A novel first exon of the *Patched1* gene is upregulated by Hedgehog signaling resulting in a protein with pathway inhibitory functions

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Abstract Patched homolog 1 (*PTCH1*) is a key component of the Hedgehog (HH) signaling pathway with three alternative first exons, but only exon 1B transcription depending on HH activation. Here, we show that in both human and mouse a novel *PTCH1* first exon (1C) is expressed. Exon 1C transcription is upregulated by HH signaling, but the resulting PTCH1-1C protein has a lower capacity for pathway inhibition than PTCH1-1B.

Keywords: *PTCH1* tumor suppressor gene; Splice variants; Alternative transcription; Hedgehog signaling; GLI1; SMO agonist

1. Introduction

Hedgehog (HH) signaling is one of the major pathways for developmental morphogenesis, and disturbances in this regulatory system may induce carcinogenesis and organismal malformation (see recent reviews, [1,2]). A number of components of this pathway are known including the *HH* gene family (*SHH*, *IHH* and *DHH*), *Smoothened* (*SMO*), the *GLI* gene family and the *Patched* genes (*PTCH1* and *PTCH2*) that act as negative regulators of HH signaling. The binding of HH proteins to PTCH allows activation of SMO and subsequently of transcription factor GLI, resulting in upregulation of target genes. Dysregulation of the inhibitory role of PTCH1 leads to malformation and carcinogenesis, exemplified by basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma [2–4]. The role of PTCH1 during embryonic development has been investigated using knock out mice [5,6]. *Ptch1*$^{-/-}$ mice have neural tube defects and die during embryogenesis while *Ptch1*$^{+/-}$ mice have skeletal defects and develop tumors, as is the case with human heterozygotes.

Recently it has been found that over 74% of human genes are characterized by alternative splicing variation, and at high frequency these events occur in the 5′ untranslated regions [7]. This suggests that a major pathway for the complexity of expression of the human proteome may depend on the utilization of alternative first exons and consequently promoter regions. In the case of the *PTCH1* gene, we have previously identified three unique first exons by the use of the 5′ RACE methodology [3,8,9]. PTCH1-1B includes the alternative first exon 1B and encodes a full-length PTCH1 protein that can completely inhibit SMO activity. PTCH1-1B does not represent a major transcript in human adult tissues but its expression is regulated by the HH signaling pathway, as exemplified by its upregulation in basal cell carcinoma [9,10]. The other first exon variants are ubiquitously expressed in human adult tissues but are not affected by HH signaling. Both PTCH1-1 and PTCH1-1A encode the same short form of PTCH1, which is translated from an ATG codon within exon 3. This short form can interact with HH family proteins just as PTCH1-1B, but is incapable of fully suppressing HH signaling [9].

Here, we present evidence for a novel first exon of *PTCH1*, named exon 1C, demonstrate that its expression is relatively abundant in comparison with the other first exon variants, and moreover is upregulated by HH signaling. Additionally PTCH1-1C can act as a pathway inhibitor. These findings may provide new clues for a better understanding of the role of PTCH1 in HH signal transduction.

2. Materials and methods

2.1. Tissue distribution analysis

Pairs of initial or nested primers were designed for each PTCH1 alternative first exon. The sequence of the primers used is given in Table 1. Multiple tissue cDNA panels from BD Biosciences (CA, USA) were used with the primers obtained from Cybergene AB (Huddinge, Sweden). Each reaction consisted of 1× buffer B (MBI Fermentas, Vilnius, Lithuania), 2 mM $MgCl_2$, 0.2 mM for each dNTP, 0.5 μM forward primers for each alternative first exon, 1.0 μM reverse primer for exon 2, 1.0 μl of *Taq* DNA polymerase (5 units/μl, MBI Fermentas), and 1 ng of cDNA in a total volume of 25 μl. Thirty cycles with 30 s at 95 °C, 30 s at 54 °C, and 1 min at 72 °C were performed on a Perkin–Elmer thermocycler. For nested PCR, 0.5 μl of the initial amplification products was used. Amplifications without exogenous cDNA were used in all sets of experiments as a negative control. The nested products were analyzed on a 4% NuSieve 3:1 agarose gel (FMC BioProducts, ME, USA). All PCR products were sequence-verified (Cybergene AB).

2.2. Cell culture and expression analysis

The human embryonic kidney cell line (Hek293) and the murine fibroblast cell line NIH3T3 were cultured as described before [10].

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Abbreviations: PTCH1, patched homolog 1; PTCH1-1, -1A, -B and -1C, PTCH1 including the alternative first exons 1, 1A, 1B and 1C, respectively; HH, Hedgehog; SMO, smoothened; SAG, SMO agonist; Hek293, human embryonic kidney cell line 293; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, haemagglutinin; GLIBS, GLI-binding site

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Table 1
Sequence of the PCR primers used

	Human	Mouse
E1-F	5′-CAGCGCCGGGGAAATG	5′-AGAGCAGCCTGCGCCCA
E1-F nested	5′-TCCCTTTGCACTCOGCT	5′-TCCTTGTGCACTCGGCT
E1A-F	5′-ACCACGTGGATCCCCAC	5′-CACCACGTGGACCTCGC
E1A-F nested	5′-GGGGGAACCCGCAATAT	5′-GACCACGGTTGCTGTAGA
E1B-F	5′-AGCGGCTGTATCGGTGC	5′-GGCCTCGGCTGGTAACG
E1B-F nested	5′-GACCGGGACTATCTGCA	5′-GACCGGGACTATCTGCA
E1C-F	5′-GGCCATGGAACTGCTTAATA	5′-GGCCATGGACCTGCTTAATA
E1C-F nested	5′-GACGACAGGGGAGACAAAG	5′-GGCGACAGGGCAGACAAAG
E2-R	5′-TGAGOAGGCCCACAACC	5′-TGAGGAGACCCACAACC
E2-R nested	5′-TTCGCTCTCAGCCACAG	5′-TTCGCTCTCAGCCACAG
GAPDH-F	5′-ACAACAGCCTCAAGATCATCA	5′-ACAACTCACTCAAGATTGTCA
GAPDH-R	5′-GTCCACCACTGACACGTTG	5′-ATCCACGACGGACACATTG

5×10^5 NIH3T3 cells were plated on 10 cm dishes. One day later, the medium was changed to DMEM containing 0.5% fetal bovine serum (FBS) with or without 100 nM SAG (SMO agonist, provided by Dr. Jan Bergman, Karolinska Institute). After incubation for 48 h, total RNA was isolated by the RNeasy kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

For reverse transcription, 5 μg of total RNA, 4 μl of a 2.5 mM dNTP mixture (2.5 mM each), and 0.5 ng of oligo $(dT)_{18}$(A/C/G)(A/C/G/T) primer in a total volume of 12 μl were denatured at 65 °C for 5 min. After cooling the mixture on ice, 4 μl of 5×RT buffer, 2 μl of 0.1 M DTT, and 1μl of Ribonuclease Inhibitor (TAKARA BIO, Shiga, Japan) were added. After incubation at 45 °C for 2 min, 1 μl of Superscript RT (Invitrogen) or water (negative control) was added. Then the mixture was incubated at 45 °C for 90 min. The reaction was stopped by heat inactivation at 75 °C for 15 min. The amount of single-stranded cDNA used for subsequent PCR amplification was equalized by monitoring the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression as a quantitative control (Table 1). PCR analysis was performed using the same conditions as for tissue distribution analysis but with mouse-specific primers (Table 1).

2.3. Expression of cDNA constructs in mammalian cells

For construction of the PTCH1-1C expression plasmid, we first performed a PCR amplification using the Expand PCR System (Roche Diagnostics, Basel, Switzerland) on IMAGE-5170834 clone (MRC-geneservice, Cambridge, UK), with PTCH1 specific primer set (5′-GCGGCTAGCCGGCCGCGGGGGGC and 5′-GCGCCTAGG-AGGTATGCTGTCCCAGACT). The PCR product and a full-length 3′-FLAG-tagged PTCH1-1B expression plasmid were digested with *Nhe*I and *Avr*II restriction enzymes and then ligated allowing the N-terminal region of PTCH1-1B to be replaced by the PCR product containing exon 1C (starting at nucleotide positions 1 of exon 1C). The construct was verified by sequencing.

The other expression constructs used have been described in previous reports, the full-length 3′-FLAG-tagged PTCH1 constructs with the alternative first exons, 1 and 1B [9], the HA (haemagglutinin)-tagged human full-length GLI1 [11], and the activated human SMO-M2 [12].

Expression constructs for PTCH1 splice variants and other pathway components were transfected into human embryonic kidney Hek293 cells and murine NIH3T3 cells using FuGENE 6 (Roche Diagnostics). Western blotting were performed as previously described [9,13]. The polyclonal antibodies to FLAG epitopes were purchased from Stratagene (CA, USA).

2.4. Reporter assays

To examine the activity of the *PTCH1* exon 1C promoter, we PCR amplified four fragments, each corresponding to part of the region around exon 1C (Fig. 2A). PCR reactions were performed using the Expand PCR System and a BAC clone (RP11-43505, BACPAC Resources, CA, USA) harboring the human *PTCH1* gene. Plasmids B1 and B2 were constructed by amplification of B1-F (5′-GCGG-CTAGCGGAGCAAATGTCAATCCGT) or B2-F (5′-GCGGCT-AGCCGGCCGCGGGGGGC), with the same reverse primer (B-R; 5′-GCGAGATCTAAGCAGTTCCATGGCCCT). Site-directed mutagenesis of the putative GLI1 binding site was carried out by replacing GACCACCCA to GACGTGGGA using mut-primer (5′-GTTCCATGGCCCTCGGCGTCCCACGTCTGCCGCG). The four PCR products were ligated into the pGL3-basic luciferase reporter vector (pGL3B, Promega, WI, USA). To generate constructs P1 and P1mut, double-stranded DNAs were prepared by annealing wild-F (5′-CTAGCTGGCGCGGCAGACCACCCACGCCGAGGGCA) to wild-R (5′-GATCTGCCCTCGGCGTGGGTGGTCTGCCGCGC-CAG) or mut-F (5′-CTAGCTGGCGCGGCAGACGTGGGACGC-CGAGGGCA) to mut-R (5′-GATCTGCCCTCGGCGTCCCA-CGTCTGCCGCGCCAG) and cloned into the *Nhe*I and *Bgl*II sites of the pGL3-promoter vector (pGL3P, Promega). All constructs were verified by sequencing. 8xGLIBS-luc and 8xmGLIBS-luc were prepared as described elsewhere [14].

Confluent cultures of NIH3T3 cells were trypsinized and plated at 1:6 dilution on 24-well plates. The next day, cells were transfected with 0.5 μg of PTCH1 exon1C-luciferase reporter plasmids (B1, B2, B1mut, B2mut, P1 or P1mut), 0.5 μg of expression constructs (HA-tagged human GLI1 or pCMV5) and 50 ng of *Renilla* luciferase (pRL-SV40) as transfection control. For the functional analysis of the PTCH1 splice variants, cells were transfected with 0.25 μg of luc reporter (8xGLIBS-luc or 8xmGLIBS-luc), 0.25 μg of expression constructs (HA-tagged human GLI1, N-terminal SHH or activated SMO-M2) and 50 ng of pRL-SV40 with or without 0.5 μg of PTCH1 expression constructs (FLAG-tagged PTCH1-1, PTCH1-1B, or PTCH1-1C). 3 μl of FuGENE 6 transfection reagent were always used. After 24 h, the medium was changed to a low-serum medium (0.5% FBS), and cells were allowed to grow for an additional 1 or 2 days before harvesting. Normalized luciferase activity was determined with the dual-luciferase reporter assay system (Promega) using the FB12 Luminometer (Berthold Detection System, Pforzheim, Germany) according to the supplier's recommendations. All experiments were repeated independently at least three times and measurements in each experiment were performed at least twice.

3. Results

3.1. In silico screening for PTCH1 splicing variations

To identify novel splice variants in HH signaling components, we screened EST sequences from the web-available databases, GRL (Gene Resource Locator, http://grl.gi.k.u-tokyo.ac.jp/) [15], EASED (Extended Alternatively Spliced EST Database, http://eased.bioinf.mdc-berlin.de/) [16], ASAP (The Alternative Splicing Annotation Project, http://www.bioinformatics.ucla.edu/ASAP/) [17] and NCBI (National Center for Biotechnology Information). Among many candidates, a human EST for *PTCH1* (IMAGE-5170834) was noted. This EST comprised *of PTCH1* sequences (NM_000264) from exon 2 to exon 5, and of unknown sequences (589 nucleotides) upstream of exon 2. The unknown sequences were found to completely match with human genomic DNA that locates about 10

kb upstream of *PTCH1* exon 2 and thus were named exon 1C (Fig. 1A). The DNA sequence of this region is highly conserved between human and mouse (Fig. 1B) and the exon-intron boundary at the 3′ end of exon 1C retains the consensus 5′ splice sequence (exon 1C ··· TG/gtgagt ··· intron). To confirm the expression of exon 1C, we made forward primers for each of PTCH1 first exon (1, 1A, 1B, and 1C) and reverse primers for exon 2 and compared the expression pattern of the PTCH1 splice variants [9] using cDNA panels (Fig. 1C). As reported previously, exons 1 and 1A were expressed ubiquitously in all eight human adult tissues tested. Since exon 1B expression is lower than that of 1 and 1A [9], it was difficult to detect it under the experimental conditions used. Exon 1C expression was observed in human brain, placenta, lung, kidney and pancreas.

In contrast to the relative similar expression of the PTCH1 variants in adult tissues, a significant tissue specificity was observed in human fetal tissues, highlighting the likely importance of exon 1 variation during early development. These results clearly demonstrated that PTCH1 can express exon 1C and that this transcript appears to be the major one in several tissues.

3.2. Regulation of expression of PTCH1 first exons by the Hedgehog signaling pathway

It is known that the expression of exon 1B is upregulated by HH signaling. To examine the relationship between the expression of exon 1C and HH signaling, we searched for consensus GLI1-binding motifs, 5′-GACCACCCA within a 2 kb genomic segment that includes exon 1C and its 5′ flanking region and identified one candidate site at position +218 to +226 (GLI-binding site, GLIBS; Fig. 2A) of the EST clone. This finding of a GLIBS in the 5′-untranslated region suggests the possibility that PTCH1-1C expression may also be regulated by HH signaling. Notably, the GLIBS sequence was found to be fully conserved in the corresponding region of mouse *Ptch1*, in line with the significant similarity of the alternative first exons in the two species. To verify that this is indeed the case, we compared the expression of the *Ptch1* first exons in NIH3T3 cells with or without SAG, a chlorobenzothiophene-containing

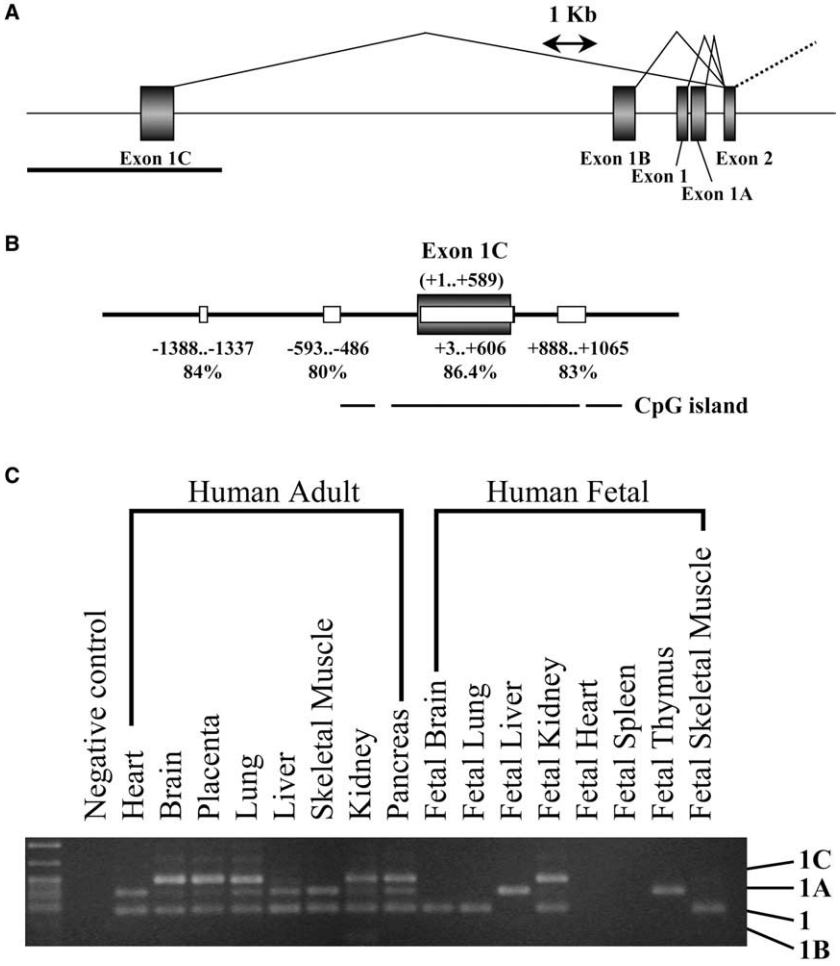

Fig. 1. Gene structure and expression of *PTCH1* exon 1C. (A) Genomic structure of the *PTCH1* region that includes the first exons and exon 2. Exons are represented by black boxes with the splicing pattern indicated. (B) Comparison of the genomic DNA sequence of exon 1C and its flanking regions (underlined in panel A) between human and mouse. White boxes represent highly conserved regions with percent identity and nucleotide position in human indicated. The position of the CpG islands identified is also shown. (C) Tissue specific expression of the alternative PTCH1 first exons. The RT-PCR methodology with primers for each of the first exons and for exon 2 was used. The predicted size of the RT-PCR fragments is as follows: Exon 1C, 233 bp; Exon 1B, 122 bp; Exon 1A, 192 bp; Exon 1, 141 bp.

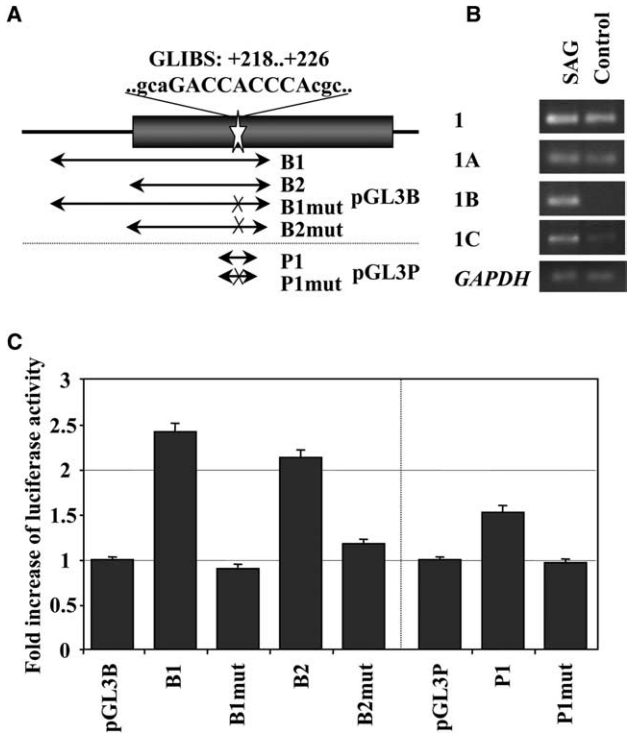

Fig. 2. Regulation of *PTCH1* exon 1C expression. (A) Presence of a GLIBS in the exon 1C region. The black box indicates the exon 1C. The putative GLIBS is shown above the black box and its nucleotide position indicated. The structure of the regulatory constructs generated (B1, B2, B1mut, B2mut, P1, and P1mut) is also shown with X indicating the mutation of the GLIBS. (B) Up-regulation of the PTCH1 mRNAs including exons 1C or 1B by SAG treatment. NIH3T3 cells were incubated with or without SAG and expression of the *Ptch1* first exons was determined by RT-PCR. (C) Effects of GLI1 expression on the exon 1C promoter activity. NIH3T3 cells were transfected with a GLI1 expression construct as well as with each of the reporter constructs of panel A.

HH pathway agonist (Fig. 2B) [18]. As expected, expression from exon 1C was upregulated after treatment with SAG, as also observed for exon 1B. The expression from either exon 1 or 1A was not affected by SAG.

To examine whether the identified GLIBS might be responsible for the HH signaling activation of this promoter, we cloned various fragments from this region upstream of the luciferase gene and performed reporter assays using NIH3T3 cells (Fig. 2A and C). As expected, constructs B1, B2, and P1 stimulated luciferase activity approximately 2-fold after overexpression of GLI1 protein, while mutations of the GLIBS (constructs B1mut, B2mut, and P1mut) suppressed the GLI1 stimulation. These results suggested that the identified GLIBS functions as a *cis*-element responsible for the signaling-dependent expression of exon 1C.

3.3. Functional properties of PTCH1 proteins with alternative N-terminal domains

The deduced amino acid sequence of PTCH1-1C indicates that translation initiates at an ATG codon at the very end of exon 1C (positions +587 to +589 of the EST clone). Therefore the putative PTCH1-1C protein lacks 67 amino acids from the N-terminal tail of PTCH1-1B. PTCH1-1 and PTCH1-1A are considered to initiate translation from an ATG codon in exon 3, and thus lack not only the N-terminal tail but also the first transmembrane region.

To better understand the biological function of the expressed first exons of the PTCH1 gene, we also made a FLAG-tagged PTCH1-1C expression construct. The PTCH1 splice variants, after transfection into Hek293 cells, were solubilized by a lysis buffer containing 1% NP-40 and Western blot analysis indicated that the level of PTCH1-1C is comparable with the other splice variants. Unexpectedly, subsequent solubilization of the pellet by a lysis buffer containing 1% SDS showed that the majority of PTCH1-1B and PTCH1-1C was localized in the NP-40 insoluble fraction and this contrasts PTCH1-1. Moreover the level of PTCH1-1B in the pellet was higher than that of PTCH1-1C (Fig. 3A).

To further investigate the localization of the PTCH1-1C protein in mammalian cells, we also transiently transfected the FLAG-tagged PTCH1 splice variants into Hek293 or NIH3T3 cells and observed the expressed proteins by immunofluorescence staining. Microscopic analysis indicated a primarily cytoplasmic localization with no major differences among the proteins tested (data not shown).

PTCH1 acts as a negative regulator of the HH signaling pathway, however the effectiveness of this inhibition depends on which first exon is expressed [9]. To compare the capacity of PTCH1-1C to downregulate signaling relative to other splice variants, we performed functional assays utilizing a 8xGLIBS-luc reporter construct and overexpressing SMO in NIH3T3 cells [9,14]. As reported previously, PTCH1-1B could fully inhibit SMO activation. PTCH1-1C could also inhibit signaling two days after transfection but lost this capacity after an additional day in cell culture, while PTCH1-1B did not (Fig. 3B). These results suggest that the N-terminal tail of PTCH1 and more specifically the 67 terminal amino acids have a critical role for the negative regulation of HH signaling.

4. Discussion

In this report, we demonstrated the presence of a novel *PTCH1* first exon conserved in both human and mouse, whose expression is regulated by the HH signaling pathway.

We have already reported the existence of three *PTCH1* first exon variants and examined their functional differences [9,10]. These three first exons are located at a narrow region, approximately 2 kb upstream of exon 2 in the human and mouse genome. PTCH1-1 and PTCH1-1A mRNAs encode short forms of PTCH1 that lack the N-terminal tail and the first transmembrane region. As shown here and in previous reports, expression of these first exons tends to be observed ubiquitously in human adult tissues. PTCH1-1B mRNA encodes the longest form of PTCH1. Its expression is regulated by the HH signaling pathway, therefore has only been observed in tissues or cells that are exposed to HH ligands. On the other hand, the novel first exon of *PTCH1*, exon 1C, is uniquely located about 10 kb upstream of *PTCH1* exon 2, and allows the synthesis of a PTCH1 protein lacking part of the N-terminal tail. Additionally, exon 1C expression is also activated by the HH signaling pathway.

Functional analysis of PTCH1 revealed dramatic differences in both the capacity of PTCH1-1, PTCH1-1B and PTCH1-1C to downregulate signaling as well as in the duration of these effects. Although we could not detect a sharp contrast in cellular localization by immunofluorescence staining, Western blot

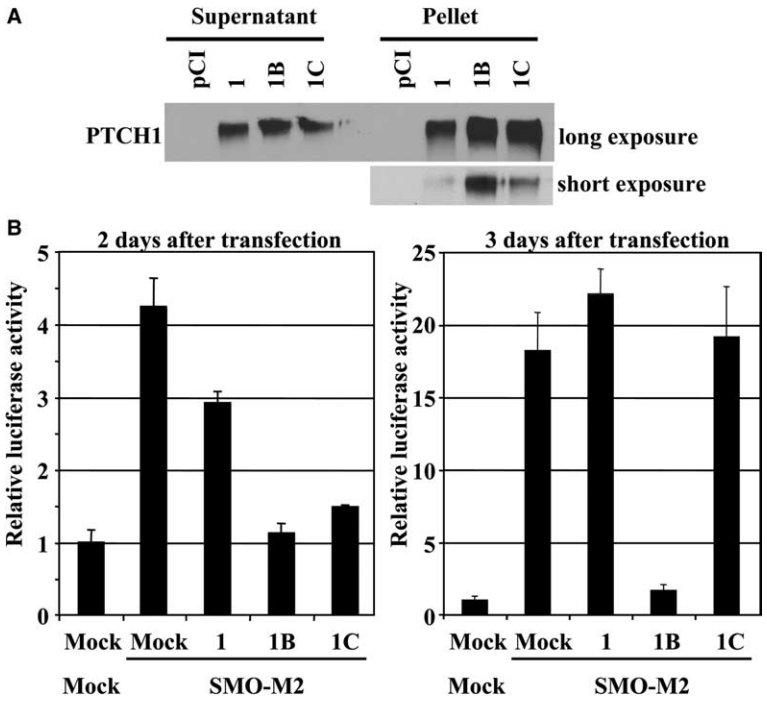

Fig. 3. Functional properties of PTCH1-1C. (A) Expression of exogenous PTCH1 splice variants in Hek293 cells. Western blotting was performed using the anti-FLAG antibody. Note that all variants are expressed at comparable levels in the NP-40 soluble fraction (supernatant) however the majority of PTCH1-IB and PTCH1-1C is present in the insoluble fraction (pellet) and is detected after subsequent solubilization by SDS. (B) Influence of overexpression of PTCH1 splice variants on SMO activation of a GLI reporter construct. The luciferase activity was measured at either 2 days (left panel) or 3 days (right panel) after transfection.

analysis indicated that the total amount and the solubility of PTCH1 are affected by the structure of the N-terminal region. Our results suggest that the role of the N-terminal domain is not only in suppressing HH signaling, but may also regulate PTCH1 solubility and expression levels. Moreover the fact that downregulation of HH signaling parallels increased protein expression and decreased solubility suggests that these properties could be interconnected. However, at this point the exact role of translational regulation, protein stability and/or inherent capacity of SMO downregulation can not be determined. In any case our data support the possibility that the expression of alternative first exon splice variants is a regulatory system for HH signaling during development in the embryo and/or homeostasis in adult tissues. An outline of the unique features of each of the PTCH1 exon 1 variants is given in Table 2.

Previously, we have reported that the *PTCH1* paralogue, *PTCH2*, expresses a splice variant lacking exon 9 and/or exon 10 [19]. This region is part of a conserved sterol-sensing domain and affects the inhibitory properties of PTCH2. PTCH1 splice variants lacking this same region are also expressed in mammalian cells [20] (Rahnama, F., et al., unpublished data). This provides additional evidence that HH signaling may be controlled by a more complex system than originally thought, involving regulated splicing variations.

In the past, despite the fact that exon 1B expression does not represent the major transcriptional output of *PTCH1* compared to exons 1 or 1 A, the role of PTCH1 in disease or developmental morphogenesis was centered only on the exon 1B transcript. Certainly, we realize that mRNA levels may not directly relate to protein levels, and moreover PTCH1-1B has the

Table 2
Schematic representation of the similarities and differences among the four unique *PTCH1* first exons and the corresponding protein products

	Exon 1	Exon 1A	Exon 1B	Exon 1C
Tissue distribution	Universal		Tissue specific	
GLI binding site	No		Yes	
Influence of HH signaling	No effect		Up-regulation	
Conservation of DNA sequence between human and mouse	High	Low	Medium	High
	PTCH1-1 (PTCH1-1A)		PTCH1-1B	PTCH1-1C
Position of 1st ATG	Exon 3		Exon 1B	Exon 1C
1st Transmembrane domain	No		Yes	
N-terminal tail	No		Yes	Yes, but lack of 67 a.a.
Subcellular localization	Cytoplasm			
Solubilization by 1% NP-40	Supernatant ≅ Pellet		Supernatant < Pellet	
Supernatant protein level	Low		Low	Low
Pellet protein level	Low		High	Medium
Inhibition of HH signaling	Weak		Strong	Intermediate

highest capacity to inhibit the HH signaling pathway. One possibility is that PTCH1-1B is the form that is involved in the feedback loop that down regulates signaling, while 1 and 1A may generally act to limit the spreading of the HH signal. PTCH1-1C could have a role in both scenarios. Downregulating signaling may represent a first response while limiting ligand spreading may reflect a secondary event. Unraveling the role of PTCH1 in development and disease will certainly require further investigations into the details of the function of each splice variant and the interplay among them.

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