

## CHAPTER 2

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# The Patched Receptor: Switching On/Off the Hedgehog Signaling Pathway

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### Abstract

The activities of the Hedgehog (Hh) protein family are central to the growth and patterning of developing tissues and organs in many different organisms. Hh proteins are secreted ligands synthesized in discrete regions. The receptor of Hh is Patched (Ptc) and it is expressed in the cells close to the source of Hh. Ptc binds the ligand and transduces a signal which is modulated depending on the context and the concentration of Hh received. Hh and several molecular components of the pathway were first identified and characterized in *Drosophila*, providing relevant milestones to our understanding on how the Hh signal is transduced. However, important gaps in the pathway still need to be elucidated. Some of these gaps converge on the Ptc receptor and its intriguing mechanisms of Hh reception and signal transduction. Mutations of Ptc that prevail both in animal and human populations are giving some clues on crucial aspects of its function. Patients bearing mutated forms of Ptc suffer a variety of serious diseases. Molecular and cellular studies in *Drosophila* have given us a clue of the function of Ptc receptor such as the normal topology and/or sorting of the receptor. Thus, a widened knowledge of the function of Ptc might help to design specific therapies for these disorders. This chapter focuses on recent advances that shed some light on how Ptc may operate in the cell.

### The Hedgehog Signaling Pathway

Several genes of the Hh pathway were first identified in the fly *Drosophila melanogaster* and later in vertebrates. Many of the names of the genes involved in the Hh signaling pathway were originally descriptive of the phenotype manifested in mutant *Drosophila* larvae. Wild-type larvae show a clearly segmented pattern due to alternate bands of denticles in ventral position, whereas the inter-band space is naked. During a screening for mutations that affected the segmental pattern, Nüsslein-Volhard and Wieschaus described a group of mutants with alterations in the patterning of the segments.<sup>1</sup> Instead of the wild-type alternate belts of denticles and naked cuticle, Hh mutants showed a continuous lawn of denticles, which gave the larva a resemblance to a hedgehog (Fig. 1B), and Ptc mutants showed patches of denticles (Fig. 1C).

The active Hh protein is a peptide that has undergone an autocatalytical processing in which the peptide is also modified at its N- and C-termini by palmitoyl and cholesterol adducts, respectively. Hh is secreted by discrete subsets of cells within a developing organ in a process that seems mediated, at least in part, by the transmembrane protein Dispatched (Disp), which has a structure very similar to Ptc. In the wing imaginal disc of *Drosophila*, Hh is

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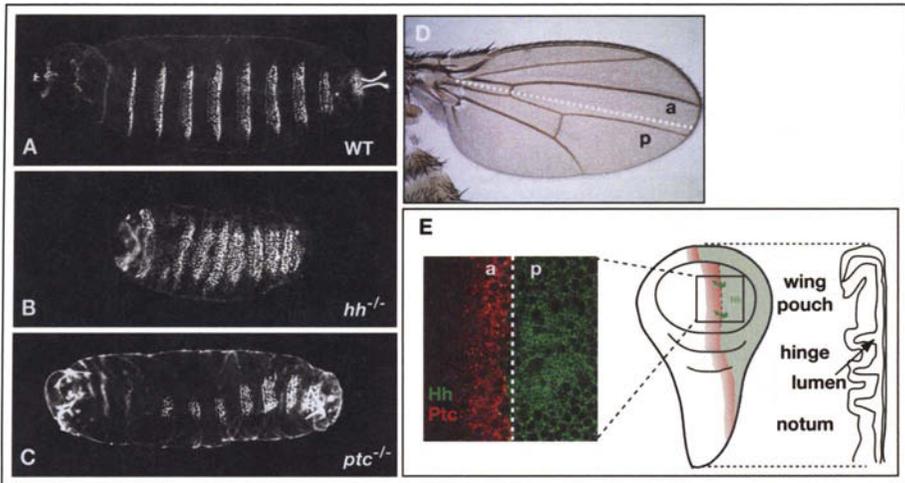


Figure 1. Two model systems in *Drosophila melanogaster*: The larva cuticle and the wing imaginal disc. A) Wild-type ventral larva cuticle showing its regular pattern of denticles. B) Hh mutant ventral cuticle. C) Ptc mutant ventral cuticle. D) Adult wing. The dashed line marks the boundary between the anterior (a) and posterior compartment (p). These compartments are already present in the wing imaginal disc, the wing primordium (E; front and lateral view) in the larva. Hh is produced in the posterior compartment (green) and signals to cells in the anterior compartment. Ptc expression is induced by Hh signaling (red). A color version of this figure is available online at <http://www.eurekah.com/chapter.php?chapid=2439&bookid=166&catid=82>.

expressed in the cells of the posterior compartment and signals to the cells of the anterior compartment, which in turn express the Ptc receptor (Fig. 1D,E). A graded and short-range response to Hh signaling occurs in the Hh receiving cells (Fig. 1E). Ptc, a 12-transmembrane protein, is a negative receptor because it keeps the Hh pathway silenced in its unliganded state.<sup>2</sup> In the absence of Hh, Ptc suppresses the activity of Smoothened (Smo), a 7-transmembrane protein that is the positive modulator of the pathway. Although Hh does not bind to Smo, without Smo there is no signaling. The target cells bind Hh by Ptc, which results in the activation of Smo, and, subsequently, in the activation of transcription factors: the Cubitus interruptus protein (Ci) in *Drosophila* and the orthologous Gli proteins in mammals (reviewed in ref. 10). One peculiarity of the Hh signaling pathway is that Ptc is up-regulated in response to increasing amounts of Hh.<sup>3-7</sup> Therefore, Hh controls both its own activity and its own spreading expressing high levels of the receptor.<sup>8</sup> By this means, a morphogenetic gradient is formed in the Hh receiving cells. Theoretical analysis of model systems built to explain how a morphogen gradient is formed indicates that this feedback is indeed required to give robustness to the gradient while maintaining its range of action.<sup>9</sup> Thus, according to one model, it is expected that fluctuations in ligand production have minor effects on the slope of the gradient, compared to a model in which the receptor has a constant, or ligand-independent level of expression.<sup>9</sup>

Since this chapter is particularly centered in the Ptc receptor, for a more general view on the Hh response network, the reader is referred to some other recent and exhaustive reviews.<sup>10-14</sup>

Although this sequence of events is well established, many molecular mechanisms connecting these and other components of the pathway remain elusive. Focusing in the case of the Ptc receptor, several fundamental questions remain unanswered: How does the Ptc receptor recognize the Hh ligand? What happens after the receptor-ligand complex is formed? Is it internalized or does it remain in the membrane before the signal is transduced? How does the unliganded Ptc receptor repress the Smo activity? And how does the liganded Ptc derepress Smo activity?

## Sequence and Functional Analysis of Ptc

A single *ptc* gene is present in *Drosophila*, whereas two are present in vertebrates (*PTCH1* and *PTCH2* in humans; *Ptc1* and *Ptc2* in mice) (reviewed in ref. 10) and several *ptc*-related genes are found in the nematode worm *Caenorhabditis elegans*.<sup>15</sup> Ptc shows homology to bacterial proton-driven transmembrane molecular transporters,<sup>16,17</sup> and presents a conserved a Sterol-Sensing Domain (SSD) that overlaps between transmembrane segment 4 to 6, and also two extracellular large loops (reviewed in ref. 18). In general, SSDs are thought to function as a regulatory domain involved in linking vesicle trafficking and protein localization with processes such as cholesterol homeostasis and cell signaling.<sup>18</sup> Concretely, the SSD of Ptc seems to mediate the intracellular trafficking of Ptc, a process that might be essential to regulate Smo activity.<sup>19,20</sup> Other functional studies have assigned particular roles to regions of the protein with no obvious similarity to conserved domains. Thus, the two extracellular large loops are required for Hh binding,<sup>21</sup> the cytoplasmic C-terminus has been involved in the transduction of the signal,<sup>22</sup> and an intracellular small loop between the SSD and the following transmembrane domain has been reported to interact with cyclin B1 to regulate cell-cycle progression in vertebrates.<sup>23</sup> However, despite this emerging body of data, we still do not know the full sequence of molecular events that involves the reception of the Hh ligand and how Ptc regulates Smo.

PTCH2 is a 12-transmembrane protein as well and the two large extracellular loops and an SSD are also conserved (reviewed in ref. 24). However, both amino- and carboxy-termini are different from PTCH1. Similar differences are found between *Ptc1* and *Ptc2* in mice. PTCH1 and PTCH2 proteins are expressed differentially during the development of the epidermis, suggesting specific roles for each protein,<sup>25</sup> although the function of PTCH2 remains unclear to date. A recent study suggests that PTCH2 may act as a Hh receptor that tunes finely the signaling in various cellular environments.<sup>26</sup>

## Hedgehog Lipid Modifications and Morphogen Distribution

The lipid modifications on the Hh protein appear to regulate its activity and distribution. Hedgehog proteins undergo two sequential lipid modifications during their posttranscriptional maturation. Following cleavage of an N-terminus signal sequence upon entry into the secretory pathway, Hh proteins undergo an autoproteolytic reaction that results in an internal cleavage inside the 45-KDa precursors, between glycine-cysteine residues from a conserved GCF motif. After this, a cholesterol molecule is covalently added at the newly generated C-terminus of the proteins.<sup>27</sup> In *Drosophila*, a construct of Hh lacking the cholesterol moiety is active in signaling but not appropriately restricted spatially in its signaling activity.<sup>28</sup> Therefore, the cholesteryl moiety restricts the spatial deployment of the signal via insertion into the lipid bilayer of the cell membrane and also functions as an essential molecular handle for a proper intracellular and extracellular trafficking and localization of the signal (reviewed in refs. 29, 30). In addition, Hh proteins are palmitoylated on a highly conserved amino-terminal cysteine residue, the first of a pentapeptide CGPGR.<sup>31</sup> In *Drosophila* this palmitoylation is a total requirement in order to produce a fully active Hh signal. Although in vitro studies in vertebrates indicated that Hh mutant forms lacking the palmitoyl adduct retained significant activity,<sup>32,33</sup> knockout mice deficient in *Skn* (the murine ortholog of the *Drosophila* *Ski*, which catalyzes Hh palmitoylation) showed that Hh acylation is absolutely required for Hh long-range signaling.<sup>24</sup>

In summary, dual lipidation of Hh protein promotes membrane affinity and allows its association to sterol-rich membrane microdomains in *Drosophila*, and to lipid rafts in mammalian cells, that function as platforms for intracellular sorting and signal transduction.<sup>34</sup> The close association of Hh proteins to the plasma membrane due to the lipid modifications could provide a mechanism for restricting the range of their activities.

## Reception of Hh

Biochemical studies have shown that Hh monomers can form multimeric complexes in which the hydrophobic moieties have been proposed to be sequestered in the interior of the multimer, making the complex soluble and diffusible.<sup>35,36</sup> This could be relevant to the movement of Hh through the extracellular matrix<sup>37</sup> and to the reception of Hh by Ptc. On one hand, the restricted diffusion of Hh through the extracellular matrix might require the formation of these multimeric complexes of lipidated Hh. On the other hand, a multimeric Hh complex might have either a higher intrinsic affinity for Ptc, or maybe is capable of eliciting greater biological responses, for instance through receptor oligomerization, or binding to coreceptor proteins. In this context, structural studies on transmembrane transporters related to Ptc determined that these proteins operate as homotrimers.<sup>38</sup> Interestingly, a mutant version of Ptc that internalizes inefficiently is, however, localized to intracellular vesicles when coexpressed with a dominant negative Ptc.<sup>39</sup> This ptc inter-allelic complementation strongly suggests that Ptc has an oligomeric structure.

Several other proteins have been identified as capable of binding to Hh, raising the question of whether they are coreceptors or adjuvants involved in the reception of Hh. Especially, recent analysis in *Drosophila* have illustrated the critical roles of heparan sulfate proteoglycans (HSPGs) in developmental signaling pathways (reviewed in ref. 40). These large macromolecules are found at the cell surface and form part of the extracellular matrix. A major feature of these proteins is the attachment of long unbranched chains of repeating and sulfated disaccharides to specific serine residues in their protein core. Dally and Dally-like (Dly), two *Drosophila* glypican members of the HSPG family, has received great attention. A specific role in Hh signaling in the embryonic epidermis has been ascribed to Dly,<sup>41</sup> while both Dally and Dly seem to be functionally redundant in the wing disc,<sup>42</sup> indicating distinct activities of these two glypicans in the embryo and the wing disc. Hh signaling might also be regulated by other HSPGs in different tissues or developmental processes. For example, Trol, the *Drosophila* ortholog of Perlecan, seems to form a complex with Hh, and mutations in the gene encoding it cause neuroblasts to undergo cell cycle arrest in the larval brain.<sup>43,44</sup> In addition, *trol* is required for the neuroblast division induced by Hh.<sup>43</sup>

Two data favour a model in which the polyanionic branches of the HSPGs would act mainly as a molecular trap to keep the morphogens in touch with epithelium surfaces. First, the strongest loss-of-function phenotypes are achieved with mutants affecting the synthesis of sugar chains of the HSPGs;<sup>42</sup> and second, the fact that the sugar chains seem to be directly involved in the movement of other morphogens.<sup>45,46</sup> Hence, the HSPGs might restrict the morphogen diffusion to the environment of the extracellular matrix, and thus preventing a massive spreading to cavities such as the imaginal disc lumen. This lateral distribution of Hh would facilitate the encounter with Ptc. This scenario does not rule out the possibility of specific regulations involving Dally or Dly. In fact, in tissue culture experiments, Dally-like protein (Dlp), but not Dally was required for Hh signaling.<sup>47</sup> In addition, it has been shown that Dlp is specifically required for Hh signaling in the embryonic epidermis.<sup>41</sup> A Dlp-mediated regulation of Hh signaling might involve Dlp acting as a coreceptor, perhaps by transferring Hh to Ptc, or by forming a Hh-Dlp-Ptc interaction or stabilizing a Hh-Ptc complex (reviewed in ref. 48). In the case of the Wingless pathway (another morphogenetic signal), it has been elegantly shown that Dlp is cleaved specifically from the cell surface by Notum, a secreted enzyme, resulting in an adequate regulation of the pathway.<sup>49</sup>

## Hh Internalization and Signal Transduction

After receiving Hh, the receptor-ligand complex is internalized and the morphogen is thus depleted from the extracellular milieu. It was proposed that this internalization has a role shaping the Hh gradient.<sup>8,50</sup> This hypothesis has been reinforced by experiments that uncoupled the Hh sequestration and the Hh signaling using a Ptc mutant that is unable to sequester Hh and yet retains a normal capacity to mediate Hh signaling.<sup>39</sup> Furthermore, the

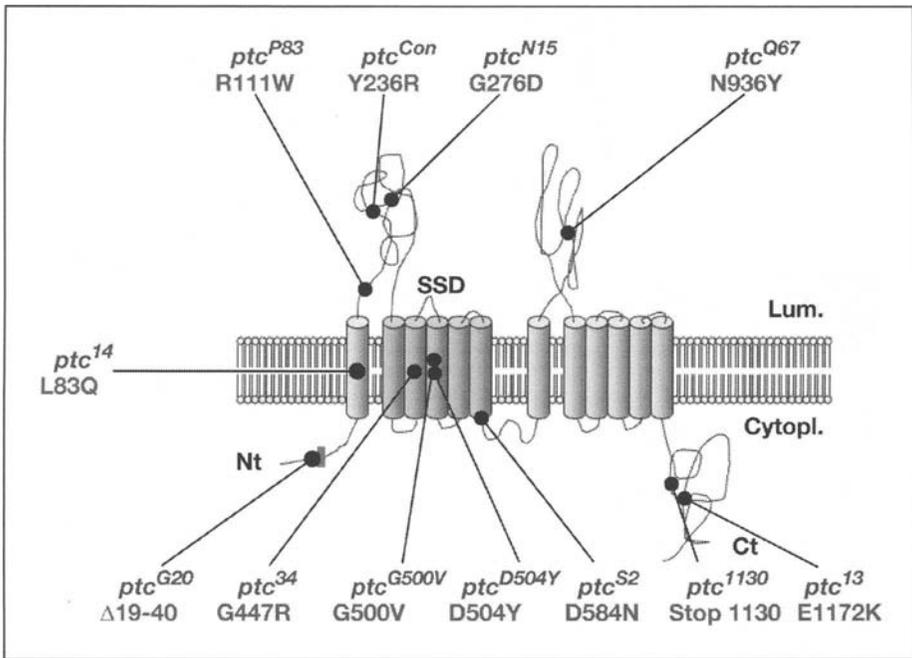


Figure 2. Ptc mutations. Ptc is a 12-transmembrane protein. The transmembrane domains highlighted in green correspond to the sterol-sensing domain (SSD). Mutations *ptc*<sup>P83</sup>, *ptc*<sup>N15</sup>, *ptc*<sup>Q67</sup> (see ref. 51) and *ptc*<sup>Con</sup> (see ref. 72) affect both Hh sequestration and signaling. Mutations *ptc*<sup>13</sup>, *ptc*<sup>34</sup> (see ref. 20), *ptc*<sup>S2</sup> (see ref. 19, 20), *ptc*<sup>1130</sup> (see ref. 20, 22), *ptc*<sup>G500V</sup>, *ptc*<sup>D504Y</sup> (see ref. 52) and *ptc*<sup>G20</sup> (unpublished data and ref. 73) affect only signaling. In the *ptc*<sup>14</sup> mutant only the sequestration is affected.<sup>39</sup>

ability to uncouple these functions suggests that Ptc-mediated internalization of Hh does not play a major role in the transduction of the signal but serves mainly to limit and control the Hh gradient.<sup>39</sup>

The analysis of different *ptc* mutants (Fig. 2) in *Drosophila* have demonstrated that the two functions of Ptc, sequestration and signalling, can be genetically separated.<sup>8,19,20,22,39,51</sup> Ptc<sup>S2</sup>, Ptc<sup>13</sup>, Ptc<sup>34</sup> and Ptc<sup>1130</sup> are defective for Hh signal transduction but sequestration properties are normal,<sup>19,20,22</sup> while the Ptc<sup>14</sup> mutant protein is defective for Hh sequestration, but normal in terms of Hh signal transduction.<sup>39</sup>

One of the most intriguing aspects of the Hh pathway is how Ptc regulates Smo activity. The mechanism is most probably indirect, since Ptc and Smo do not need to bind or to colocalize to control signaling and, in addition, Ptc acts substoichiometrically to inhibit Smo, possibly through changes in the distribution or concentration of an as yet uncharacterized small molecule (Fig. 3).<sup>52</sup> A recent study<sup>53</sup> suggests a mechanism by which the ratio of unliganded to liganded Ptc determines the cellular response (reviewed in ref. 54). In this context, several alternative models are possible. For example, unliganded Ptc might import a Smo antagonist, while liganded Ptc might promote its export. Alternatively, the functional Hh receptor may comprise a multimer of Ptc proteins and the binding of Hh to one Ptc subunit in a receptor complex may block the ability of the multimer to inhibit Smo activity.<sup>53,54</sup>

### Smo Regulation by Ptc

When Hh binds Ptc, Smo is somehow liberated to start the process of activating target gene transcription. The mechanism in which Ptc inhibits and Hh activates Smo protein remains

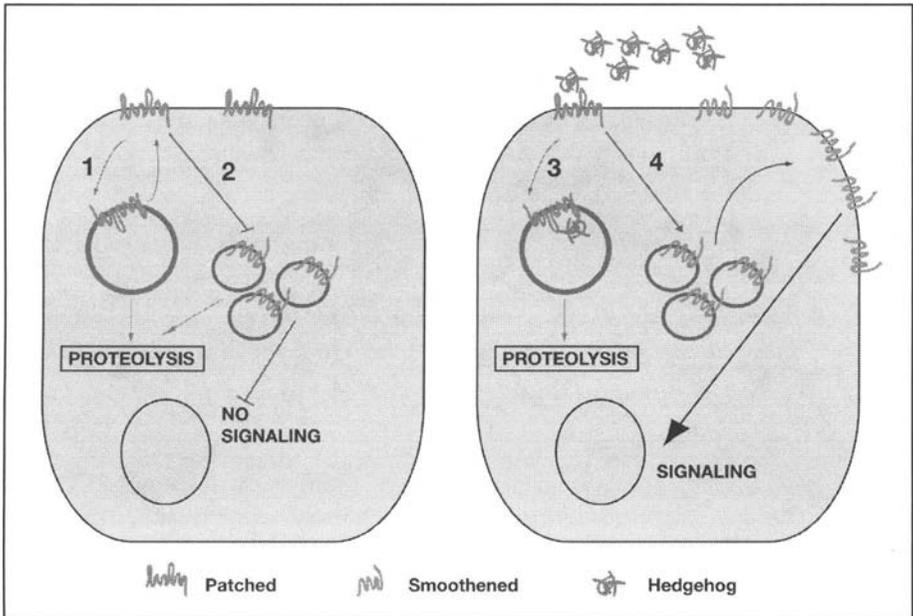


Figure 3. Model of Hh signal activation. 1) In the absence of Hh (left panel), Ptc is constantly recycling from the plasma membrane to vesicles inside the cells. Occasionally, these vesicles enter the proteolytic pathway. 2) At the same time, Smo is kept inactive in an intracellular compartment, unable to signal. Smo enters as well the degradative pathway under this situation. 3) Upon the reception of Hh (right panel), the receptor-ligand complex is internalized, contributing to the slope of the gradient of Hh. 4) At the same time, Ptc no longer represses Smo, which is stabilized and traffics to the plasma membrane preferentially. This plasma membrane stabilization of Smo correlates with signal activation.

unknown. *Smo* is transcribed in a generalized pattern not transcriptionally regulated by the Hh signal. Smo protein is posttranscriptionally regulated by phosphorylation, becoming more stable, and by moving to the cell surface after a Hh signal is received.<sup>55-60</sup> A detailed study has shown that Smo accumulates in the plasma membrane of cells in which Ptc activity is abrogated by Hh but is targeted to the degradative pathway in cells where Ptc is active.<sup>61</sup> Thus, Ptc could regulate Smo activity through inhibition of its accumulation in the plasma membrane, targeting instead to lysosomal compartments. In this view, Ptc might regulate Smo activity simply by modulating the levels of protein present in the cell. Alternatively, it may be that sub-cellular location of Smo is critical for its activation, the plasma membrane providing an environment for Smo to be accessible to an intracellular agonist (see Fig. 3 for details).<sup>61</sup>

### Ptc and Human Disease

Genetic studies in various model organisms are beginning to elucidate the factors that are likely candidates for the causes of early embryonic defects in humans (reviewed in refs. 62, 63). Thus, detailed knowledge of the Hh signaling pathway is fundamental to an understanding of vertebrate development as well as several birth defects in humans.

Since the unliganded Ptc receptor exerts a repression on the Hh pathway, mutations affecting Ptc are frequently associated to a constitutive activation of the Hh signaling pathway. This is in agreement with the fact that most of the mutations characterized to date in *PTCH1* result in protein truncation.<sup>24</sup> The spectrum of human *PTCH1* mutations also includes deletions, insertions, splice site alterations, and nonsense and missense mutations distributed throughout the gene (for an example, see Table 1).<sup>64-66</sup>

**Table 1. Mutations of the PTCH1 gene detected in patients with Gorlin syndrome and BCC (see refs. 64, 65, 74)**

Mutation	Exon	Nucleotide Change	Effect on Protein (When Reported)	Disease Associated	Ref.
1	2	260del5TinsAA	L87del, stop at 87	GS	64
2	2	280insA	94 frameshift, stop at 138	GS	64
3	2	269insT		GS	65
4	2	277AA→C		GS	65
5	2	238		BCC	74
6	3	531del ACA	Q177del	GS	64
7	3	429		BCC	74
8	5	C672A	Y224X	GS	64
9	5	742delCTinsGGAG	248 frameshift, stop at 249	GS	64
10	intron 5	IVS5+3 del AA		GS	64
11	6	853insC		GS	65
12	8	1194delCinsATATG	398 frameshift, stop at 436	GS	64
13	8	1208delAT	403 frameshift, stop at 435	GS	64
14	8	C1081T	Q365X	GS	65
15	intron 9	1336-135		BCC	74
16	10	G1450A	G484R	GS	64
17	11	C1511T	P504L	GS	64
18	11	G1513	G509R	GS	65
19	11	G1514T	G509V	GS	65
20	11	1552		BCC	74
21	12	1665		BCC	74
22	12	1686		BCC	74
23	13	2000insC		GS	65
24	13	2047insCT		GS	65
25	14	C1941A	S647R	GS	64
26	14	2178insC	721 frameshift, stop at 1048	GS	64
27	14	2434del3	Q815del	GS	65
28	14	2199		BCC	74
29	15	T2465C	L822P	GS	64
30	intron 15	2560+9		BCC	74
31	16	C2619A	Y873X	GS	64
32	16	2875+1G→C		GS	65
33	18	3042delC	1014 frameshift, stop at 1048	GS	64
34	19	C3383A	S1132Y	GS	65
35	17	T2776C	W926R	GS	64
36	21	A3583T	T1195S	GS	64
37	23	3944		BCC	74

*PTCH1* deregulation in the epidermis is sufficient to induce Basal Cell Carcinomas (BCCs) of the skin. 30-40% of Gorlin syndrome patients (GS; also known as Nevoid basal cell carcinoma syndrome) have familial loss-of-function mutations in the *PTCH1* gene. GS is an autosomal dominant disease with nearly complete penetrance and variable expressivity because mutations in *PTCH1* seem to be haploinsufficient in humans (a mutation in only one of the two alleles is enough to show a phenotype). Clinically, GS patients present congenital abnormalities that includes skeletal defects (polydactyly, fused or bifid ribs), early onset of multiple BCCs and an increased rate to develop other tumors, including medulloblastomas of the cerebellum (reviewed in refs. 67-69). A limited number of mutations in *PTCH1* have been linked to Holoprosencephaly (HPE).<sup>70</sup> HPE affects the forebrain and face to various degrees, from the most extreme lethal alobar type to milder microforms that include small midline facial defects. However, since HPE arises from loss of SHH signaling, these mutations might rather reflect an impaired ability of PTCH1 to interact with SHH, thus permanently shutting down the pathway. In fact, two out of four rare *PTCH1* missense mutations that have been reported to be associated to HPE, were localized in the extracellular loops of PTCH1 required for SHH binding.<sup>70</sup>

There is a limited number of missense mutations described for *PTCH1*. Missense mutations do not occur as frequently as frameshift or nonsense mutations. In a clinical study on French patients, missense mutations span the second group of six transmembrane domains of the protein.<sup>64</sup> Unfortunately, no data are available to interpret the pathologic value of the missense mutations described in humans.<sup>64</sup> So far, only the molecular and cellular studies of *Drosophila ptc* mutations indicate an alteration of the correct topology and/or sorting of the Ptc receptor. We propose *Drosophila* as an ideal model system to analyze the functional alterations of the missense mutations found in the PTCH1 receptor. In fact, a recent study analyses in the fly the function of an artificially mutated form of Ptc analog to a mutated PTCH1 previously characterized in GS patients.<sup>71</sup> Therefore, understanding the molecular mechanisms of Hh signal reception will allow identifying potential drug targets in order to devise strategies for the treatment of BCC.

## Concluding Remarks

More genes of the Hh pathway are going to be uncovered in the near future and *Drosophila* genetics will undoubtedly help in to resolve this issue. Hence, concerning the function of Ptc receptor, an unresolved question is how Ptc is able to repress the activity of Smo, and whether there are intermediate elements. Although other molecules have been found as possible candidates in the reception of Hh in vertebrates such as Megalin, Hip or Gas1, mutagenesis screens need to be done in *Drosophila* to find more proteins implicated in Hh reception. The study of their function will help to understand how several cellular and molecular processes drive and regulate the pathway. Another important unresolved issue in the Hh pathway is the role of cell polarity in the transduction of the signal, or the endocytic and exocytic routes involved. Also questions remain regarding how a morphogen gradient is formed, how the cytoskeleton affects the pathway, or how the transcription of target genes is regulated. New model systems in vertebrate genetics, cellular biology and biochemistry will help to elucidate some of these questions. Definitely, a huge biochemical and cell biology effort needs to be done in order to solve the role of this kind of proteins in Hh signaling pathway. This knowledge will undoubtedly help to design specific therapies to serious human diseases caused by mutations on genes of this signaling pathway.

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