized and used for RT-PCR. All tissues were obtained from a 1-month-old mouse. (B) Whole mount *in situ* hybridization on mouse embryos. Digoxigenin-labeled RNA probes were synthesized in both orientations, sense (S) and antisense (AS), and used on embryos at E10.5. The arrow indicates the position of the heart.

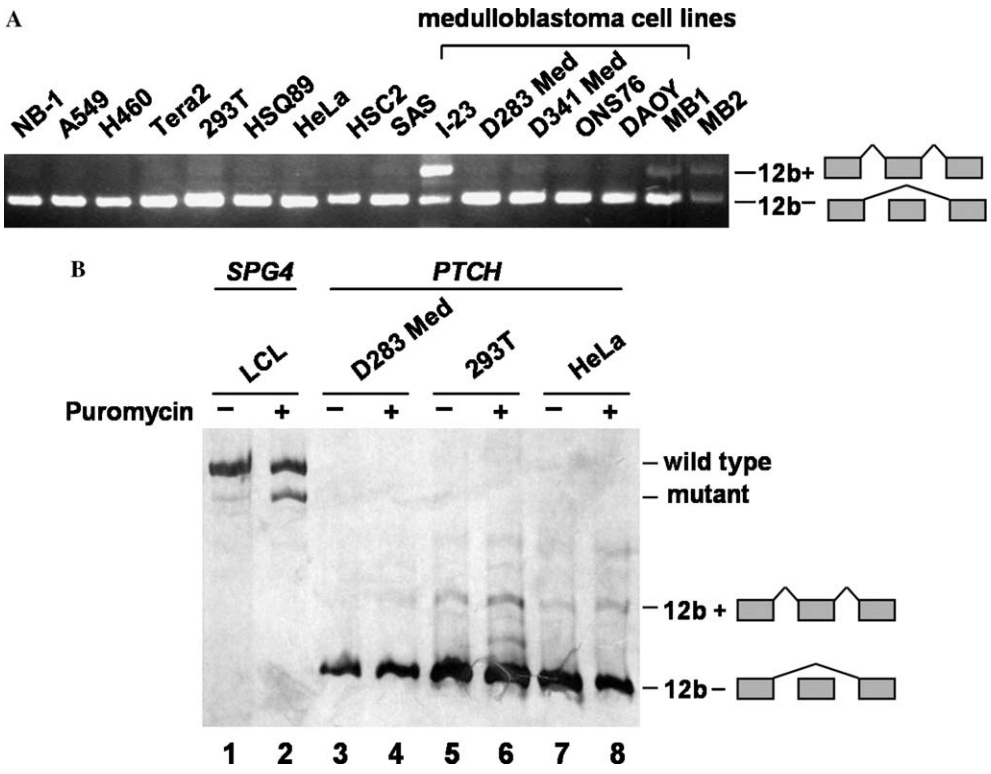

Fig. 3. Expression of exon12b in human cell lines and medulloblastomas. (A) RT-PCR analysis was performed using specific primers for exon 11 and exon 13. RNAs extracted from various cell lines and medulloblastoma samples (MB1, MB2) were used as templates. (B) *PTCH12b* is subjected to NMD to a small extent. Cell lines indicated at the top were grown in the presence or absence of 100 μg/ml puromycin for 6 h. Total RNA was extracted and subjected to RT-PCR. The RT-PCR products were run on a 3.5% polyacrylamide gel to emphasize the difference in size between transcripts from wild type allele and mutant allele of the *SPG4* gene.

PTCH12b isoform undergoes NMD to a small extent

It is known that spliced transcripts with PTCs, such as *Ptc12b* or *PTCH12b*, can potentially activate transcript degradation via the process of nonsense-mediated mRNA decay (NMD) [18]. NMD is important for the removal of PTC-containing transcripts encoding nonfunctional or potentially dominant negative proteins. In order to investigate this possibility, D283 Med, 293T, and HeLa cells, which express barely detectable levels of *PTCH12b*, were cultured in the presence or absence of an NMD inhibitor, puromycin, and subjected to RT-PCR as described above. A lymphoblastoid cell line (LCL) established from a patient in which a PTC is created due to the mutation in the *SPG4* gene (unpublished data by H. U., K. F. and T. M.) was employed as a positive control for NMD. Compared with the positive control where the levels of the transcript containing PTC were markedly increased upon the treatment with puromycin (Fig. 3B, lane 2, mutant), the transcripts of *PTCH12b* were only marginally elevated upon the treatment (Fig. 3B, lanes 4, 6, and 8). Similar results were obtained using another NMD inhibitor, cycloheximide (data not shown). This implies that this isoform undergoes NMD to a limited extent and is already expressed at low abundance independently of NMD in most tissues.

PTCH12b functions as a dominant negative isoform

We performed a functional analysis of PTCH12b using a GLI-responsive luciferase reporter in I-23 medulloblastoma cells. The binding of Shh to its receptor activates a signaling cascade that ultimately leads to an increased activity of the GLI family of transcription factors. The luciferase activities were suppressed when I-23 cells were transfected with plasmids for PTCH, but not with a plasmid for PTCH12b, consistent with PTCH being a suppressive component of the Shh receptor. This also implies that there is a basal level of leakage activity of Smo that excess PTCH prevents in the apparent absence of Shh. However, this suppression by PTCH was relieved when cotransfected with a plasmid for PTCH12b (Fig. 4A). Taken together, these results imply that the PTCH12b protein has a dominant negative effect on PTCH. In order to investigate the subcellular localizations, PTCH12b, as well as PTCH, both tagged with myc at their N-terminal ends, was expressed in HeLa cells and stained with an anti-myc antibody followed by confocal microscopy. PTCH was mainly localized in cytoplasmic vesicular structures as previously reported [19], and no significant difference in localization was observed between PTCH and PTCH12b (Fig. 4B and C). Therefore, it is unlikely that the dominant negative function of PTCH12b is due to its subcellular localization dis-

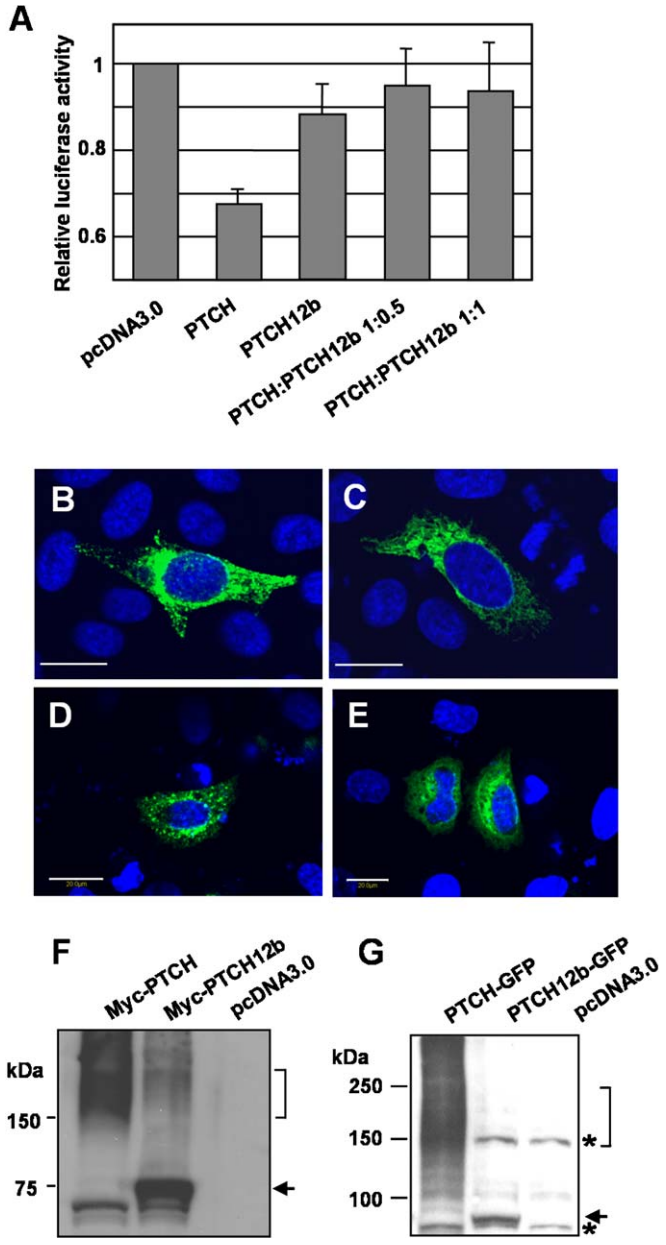

Fig. 4. Functional analysis of *PTCH12b*. (A) I-23 cells were transfected with various combinations of expression plasmids indicated at the bottom, together with a construct for a GLI-responsive luciferase reporter, 8 × GLI-Luc. Twenty-four hours after the transfection, cells were harvested and subjected to a luciferase assay. Shown are the representative data obtained in two independent experiments with triplicates in each experiment. (B–E) Subcellular localization of PTCH proteins. The expression patterns of myc-tagged (B and C) and EGFP-tagged (D and E) PTCH (B and D) or PTCH12b (C and E) in HeLa cells were examined using confocal microscopy. The nuclei were counterstained with Hoechst33342. Bar, 20 μm. (F and G) Western blotting was performed using protein samples obtained from the HeLa cells described above. Anti-c-myc (F) and anti-GFP (G) antibodies were used as a primary antibody. Asterisks indicate non-specific bands.

tinct from PTCH. Similar results were obtained when the both isoforms tagged with enhanced green fluorescent protein (EGFP) at their C-terminal ends were expressed (Fig. 4D and E). Comparative levels of protein expressions of both PTCH and PTCH12b with expected sizes were confirmed by Western blotting (Fig. 4F and G), indicating that the stability of the PTCH12b protein is similar to that of PTCH.

Discussion

Exon 12b in the human *PTCH* gene is conserved in mice and expressed in a brain- and heart-specific manner in both species. According to a recent report by Pan et al., alternative exons with the potential to introduce PTCs upon exon inclusion are not usually conserved between humans and mice [20], suggesting some biological significance of exon 12b.

The precise mechanism of how Ptc12b/PTCH12b functions as a dominant negative isoform remains to be elucidated. Recently, two mutant forms of the Ptc1 protein, G509V and 1130X, have been reported to be dominant negative forms, at least in *Drosophila* [21–23]. Whereas 1130X accumulated strongly along the plasma membrane, G509V and wild type Ptc1 protein localized mainly in the cytoplasmic vesicles, indicating that their mode of action is different [22,23]. Our isoform localized in the cytoplasm. We failed to detect a significant difference in the subcellular localization between PTCH and PTCH12b. It would be interesting to see the *in vivo* function of Ptc12b/PTCH12b using animal models.

The important question would be whether truncated PTCH proteins generally function in a dominant negative manner, because most of the mutations found in patients with NBCCS lead to the truncation of the PTCH protein due to the frameshift or nonsense mutations [24,25]. *Ptc1*$^{-/-}$ mice are embryonic lethal due to the failure of neural tube closure and abnormal development of the heart [26]. Therefore, if truncated PTCH proteins are generally dominant negatives, then the patients with NBCCS should have a phenotype similar to that of *Ptc1*$^{-/-}$ mice, which is not the case. There are at least three explanations regarding this issue. First, mRNA with a frameshift or nonsense mutation may be expressed less than the wild type through NMD-dependent and/or independent mechanisms [20]. Second, at least truncated proteins with a large C-terminal deletion are unlikely to function as a dominant negative. Third, the sensitivity to the perturbation of SHH signaling may be species dependent. For example, mutations in the *SHH* gene are found in some of the children with autosomal dominant holoprosencephaly [27,28], whereas a phenotype resembling human holoprosencephaly is found in *Shh*$^{-/-}$ mice, but not in *Shh*$^{+/-}$ mice [29].

Synthesis of large amounts of C-terminally truncated polypeptides encoded by PTC-containing mRNA is avoided by a splicing- and translation-dependent NMD. Therefore, we wondered why *Ptc12b*/*PTCH12b* is expressed at high levels in certain tissues. Since NMD inhibition resulted in a limited amount of increase in expression of *PTCH12b*, we concluded that this isoform is already present at low levels independently from NMD in most tissues,

and that in the brain and some of the medulloblastomas it is abundantly expressed through a mechanism distinct from the inhibition of NMD. This conclusion is not surprising considering the recent report that only a fraction of PTC-introducing AS events are significantly regulated by NMD [20].

Although a relatively low frequency (10–20%) of sporadic medulloblastomas carry *PTCH* mutations [8], microarray analysis revealed that almost all medulloblastomas with desmoplastic histology are characterized by activation of the SHH signaling pathway [30]. Given the dominant negative function of PTCH12b and the detection of this isoform in the cerebellum and medulloblastoma, it is intriguing to speculate that *PTCH12b* plays an important role in the development of medulloblastoma. In our experiment, 1 out of 5 medulloblastoma cell lines expressed this isoform, whereas 2 out of 2 medulloblastoma tissues expressed this isoform. This may reflect the recent report that Shh activity is down-regulated in cultured medulloblastoma cells [31]. Tumor-specific AS is not a rare event based on a genome-wide computational screen [32]. However, the functional significance of respective protein isoforms generated by these ASs in oncogenesis has yet to be clarified. In NBCCS patients, 65 out of 132 *PTCH* mutations (49%) are localized in the second half of the protein (exon 13 or more downstream). Interestingly, in sporadic medulloblastomas, 16 out of 23 mutations (70%) are found in this region [33], implying that the gene structure encoding *PTCH12b* is more frequently preserved in sporadic medulloblastomas. Although more cases are needed to be investigated, consideration of not only the total expression levels of *PTCH* but also the expression of this particular *PTCH* isoform may help classify medulloblastomas and predict the clinical outcome of the children with medulloblastoma. Lastly, it should be noted that 3% of the individuals with NBCCS are known to have cardiac fibromas [34] and the heart is another tissue where *PTCH12b* is expressed.

Acknowledgments

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