Molecular Cell, Vol. 6, 457–465, August, 2000, Copyright ©2000 by Cell Press

Posttranscriptional Regulation of Smoothened Is Part of a Self-Correcting Mechanism in the Hedgehog Signaling System

Joy Alcedo,[†‡] Yu Zou,[†] and Markus Noll*
Institute for Molecular Biology
University of Zürich
Winterthurerstrasse 190
CH-8057 Zürich
Switzerland

Summary

Hedgehog signaling, mediated through its Patched-Smoothened receptor complex, is essential for pattern formation in animal development. Activating mutations within Smoothened have been associated with basal cell carcinoma, suggesting that *smoothened* is a protooncogene. Thus, regulation of Smoothened levels might be critical for normal development. We show that Smoothened protein levels in *Drosophila* embryos are regulated posttranscriptionally by a mechanism dependent on Hedgehog signaling but not on its nuclear effector Cubitus interruptus. Hedgehog signaling upregulates Smoothened levels, which are otherwise downregulated by Patched. Demonstrating properties of a self-correcting system, the Hedgehog signaling pathway adjusts the concentrations of Smoothened and Patched to each other and to that of the Hedgehog signal, which ensures that activation of Hedgehog target genes by Smoothened signaling becomes strictly dependent on Hedgehog.

Introduction

The *smoothened* (*smo*) gene is essential for pattern formation and morphogenesis of most multicellular organisms. Its product, a seven-pass membrane protein (Alcedo et al., 1996; van den Heuvel and Ingham, 1996), constitutes together with the Patched (Ptc) multipass membrane protein the receptor for the Hedgehog (Hh) signal (Chen and Struhl, 1996; Stone et al., 1996; Alcedo and Noll, 1997). Ptc has been shown to bind both Hh (Marigo et al., 1996; Stone et al., 1996) and Smo (Stone et al., 1996), whose activity, in the absence of Ptc, is independent of the Hh ligand and constitutively activates the Hh signaling pathway (Hooper, 1994; Alcedo et al., 1996). Hence, Ptc has been proposed to be a ligand-regulated inhibitor of a constitutive signaling moiety, Smo, in the Hh receptor complex (Chen and Struhl, 1996; Stone et al., 1996; Alcedo and Noll, 1997). Loss of *ptc* function (Hahn et al., 1996; Johnson et al., 1996) and activating *smo* mutations (Xie et al., 1998) have been associated with basal cell carcinoma, which suggests that Ptc acts as a tumor suppressor by repressing the oncogenic activity of Smo in the transduction of the Hh signal (Hahn et al., 1996; Johnson et al., 1996; Xie et al., 1998). Since *smo* can be classified as a protooncogene, regulation of the intrinsic constitutive activity of its product is expected to be crucial for its proper function in transducing the Hh signal and thereby in normal development and in the circumvention of cancer.

Here, we analyze the expression patterns of the Smo protein in *Drosophila* embryos and demonstrate that they are regulated by a mechanism dependent on Hh signaling. The regulation of Smo levels does not depend on the nuclear effector Cubitus interruptus (Ci) of Hh signaling but occurs posttranscriptionally and depends on protein kinase A (PKA). Since Smo signaling is required for the transcription of *ptc,* whose product downregulates Smo protein in the absence of Hh, this mechanism ensures that high Smo signaling becomes strictly dependent on the presence of Hh. Consequently, high levels of Smo and hence of constitutive Smo signaling in regions of low or no Hh are deleterious to the animal. Thus, we propose that Hh and its Ptc-Smo receptor have developed the properties of a self-correcting system in which the Hh signal adjusts the concentration of its receptor to its own concentration.

Results

Smo Protein Expression in Wild-Type Embryos

The expression of Smo before cellular blastoderm is weak and extends mainly between 15% and 75% EL (egg length, measured from the posterior pole), corresponding approximately to the segmental anlage (data not shown). Smo levels increase in these cells by early gastrulation (Figure 1A) and show no modulation along the anteroposterior axis (Figure 1B). Subsequently, Smo protein continues to accumulate, and its expression begins to be segmentally regulated at stage 8 (Figure 1C). By stages 10 and 11, Smo expression is clearly enhanced in a segmentally modulated pattern of stripes (Figures 1D and 1E). Each Smo stripe overlaps with Engrailed (En) expression in the posterior compartment of each segment but extends approximately two cells posterior and one cell anterior to En (Figure 1F). Thus, Smo and Wingless (Wg) stripes coincide at their anterior boundaries (Figure 3C). Since Hh, which is secreted by En-expressing cells, signals to both anterior and posterior cells, activating *ptc* and *wg* anteriorly (DiNardo et al., 1988; Hooper and Scott, 1989; Nakano et al., 1989; Hidalgo and Ingham, 1990; Hidalgo, 1991; Ingham and Hidalgo, 1993; Tabata and Kornberg, 1994; Thérond et al., 1999) and *ptc* posteriorly (Hooper and Scott, 1989; Nakano et al., 1989; Hidalgo and Ingham, 1990; Tabata and Kornberg, 1994; Thérond et al., 1999), all cells known to respond to the Hh signal express Smo at high levels.

The Hh Signal Regulates Smo Protein Levels

During germband extension, Hh and Wg maintain each other's expression in neighboring cells on opposite sides of the compartment boundary to establish signaling centers and organize the segmental pattern of the epidermis (reviewed by Perrimon, 1994). The following

*To whom correspondence should be addressed (e-mail: noll@molbio.unizh.ch).
[†]These authors contributed equally to this work.
[‡]Present address: Department of Biochemistry and Biophysics, University of California, 513 Parnassus Avenue, San Francisco, California 94143.

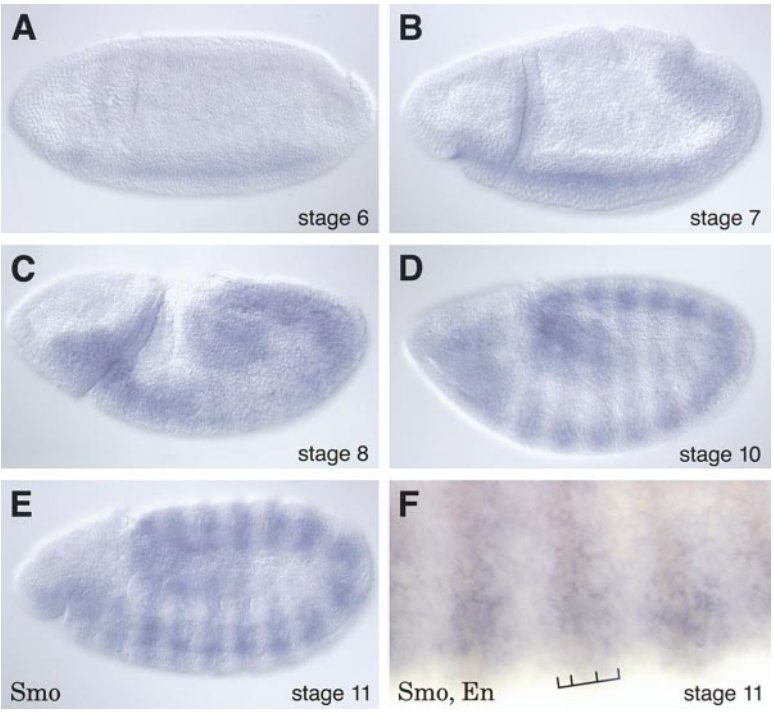

Figure 1. Expression of Smo Protein in Wild-Type Embryos

(A–E) *Oregon-R* embryos of different stages ([A], early gastrulation, stage 6; [B], stage 7; [C], stage 8; [D], early stage 10; and [E], stage 11) were immunostained with rabbit anti-Smo antiserum.

(F) Smo protein stripes (blue), observed on cell membranes, completely overlap with stripes of nuclear En protein (brown) in stage 11 wild-type embryos, as shown at higher magnification for the ventral portions of parasegments 3 to 6. Higher Smo levels extend about one cell anterior and two cells posterior to En, as indicated by brackets. Embryos in this and subsequent figures are oriented with their anterior to the left and dorsal side up. All embryos were photographed under Nomarski optics.

lines of evidence demonstrate that Smo protein levels depend on Hh rather than Wg. (1) Smo levels are considerably reduced in *hh* mutants (Figures 2C and 2D) compared to wild-type (Figures 2A and 2B), while their decay is clearly delayed in *wg* mutants (Figures 2E and 2F). (2) Even more striking than the reduced levels of Smo protein is the absence of a modulated expression of Smo in *hh* as compared to *wg* mutants (Figures 2C–2F), which suggests that both high levels of Smo and its segmental modulation depend on Hh. (3) Ubiquitous expression of Wg under a heat-shock promoter in *hh* mutants does not rescue Smo expression in the ventral epidermis of stage 11 embryos (data not shown). (4) Upon ubiquitous Hh expression, Smo protein levels are upregulated in the anterior compartment, and hence their segmental modulation is much less pronounced (Figure 2G). (5) The Smo concentration is uniformly high in embryos mutant for *ptc* (Figure 2H), which indicates that a functional Ptc protein reduces Smo levels in the central region of the anterior compartment where the Hh concentration is low. (6) The upregulation of Smo in *ptc* embryos does not depend on the Wg signal, as Smo expression in *ptc* embryos resembles that in *wg ptc* double mutants (Figure 2I). (7) Finally, ubiquitous overexpression of Ptc, expressed under the indirect control of an actin promoter, has the opposite effect and strongly reduces Smo levels (Figure 2J). Thus, Smo levels are upregulated by Hh and downregulated by Ptc. These results are consistent with the model of Hh signal transduction, in which Hh mediates its effect entirely through Ptc, which inhibits constitutive signaling of Smo (Chen and Struhl, 1996; Stone et al., 1996; Alcedo and Noll, 1997).

Smo Protein Expression Does Not Depend on Ci

The dependence of Smo levels on the Hh signal is independent of the Ci protein, the *Drosophila* homolog of the vertebrate Gli transcription factors and the only known nuclear effector of the Hh pathway (reviewed by Aza-Blanc and Kornberg, 1999). Smo protein does not decay in *ci* null (Figure 3A) or dominant negative mutants (data not shown), and its striped pattern is indistinguishable from that in wild-type embryos (Figures 1E and 3C). Moreover, the uniformly high levels of Smo in *ptc* mutants (Figures 2I and 2J) remain unaltered in the absence of Ci in *ptc ci* double mutants (Figure 3B). Evidently, Ci is not required to upregulate Smo. It is possible that Hh controls Smo expression through a transcriptional activator different from Ci since Smo is also expressed in the posterior compartment of each segment (Figures 1F and 3C), where Ci is not present (reviewed by Aza-Blanc and Kornberg, 1999). No Ci consensus binding sites are found in the upstream, intronic, or downstream sequences of *smo*, while in vertebrates, a factor distinct from Gli has been reported to mediate Hh signaling to one of its target genes (Krishnan et al., 1997). In addition, a Hh-responsive element of the *wg* gene in *Drosophila* contains no consensus Ci binding sites (Lessing and Nusse, 1998). However, the Ci independence of this regulatory region has not been conclusively shown.

Alternatively, the Ci independence of Smo expression might suggest that Smo is regulated posttranscriptionally and that this regulation is dependent on Hh. Consistent with this alternative hypothesis, we observed that *smo* RNA patterns in *hh* mutants are indistinguishable from those in wild-type embryos (data not shown), which suggests that regulation of Smo concentration occurs at the translational or posttranslational level.

Smo Is Regulated Posttranscriptionally by the Hh Signaling Pathway

To test further if Smo levels are regulated posttranscriptionally and if the segmentally modulated expression of Smo is important for normal development, we uniformly expressed a hemagglutinin (HA)-tagged Smo protein (Smo-

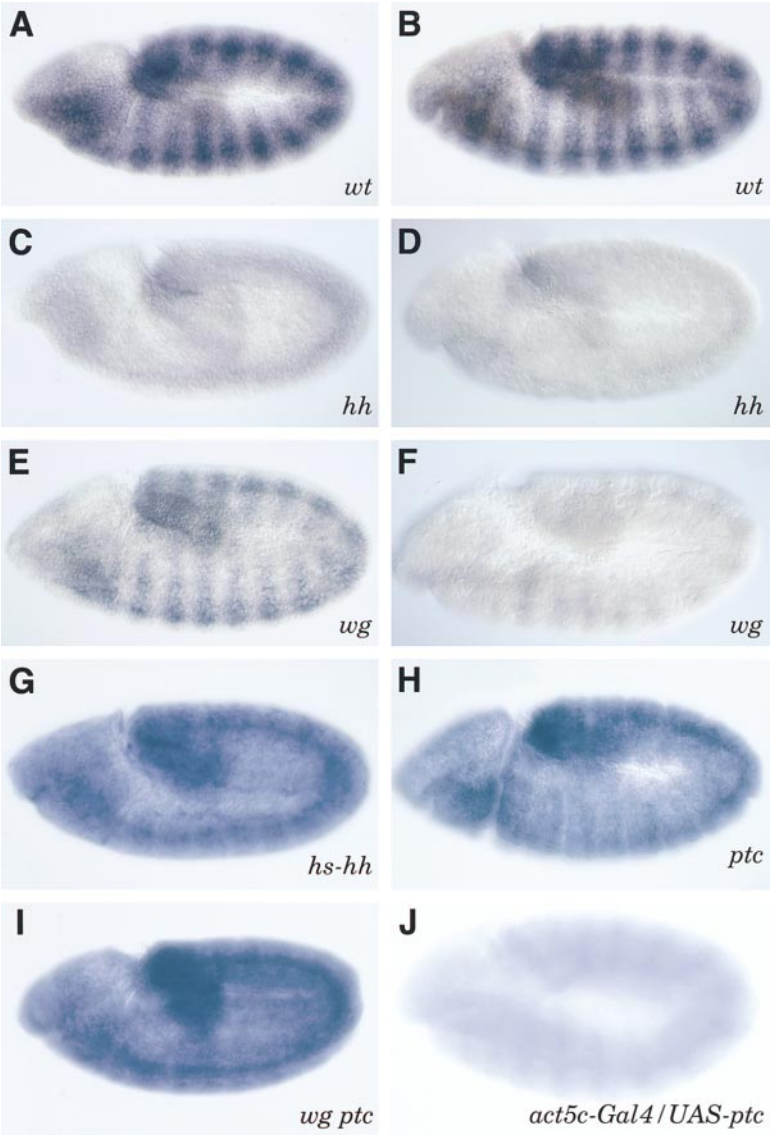

Figure 2. Expression and Segmental Modulation of Smo Depends on the Hh but Not the Wg Signal

Smo expression (blue) in wild-type embryos (A and B) is compared to that in hh^{13C} (C and D), wg^{IG22} (E and F), $ptc^{Df(2R)44CE}$ (H), and wg^{IG22} ptc^{7} (I) mutant embryos at stages 10 (A, C, and E) and 11 (B, D, F, H, and I) at 25°C. Heterozygous mutant stocks, with the exception of the *wg ptc* double mutant, were prepared over *actin*-LacZ-marked balancers, and mutant embryos were identified by the absence of staining for LacZ, whereas wild-type embryos (A and B) were identified by its presence (brown).

(G) Ubiquitous expression of Hh under heat-shock control results in increased levels of Smo protein in stage 11 embryos.

(J) Ubiquitous expression of Ptc in *act5c-Gal4/UAS-ptc* embryos results in low Smo levels at stage 11.

All photographs were taken under Nomarski optics except (J), which was photographed under bright-field illumination.

HA) under the control of the constitutive *tubulin*α*1* promoter from a *tub-smo-HA* transgene. Despite the constitutive and uniform transcription of its RNA, Smo-HA is expressed in the same temporal and spatial pattern of stripes as endogenous Smo (Figures 3C and 3D), which confirms that Smo protein levels are posttranscriptionally regulated. In addition, as with the endogenous Smo protein, high levels of Smo-HA depend on the Hh signal (Figure 3E) and are downregulated by excess Ptc expressed under the indirect control of a *paired* (*prd*) enhancer in every other segment (Figure 3F). Moreover, since one copy of the *tub-smo-HA* transgene is able to rescue homo- or transheterozygous smo^{1}, smo^{2}, and smo^{3} embryos (Table 1) to wild-type adults, *tub-smo-HA* is also functionally equivalent to the endogenous wild-type *smo* gene. As Smo-HA protein levels are segmentally modulated even though they arise from nonmodulated levels of its mRNA, it appears that the segmental modulation of Smo-HA and thus of Smo protein levels is crucial for normal development. We further conclude that both Smo and Smo-HA are regulated by the same posttranscriptional mechanism, which depends on the activation of a Ci-independent Hh signaling pathway.

Because Smo levels are upregulated by Hh and downregulated by Ptc, and since in the absence of Ptc high Smo levels are independent of Hh (as suggested by the indistinguishable expression patterns of Smo in $Df(2R)ptc^{44CE}$; hh^{AC} double mutant and $Df(2R)ptc^{44CE}$ single mutant embryos at the extended germband stage; our unpublished data), a Ci-independent posttranscriptional regulation of Smo could be explained in two different ways: (i) Hh-dependent or constitutive Smo signaling is required to upregulate Smo levels, either by inhibiting the degradation or stimulating the synthesis of Smo protein; (ii) Ptc does not destabilize Smo by inhibiting Smo signaling but rather through a mechanism that is independent of Smo signaling. We find that the missense Smo^{1} and Smo^{2} and the truncated Smo^{3} mutant proteins (Table 1) are expressed only at low levels both in the single and in the *smo ptc* double mutants (Figures 4A and 4B; data not shown), which suggests that high levels

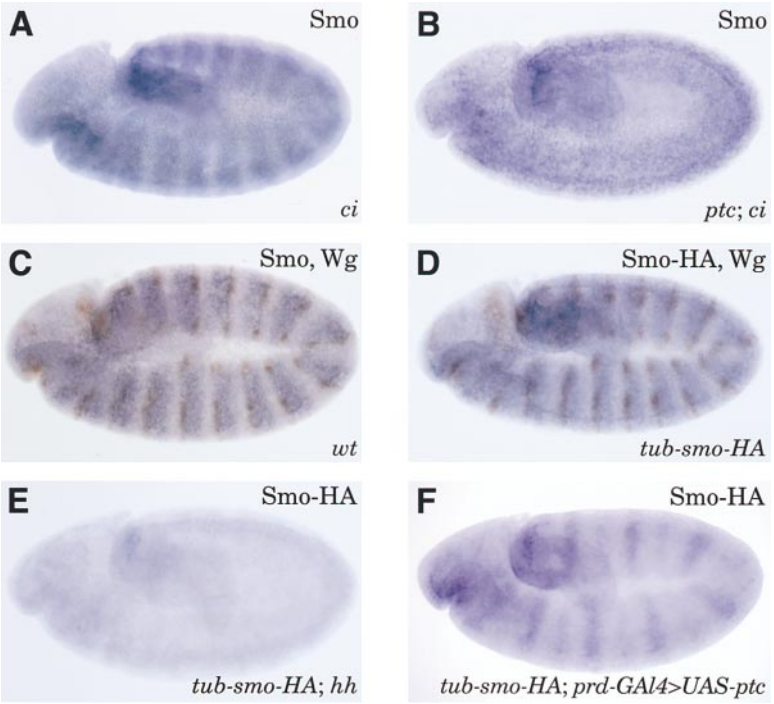

Figure 3. Smo Expression Is Independent of Ci and Regulated Posttranscriptionally by the Hh-Signaling Pathway

(A) Smo protein expression in stage 11 amorphic ci^{94} embryos is indistinguishable from that in wild-type embryos derived from the same cross (data not shown; but cf. [C]). ci^{94} embryos were identified by the absence of staining for Ci.

(B) Smo protein expression in stage 11 ptc^{16}; ci^{94} embryos is indistinguishable from that in ptc^{-} embryos derived from the same cross (data not shown; but cf. Figure 2H). ptc^{16}; ci^{94} embryos were identified by the absence of staining with anti-LacZ mouse monoclonal antibody for the *hb-lacZ* marker on the *CyO* balancer chromosome and for the ci^{D}-*lacZ* marker on the ci^{+} fourth chromosome.

(C) Anterior boundaries of the broad Smo protein stripes (blue) coincide with those of the narrow Wg protein stripes (brown) in stage 11 wild-type embryos.

(D) Smo-HA (blue), expressed under the control of the tubulin promoter (*tub*) from a *tub-smo-HA* transgene, shows the same expression pattern as endogenous Smo protein (C) in stage 11 embryos, which are also stained for Wg (brown).

(E) Smo-HA decays like endogenous Smo protein in stage 11 hh^{13C} embryos (Figure 2D). hh^{13C} embryos were identified by the absence of staining with anti-lacZ for the *hb-lacZ* marker on the *TM3*, *Sb Ser* balancer chromosome.

(F) Smo-HA levels are downregulated by Ptc, which is expressed from a Gal4-responsive transgene under the indirect control of a *prd* enhancer in every other segment of *tub-smo-HA/UAS-ptc*; *prd-Gal4/+* embryos.

All embryos were photographed under bright-field illumination.

of Smo depend on Smo signaling. These low levels of mutant Smo are not caused by a dependence of *smo* transcription on Smo signaling because no differences in *smo* RNA patterns are observed among *smo* mutant, *smo ptc* double mutant, and wild-type embryos (data not shown). Since it is possible that the intrinsic stabilities of all mutant Smo proteins tested are drastically reduced as compared to the stability of wild-type Smo, we cannot strictly rule out that Ptc affects the stability of Smo by a mechanism that is independent of Smo signaling. However, in the absence of conclusive evidence for such a model, we favor a mechanism by which Ptc destabilizes Smo through the inhibition of Smo signaling because it is not only more probable but also a simpler hypothesis. Hh signaling, which is mediated through Smo, has been shown to antagonize PKA and thereby results in the activation of Hh target genes (Jiang and Struhl, 1995; Johnson et al., 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). Similarly, as shown below, Hh signaling antagonizes PKA activity and thus leads to enhanced Smo levels. Therefore, the simplest model, consistent with existing

Table 1. Mutations in Three *smo* Alleles

Allele	73	226	593	815	1089	1240	1518	1783	2197	3022	3686	4161
wt	T	T	C	G	G	T	G	G	C	G	C	T
smo^{1}	–	G	G	T	G	C	**A**	G	T	A	T	A
smo^{2}	–	G	G	T	**A**	C	G	G	T	A	T	A
smo^{3}	T	T	C	G	G	T	G	**A**				
Effect	5′ UTR	5′ UTR	A96G	intron 2	C155Y	N205N	C298Y	W366Stop	A504A	S735N	T956T	3′ UTR
Domain			N-extra		N-extra		TM-2	IL-2		C-intra		
Mutation			polym.		smo^{2}		smo^{1}	smo^{3}		polym.		

smo^{1} and smo^{2} mutations are G to A transitions, resulting in the missense mutations C298Y in the middle of the second transmembrane domain (TM-2) of Smo and C155Y in the conserved N-terminal cysteine-rich extracellular domain (N-extra) of Smo, respectively. In addition, both these alleles display the polymorphisms A96G (due to a C to G transversion) in the N-terminal extracellular domain and S735N (due to a G to A transition) in the C-terminal intracellular domain (C-intra). Additional polymorphisms of smo^{1} and smo^{2}, as compared to the wild-type sequence (Alcedo et al., 1996), are found in the coding region without changing the amino acid identity, in the 5′UTR, 3′UTR, or intron 2. Dash indicates deletion of the nucleotide at position 73. Finally, in agreement with an earlier report (Chen and Struhl, 1998), smo^{3} is a G to A transition, resulting in the nonsense mutation W366Stop, which truncates the Smo protein in the second intracellular loop (IL-2). The smo^{2} mutation turns out to be identical to that found in the smo^{F3} and smo^{F11} alleles (Chen and Struhl, 1998). Numbers of nucleotides (top row) and amino acids (effect) refer to the positions in the unspliced primary *smo* transcript and the open reading frame encoding Smo (Alcedo et al., 1996), as found under the GenBank accession number L79947.

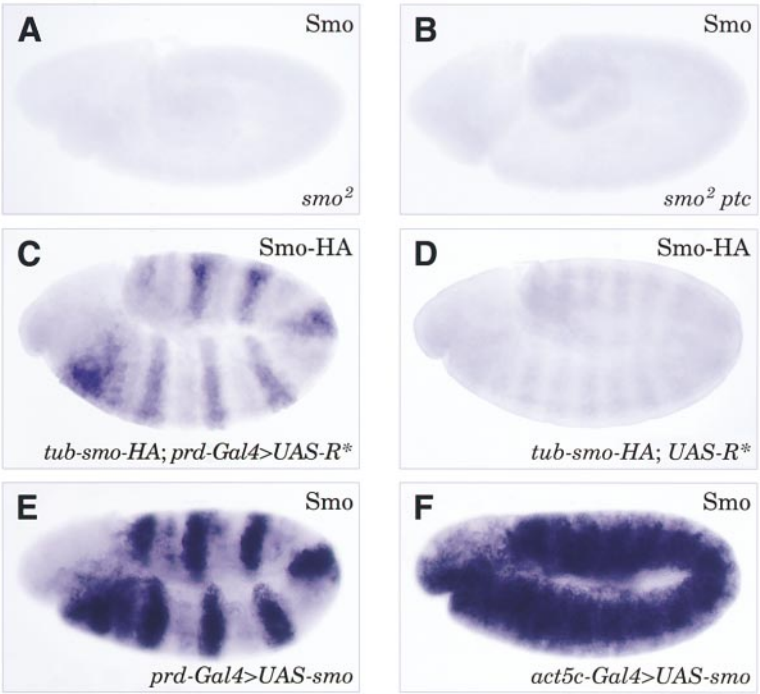

Figure 4. Smo Protein Levels Are Upregulated by Smo Signaling and Downregulated by PKA and Must Be Strictly Dependent on Hh

(A and B) Smo^2 levels are considerably reduced in *smo*^{2} (A) and *smo*^{2} *ptc*$^{Df(2R)44CE}$ (B) embryos by stage 10 at 18°C. Similar results are obtained for Smo^3 in *smo*^{3} and *smo*^{3} *ptc*$^{Df(2R)44CE}$ embryos.

(C and D) Smo-HA is strongly upregulated by stage 10 (shown is stage 11) in alternating segments expressing R* of *tub-smo-HA/+*; *prd-Gal4/UAS-R** embryos (C) but not in *tub-smo-HA/+*; *+/UAS-R** embryos that lack the *prd-Gal4* driver (D).

(E and F) Enhanced Smo levels in regions of high Hh in alternating segments of *UAS-smo/+* (or Y); *prd-Gal4/+* embryos (E) do not affect development, whereas ubiquitous overexpression of Smo in *UAS-smo/+* (or Y); *act5c-Gal4* embryos (F) is lethal by stage 14. All embryos were photographed under bright-field illumination.

data on Hh signaling, postulates that Hh antagonizes PKA through Smo signaling in both processes: the activation of Hh target genes and the stabilization of Smo protein.

We also observed that the decay of Smo is delayed in *hh* embryos as compared to that of Smo^1, Smo^2, and Smo^3 in *smo* embryos. While Smo levels in *hh* and wild-type embryos are similar up to stage 8, mutant Smo protein levels already begin to decline by the end of gastrulation and are barely detectable by stage 10 (data not shown). This observation suggests that the ubiquitous expression of Smo before stage 9 is largely independent of Hh but might already depend on Smo signaling.

Smo Protein Levels Are Downregulated by PKA

In agreement with the requirement for Hh-dependent or constitutive Smo signaling to upregulate Smo protein, inhibition of PKA, an antagonist of Hh and Smo signaling (Jiang and Struhl, 1995; Johnson et al., 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995), upregulates Smo-HA levels dramatically in alternating segments of *tub-smo-HA/+*; *prd-Gal4>UAS-R** embryos (Figure 4C). These embryos express in every other segment a mutant regulatory subunit of PKA, R*, under the indirect control of a *prd* enhancer, which blocks PKA activity in a dominant-negative fashion since R* binds cAMP poorly (Li et al., 1995; Ohlmeyer and Kalderon, 1997). Evidently, this inhibition of PKA by R* is much more effective than endogenous Hh signaling in upregulating Smo-HA, since Smo-HA levels are considerably lower in segments that do not express R* (Figure 4C) or in control embryos that lack the *prd-Gal4* driver (Figure 4D). In addition, ubiquitous inhibition of PKA by R*, expressed under the indirect control of an actin promoter, uniformly upregulates Smo-HA (data not shown). Since it is extremely unlikely that PKA regulates the levels of Smo-HA through the *tubulin* promoter, PKA probably regulates Smo levels posttranscriptionally, which is consistent with our earlier conclusions.

Effects of Smo Overexpression on Animal Survival

Since normal development seems to depend on the segmental modulation of Smo expression as shown above, we would predict that constitutive Smo signaling in regions of low Hh concentrations is deleterious. To test this prediction, we examined the effect on survival of Smo overexpression under the control of *prd-Gal4* in alternating embryonic segments (Figure 4E) or of ubiquitous *UAS-smo* expression driven by *act-Gal4* (Figure 4F). While all *prd-Gal4>UAS-smo* embryos, in which Smo overexpression occurs only in regions that normally express high levels of Smo, develop into wild-type adults, all *act-Gal4>UAS-smo* embryos die by stage 14. Since Gal4 is not subject to negative feedback control and presumably considerably more stable than Smo, the steady-state levels of Smo observed in these embryos are much higher than those found in wild-type embryos. This contrasts with Smo-HA levels observed in *tub-smo-HA* embryos, which are susceptible to the same negative feedback regulation as Smo and hence are similar to wild-type.

Discussion

Regulation of Smo protein levels might be crucial for the normal development of the animal since basal cell carcinoma has been associated with the constitutive activation of Smo, which suggests that *smo* is a protooncogene (Xie et al., 1998). We have shown that Smo protein levels in *Drosophila* embryos are regulated posttranscriptionally and that this regulation is dependent on Hh signaling but not on its nuclear effector Ci. Moreover, we have shown that the posttranscriptional regulation of Smo depends on PKA, an antagonist of the Hh signaling

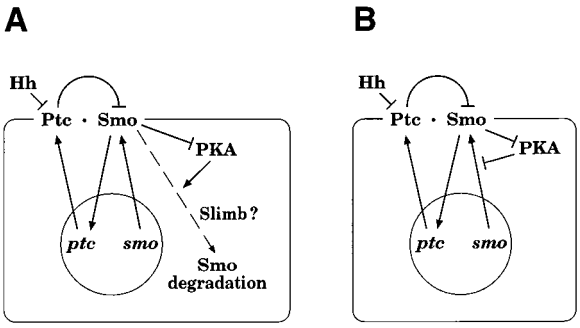

Figure 5. Model of Hh Signaling, a Self-Correcting System

Smo levels are regulated by PKA, which promotes degradation (A) or inhibits synthesis (B) of Smo. For reasons discussed in the text, we favor the model in (A). Although Smo activity is indicated to inhibit PKA, we do not wish to imply that Smo acts directly on PKA, since Smo signaling does not inhibit PKA through the regulation of cAMP levels in embryos (Ohlmeyer and Kalderon, 1997). Indeed, Smo might antagonize PKA by activating a phosphatase that dephosphorylates PKA sites (Chen et al., 1999). Moreover, PKA might also not act directly on Smo protein to promote its proteolytic processing even though this might be suggested by the presence of 5 PKA sites in the cytoplasmic C terminus of Smo (Alcedo et al., 1996).

pathway (Jiang and Struhl, 1995; Johnson et al., 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). Thus, PKA activity regulates Smo levels either by stimulating the degradation of Smo (Figure 5A) or by reducing its rate of synthesis (Figure 5B). We favor the mechanism by which Hh regulates the stability of the Smo protein through PKA-dependent proteolysis (Figure 5A) for two reasons: there is no evidence for PKA-dependent regulation of the rate of synthesis of any protein (Figure 5B), while on the other hand it is known that Hh signaling inhibits PKA-dependent proteolytic processing of its nuclear effector Ci (Aza-Blanc et al., 1997; Chen et al., 1998; Méthot and Basler, 1999; Price and Kalderon, 1999). Full-length Ci, which acts as transcriptional activator Ci^A in Hh signaling, is phosphorylated by PKA in the absence of Hh to promote its proteolytic conversion into the Ci^R repressor form, a process that further depends on Slimb (Jiang and Struhl, 1998; Ohlmeyer and Kalderon, 1998; Theodosiou et al., 1998; Wang et al., 1999). Moreover, Jiang and Struhl (1998), to explain the high levels of full-length Ci accumulating in *slimb (slmb)* mutant clones, have proposed that Slimb, a Cdc4-related ubiquitin targeting protein, targets Ci for processing to the ubiquitin/proteasome pathway. This proposal is further supported by the finding that Ci processing does depend on proteasome activity (Chen et al., 1999; reviewed by Maniatis, 1999). Since several consensus PKA phosphorylation sites are found in the cytoplasmic portions of Smo (Alcedo et al., 1996), PKA might also exert its effect directly on Smo. The phosphorylated form of Smo might be targeted by Slimb to the ubiquitin-ligase complex prior to its proteasome-mediated degradation, a mechanism inhibited by Hh and constitutive Smo signaling (Figure 5A). Alternatively, PKA does not act directly on Smo but affects the stability of Smo by activating a protein that destabilizes Smo or by inhibiting a protein that stabilizes Smo.

A test if Smo levels are uniformly elevated after reducing or completely removing the zygotic Slimb activity in homozygous or transheterozygous $slmb^2$ (Jiang and Struhl, 1998) and $slmb^{e4-1}$ (Theodosiou et al., 1998) embryos was negative, presumably because of the presence of sufficient wild-type maternal Slimb (Jiang and Struhl, 1998). After reducing the maternal Slimb activity in hypomorphic $slmb^1$ (Jiang and Struhl, 1998) germline clones, $slmb^1$ embryos ceased to develop by stage 6 and hence could not be tested, as Smo levels are still very low at this stage (Figure 1A).

A Model of Hh Signaling: A Self-Correcting System

Why do Hh and Smo signaling upregulate the two Hh-receptor components, Ptc and Smo, at the transcriptional and posttranscriptional level, respectively? What are the advantages of this Hh and Smo signaling system in which Hh inhibits Ptc, which otherwise suppresses Smo signaling and hence downregulates both Smo and Ptc? For convenience, we assume in the following a model in which Smo signaling is activated by Hh binding to Ptc as part of a Ptc-Smo receptor complex so far only demonstrated in mammals (Stone et al., 1996). Yet none of our considerations are affected by the assumption of such a complex because they are independent of whether Ptc inhibits Smo signaling directly or indirectly. As emphasized above, the constitutive activation of the Hh signaling pathway in the absence of Hh is oncogenic (Hahn et al., 1996; Johnson et al., 1996; Xie et al., 1998). Hence, it is crucial that Smo signaling strictly depends on the presence of Hh and that, in the absence of Hh, constitutive Smo signaling is restricted by Ptc below a threshold necessary for the transcriptional control of Hh target genes (for a derivation of the equations illustrating how the concentrations of Ptc and Smo depend on each other and on Hh, see the Experimental Procedures). When Hh levels decrease, Smo is destabilized because of the inhibition of Smo signaling by Ptc. The concentration of Smo will be reduced more rapidly than that of Ptc, which continues to be translated from a decreasing concentration of its mRNA, and eventually Smo will reach a reduced steady-state concentration, which is lowest in regions where Hh is absent. When the Ptc concentration falls below a threshold, Smo signaling begins to inhibit its own degradation and to activate transcription of *ptc*, whose product suppresses Smo signaling and thus again downregulates itself and Smo. Hence, a new steady state is reached at which the levels of Ptc and Smo are reduced to a level corresponding to the low Hh concentration. The sequence of events are expected to be reversed, if the Hh concentration is again increased (compare equations given in Experimental Procedures). Thus, the Hh signaling pathway has the properties of a self-correcting system, since an imbalance between Ptc and Smo or between Hh and the Ptc-Smo receptor is readjusted to equilibrium.

Although this self-correcting Hh signaling system may appear complex, its properties are probably the simplest solution in ensuring that Smo signaling strictly depends on the concentration of Hh (a similar argument, based on the premise that evolution selects for simplicity, has been made by M. N. as cited in Basler (2000) to explain the logic behind the mechanism that generates the anteroposterior organizer in the wing disc and in which Hh signaling plays a key role), as apparent from the following considerations. Since Smo signals constitutively in the absence of Ptc (Hooper, 1994; Alcedo et al., 1996), Smo signaling must activate *ptc* to inhibit its constitutive activity. To avoid an imbalance between the two Hh-receptor moieties, Smo signaling must also upregulate

Smo. If Smo levels were independent of Smo signaling, Smo would reach a uniformly high level while the concentration of Ptc would oscillate around an equilibrium since Ptc inhibits Smo signaling on which its synthesis depends. However, in this case Smo would signal even in the absence or at low levels of Hh, which is not what we observe (Figures 2C and 2D). Therefore, to ensure that Ptc and Smo reach an equilibrium at which Ptc completely inhibits Smo signaling most rapidly in the absence of Hh, Smo regulates its own breakdown. The alternative possibility, that Smo regulates its own concentration at the transcriptional level, would be not only a much slower mechanism, but also less safe. In this case, an excess of Ptc over Smo could result in the loss of both *ptc* and *smo* transcription, which would lead to a decline of both Ptc and Smo. This may result in the complete disappearance of the Hh receptor rather than in its reduced synthesis, and the self-correcting property of the system to adjust its receptor concentration to that of its signal would be lost (compare equations given in Experimental Procedures).

Our model (Figure 5A) is in excellent agreement with the observed patterns of Smo and Ptc expression. The early Smo expression is uniform because it results from an incomplete inhibition by Ptc of constitutive Smo signaling and hence is still largely independent of Hh, as argued above. The observation that segmental modulation of Smo levels requires a dependence on Hh is consistent with the uniform Smo levels in *hh* (Figures 2C and 2D) and *ptc* mutant embryos (Figures 2H and 2I). In contrast, the modulation persists in *wg* embryos until Hh has decayed (Figures 2E and 2F). In wild-type embryos, Smo becomes segmentally modulated when *ptc* is no longer activated in the posterior compartment and is upregulated in the anterior compartment. Therefore, Smo becomes upregulated in the posterior compartment and its neighboring cells that are exposed to high Hh concentrations. In the posterior compartment, Ptc is absent because *ci*, whose product is required to activate *ptc* transcription (Hidalgo and Ingham, 1990; Tabata and Kornberg, 1994; Thérond et al., 1999), is repressed by En (Eaton and Kornberg, 1990). Because its effector Ci is absent, there is no need to downregulate the resulting constitutive signaling activity of Smo in the posterior compartment. It should be noted that in *hh* embryos, *ci* is derepressed in the posterior compartment because En decays. Consequently, *ptc* is activated and its product inhibits Smo signaling, which results in the decay of Smo in *hh* embryos. In addition, since cells in the middle of the anterior compartment of wild-type embryos are shielded from high Hh levels by Ptc (Chen and Struhl, 1996), they downregulate their Ptc expression and, accordingly, their Smo levels (Figures 1D and 1E).

Finally, our model is also consistent with our observation that constitutive Smo signaling in regions of low Hh concentrations is harmful to the animal (Figure 4F), whereas constitutive Smo signaling in regions of high Hh concentrations is not (Figure 4E). Thus, only strong external interference is able to abolish the strict dependence of Smo signaling on Hh, whereas the Hh signaling system resists small perturbations because of its self-correcting properties.

Experimental Procedures

Fly Strains

The amorphic ci^{94}/ey^{D} stock (Slusarski et al., 1995; Méthot and Basler, 1999) was provided by R. Holmgren (Evanston, IL), while *w*; *P{hs-hh w⁺}/TM3*, *Ser* flies (Ingham, 1993) were obtained from P. Ingham (Sheffield, UK). The *w*; *P{UAS-R* w⁺}* stock (Li et al., 1995; Ohlmeyer and Kalderon, 1997) was provided by D. Kalderon (Columbia University, NY), while the unpublished *y w*; *P{tub-smo-HA w⁺}* stock was kindly provided by C. von Mering and K. Basler (Zürich, Switzerland). The unpublished *w*; *P{UAS-ptc w⁺}* stock was kindly provided by R. Burke and K. Basler, and the *UAS-ptc* chromosome was derived from *P{UAS>CD2, y⁺>ptc}* (Chen and Struhl, 1996). The unpublished *w P{UAS-smo w⁺}* stock was kindly provided by E. Frei (Zürich, Switzerland). All other stocks were obtained from the Bloomington and Umea Stock Centers.

smo^{2} $ptc^{Df(2R)44CE}$ and smo^{3} $ptc^{Df(2R)44CE}$ double mutant stocks were prepared by recombination between single mutant chromosomes and identified by control crosses.

Heat-Shock Treatment and Immunostaining of Embryos

w; *P{hs-hh w⁺}/TM3*, *Ser* embryos were collected for 2 hr and aged for 3 hr at 25°C, heat-shocked at 37°C for two 30 min periods separated by 45 min at 25°C, and then allowed to recover for 7 min at 25°C, fixed, and assayed for Smo protein levels.

Detection of Smo protein was performed by staining embryos overnight at room temperature with rabbit anti-Smo antiserum at a dilution of 1:100. The anti-Smo antiserum was generated and affinity-purified against a histidine-tagged Smo peptide, which consisted of the amino acids 48 to 245 (Alcedo et al., 1996), in a pET19b vector. En immunohistochemistry used the 4D9 monoclonal antibody (gift of T. Kornberg, San Francisco, CA), while Wg immunohistochemistry used a mouse monoclonal anti-Wg antibody (gift of S. Cohen, Heidelberg, Germany). Ci protein was detected by using the 2A1 mouse monoclonal antibody (gift of R. Holmgren), while the LacZ protein was detected by using mouse monoclonal antibodies from Boehringer-Mannheim. The HA protein was detected using the 3F10 rat anti-HA monoclonal antibody from Boehringer-Mannheim.

Antibodies were detected with the Vectastain ABC detection system (Vector Laboratories, Burlingame, CA). Double-labeling of proteins was carried out according to Lawrence et al. (1987).

Sequence Analysis of *smo* Mutant Alleles

Homozygous smo^{1} and smo^{2} embryos were selected from wild-type and heterozygous embryos by staining with X-Gal for the presence of the *lacZ*-marked balancer chromosome. Homozygous smo^{3} flies were obtained from a stock carrying a wild-type *smo* transgene kindly provided by K. Basler. All homo- or heteroallelic combinations of these three *smo* alleles are embryonic lethal at 18°C and rescuable to wild-type adults by a single copy of a *smo* transgene (Alcedo et al., 1996). DNA was prepared from the homozygous *smo* mutants according to standard procedures, and the mutant alleles were amplified by long-range PCR with the use of the Expand Long Template PCR System (Boehringer). The mutant DNA fragments were purified according to standard procedures and sequenced on both strands of the entire transcribed *smo* region (Alcedo et al., 1996) by the dideoxynucleotide method with a DNA sequencer Model 373A using dye terminators (Applied Biosystems, Inc.). The sequencing results are summarized in Table 1.

Partial Differential Equations Describing the Coupling of the Protein Concentrations of Smo, Ptc, and Hh

The elegance and advantages of the Hh signaling system become apparent if one considers how the concentrations of Smo and Ptc change with time in regions of high and low Hh concentrations. A derivation of how the concentrations of Ptc, P, and Smo proteins, S, depend on each other, on Hh, and on time leads to nonlinear coupled differential equations for P and S that can be solved only numerically: $\partial P/\partial t = k_3/k_2\,[1 - \exp(-k_2 t)]\,f(S) - k_4 P$, in which the k's are rate constants and f(S), the rate constant of *ptc* mRNA synthesis, is a nonlinear function of S (and Hh) in the anterior compartment; $\partial S/\partial t = k_5\,k_7/k_6\,[1 - \exp(-k_6 t)] - f(P)\,S$, in which the k's are rate constants and f(P) is a nonlinear function of P in the anterior compartment. In the posterior compartment, $P = 0$ and f(P) is replaced by the rate constant k_8 to yield an uncoupled differential equation for S. At equilibrium, in the anterior compartment, $P_{eq} = \kappa_1\,[1 - \exp(-S_f/S_b)]$, in which κ_1 is constant and S_f and S_b are the equilibrium concentrations of free Smo and Smo bound to Ptc; and $S_{eq} = \kappa_2/f(P)$, in which κ_2 is a constant and f(P) is the rate constant

of Smo protein degradation, which depends on P_{eq} or S_f (or, what is equivalent, on the ratio of inactive to active PKA, or on the ratio of dephosphorylated to phosphorylated critical PKA sites on Smo, if S_f activates a phosphatase that competes with PKA). The derivation of these equations may be obtained from the corresponding author upon request.

Acknowledgments

We thank C. von Mering, R. Burke, K. Basler, and E. Frei for providing their unpublished *tub-smo-HA*, *UAS-ptc*, and *UAS-smo* stocks, and T. Gutjahr for assistance in the purification of the anti-Smo antiserum. We are indebted to E. Frei for discussions and K. Basler for his suggestion to use *tub-smo-HA*. We are grateful to T. Kornberg for anti-En, S. Cohen for anti-Wg, R. Holmgren for anti-Ci mouse monoclonal antibodies and the ci^{94} stock, the Bloomington Stock Center for the *act5c-Gal4*, *prd-Gal4*, and other mutant stocks, D. Kalderon for the *UAS-R** stock, and D. Casso and T. Kornberg for the *ptc hh* double mutant stock. We thank Fritz Ochsenbein for his expertise and help in the prepararion of the table and figures, and K. Basler, N. Méthot, M. Levine, E. Frei, T. Kornberg, P.-T. Chuang, F. Cifuentes, F.-A. Ramírez-Weber, and Hans Noll for comments on the manuscript. This work was supported by the Swiss National Science Foundation grants 31-40874.94 and 31-56817.99 and by the Kanton Zürich.

Received June 1, 2000; revised July 10, 2000.

References

Alcedo, J., and Noll, M. (1997). Hedgehog and its Patched-Smoothened receptor complex: a novel signalling mechanism at the cell surface. Biol. Chem. *378*, 583–590.

Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M., and Hooper, J.E. (1996). The *Drosophila smoothened* gene encodes a seven-pass membrane protein, a putative receptor for the Hedgehog signal. Cell *86*, 221–232.

Aza-Blanc, P., and Kornberg, T.B. (1999). Ci, a complex transducer of the Hedgehog signal. Trends Genet. *15*, 458–462.

Aza-Blanc, P., Ramírez-Weber, F.-A., Laget, M.-P., Schwartz, C., and Kornberg, T.B. (1997). Proteolysis that is inhibited by Hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. Cell *89*, 1043–1053.

Basler, K. (2000). Waiting periods, instructive signals and positional information. EMBO J. *19*, 1169–1175.

Chen, Y., and Struhl, G. (1996). Dual roles for Patched in sequestering and transducing Hedgehog. Cell *87*, 553–563.

Chen, Y., and Struhl, G. (1998). In vivo evidence that Patched and Smoothened constitute distinct binding and transducing components of a Hedgehog receptor complex. Development *125*, 4943–4948.

Chen, Y., Gallaher, N., Goodman, R.H., and Smolik, S.M. (1998). Protein kinase A directly regulates the activity and proteolysis of *cubitus interruptus*. Proc. Natl. Acad. Sci. USA *95*, 2349–2354.

Chen, C.-H., von Kessler, D.P., Park, W., Wang, B., Ma, Y., and Beachy, P.A. (1999). Nuclear trafficking of Cubitus interruptus in the transcriptional regulation of Hedgehog target gene expression. Cell *98*, 305–316.

DiNardo, S., Sher, E., Heemskerk-Jongens, J., Kassis, J.A., and O'Farrell, P.H. (1988). Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis. Nature *332*, 604–609.

Eaton, S., and Kornberg, T.B. (1990). Repression of *ci-D* in posterior compartments of *Drosophila* by *engrailed*. Genes Dev. *4*, 1068–1077.

Hahn, H., Wicking, C., Zaphiropoulos, P.G., Gailani, M.R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A.B., Gillies, S., et al. (1996). Mutations of the human homolog of *Drosophila patched* in the nevoid basal cell carcinoma syndrome. Cell *85*, 841–851.

Hidalgo, A. (1991). Interactions between segment polarity genes and the generation of the segmental pattern in *Drosophila*. Mech. Dev. *35*, 77–87.

Hidalgo, A., and Ingham, P. (1990). Cell patterning in the *Drosophila* segment: spatial regulation of the segment polarity gene *patched*. Development *110*, 291–301.

Hooper, J.E. (1994). Distinct pathways for autocrine and paracrine Wingless signalling in *Drosophila* embryos. Nature *372*, 461–464.

Hooper, J.E., and Scott, M.P. (1989). The *Drosophila patched* gene encodes a putative membrane protein required for segmental patterning. Cell *59*, 751–765.

Ingham, P.W. (1993). Localized *hedgehog* activity controls spatial limits of *wingless* transcription in the *Drosophila* embryo. Nature *366*, 560–562.

Ingham, P.W., and Hidalgo, A. (1993). Regulation of *wingless* transcription in the *Drosophila* embryo. Development *117*, 283–291.

Jiang, J., and Struhl, G. (1995). Protein kinase A and Hedgehog signaling in *Drosophila* limb development. Cell *80*, 563–572.

Jiang, J., and Struhl, G. (1998). Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. Nature *391*, 493–496.

Johnson, R.L., Grenier, J.K., and Scott, M.P. (1995). *patched* overexpression alters wing disc size and pattern: transcriptional and post-transcriptional effects on *hedgehog* targets. Development *121*, 4161–4170.

Johnson, R.L., Rothman, A.L., Xie, J., Goodrich, L.V., Bare, J.W., Bonifas, J.M., Quinn, A.G., Myers, R.M., Cox, D.R., Epstein, E.H., Jr., and Scott, M.P. (1996). Human homolog of *patched*, a candidate gene for the basal cell nevus syndrome. Science *272*, 1668–1671.

Krishnan, V., Pereira, F.A., Qiu, Y., Chen, C.-H., Beachy, P.A., Tsai, S.Y., and Tsai, M.-J. (1997). Mediation of Sonic Hedgehog-induced expression of COUP-TFII by a protein phosphatase. Science *278*, 1947–1950.

Lawrence, P.A., Johnston, P., Macdonald, P., and Struhl, G. (1987). Borders of parasegments in *Drosophila* embryos are delimited by the *fushi tarazu* and *even-skipped* genes. Nature *328*, 440–442.

Lepage, T., Cohen, S.M., Diaz-Benjumea, F.J., and Parkhurst, S.M. (1995). Signal transduction by cAMP-dependent protein kinase A in *Drosophila* limb patterning. Nature *373*, 711–715.

Lessing, D., and Nusse, R. (1998). Expression of *wingless* in the *Drosophila* embryo: a conserved *cis*-acting element lacking conserved Ci-binding sites is required for *patched*-mediated repression. Development *125*, 1469–1476.

Li, W., Ohlmeyer, J.T., Lane, M.E., and Kalderon, D. (1995). Function of protein kinase A in Hedgehog signal transduction and Drosophila imaginal disc development. Cell *80*, 553–562.

Maniatis, T. (1999). A ubiquitin ligase complex essential for the NF-κB, Wnt/Wingless, and Hedgehog signaling pathways. Genes Dev. *13*, 505–510.

Marigo, V., Davey, R.A., Zuo, Y., Cunningham, J.M., and Tabin, C.J. (1996). Biochemical evidence that Patched is the Hedgehog receptor. Nature *384*, 176–179.

Méthot, N., and Basler, K. (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. Cell *96*, 819–831.

Nakano, Y., Guerrero, I., Hidalgo, A., Taylor, A., Whittle, J.R.S., and Ingham, P.W. (1989). A protein with several possible membrane-spanning domains encoded by the *Drosophila* segment polarity gene *patched*. Nature *341*, 508–513.

Ohlmeyer, J.T., and Kalderon, D. (1997). Dual pathways for induction of *wingless* expression by protein kinase A and Hedgehog in *Drosophila* embryos. Genes Dev. *11*, 2250–2258.

Ohlmeyer, J.T., and Kalderon, D. (1998). Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. Nature *396*, 749–753.

Pan, D., and Rubin, G.M. (1995). cAMP-dependent protein kinase and *hedgehog* act antagonistically in regulating *decapentaplegic* transcription in Drosophila imaginal discs. Cell *80*, 543–552.

Perrimon, N. (1994). The genetic basis of patterned baldness in *Drosophila*. Cell *76*, 781–784.

Price, M.A., and Kalderon, D. (1999). Proteolysis of Cubitus interruptus in *Drosophila* requires phosphorylation by protein kinase A. Development *126*, 4331–4339.

Slusarski, D.C., Motzny, C.K., and Holmgren, R. (1995). Mutations that alter the timing and pattern of *cubitus interruptus* gene expression in *Drosophila melanogaster*. Genetics *139*, 229–240.

Stone, D.M., Hynes, M., Armanini, M., Swanson, T.A., Gu, Q., Johnson, R.L., Scott, M.P., Pennica, D., Goddard, A., Phillips, H., et al. (1996). The tumour-suppressor gene *patched* encodes a candidate receptor for Sonic hedgehog. Nature *384*, 129–134.

Strutt, D.I., Wiersdorff, V., and Mlodzik, M. (1995). Regulation of furrow progression in the *Drosophila* eye by cAMP-dependent protein kinase A. Nature *373*, 705–709.

Tabata, T., and Kornberg, T.B. (1994). Hedgehog is a signaling protein with a key role in patterning Drosophila imaginal discs. Cell *76*, 89–102.

Theodosiou, N.A., Zhang, S., Wang, W.-Y., and Xu, T. (1998). *slimb* coordinates *wg* and *dpp* expression in the dorsal-ventral and anterior-posterior axes during limb development. Development *125*, 3411–3416.

Thérond, P.P., Limbourg-Bouchon, B., Gallet, A., Dussilol, F., Pietri, T., van den Heuvel, M., and Tricoire, H. (1999). Differential requirements of the Fused kinase for Hedgehog signalling in the *Drosophila* embryo. Development *126*, 4039–4051.

van den Heuvel, M., and Ingham, P.W. (1996). *smoothened* encodes a receptor-like serpentine protein required for *hedgehog* signalling. Nature *382*, 547–551.

Wang, G., Wang, B., and Jiang, J. (1999). Protein kinase A antagonizes Hedgehog signaling by regulating both the activator and repressor forms of Cubitus interruptus. Genes Dev. *13*, 2828–2837.

Xie, J., Murone, M., Luoh, S.-M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J.M., Lam, C.-W., Hynes, M., Goddard, A., et al. (1998). Activating *Smoothened* mutations in sporadic basal-cell carcinoma. Nature *391*, 90–92.

Note Added in Proof

After submission of this manuscript, we learned that similar results consistent with our model, which is based on studies in the embryo, have been obtained in imaginal discs by Denef et al. (Hedgehog Induces Opposite Changes in Turnover and Subcellular Localization of Patched and Smoothened [Cell *102*, 521–531]) http://www.cell.com/cgi/content/full/102/4/521/.