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Smoothened translates Hedgehog levels into distinct responses

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SUMMARY

In the *Drosophila* wing, Hedgehog is made by cells of the posterior compartment and acts as a morphogen to pattern cells of the anterior compartment. High Hedgehog levels instruct L3/4 intervein fate, whereas lower levels instruct L3 vein fate. Transcriptional responses to Hedgehog are mediated by the balance between repressor and activator forms of Cubitus interruptus, CiR and CiA. Hedgehog regulates this balance through its receptor, Patched, which acts through Smoothened and thence a regulatory complex that includes Fused, Costal, Suppressor of Fused and Cubitus interruptus. It is not known how the Hedgehog signal is relayed from Smoothened to the regulatory complex nor how responses to different levels of Hedgehog are implemented. We have used chimeric and deleted forms of Smoothened to explore the signaling functions of Smoothened. A Frizzled/Smoothened chimera containing the Smo cytoplasmic tail (FFS) can induce the full spectrum of Hedgehog responses but is regulated by Wingless rather than Hedgehog. Smoothened whose cytoplasmic tail is replaced with that of Frizzled (SSF) mimics *fused* mutants, interfering with high Hedgehog responses but with no effect on low Hedgehog responses. The cytoplasmic tail of Smoothened with no transmembrane or extracellular domains (SmoC) interferes with high Hedgehog responses and allows endogenous Smoothened to constitutively initiate low responses. SmoC mimics *costal* mutants. Genetic interactions suggest that SSF interferes with high signaling by titrating out Smoothened, whereas SmoC drives constitutive low signaling by titrating out Costal. These data suggest that low and high signaling (1) are qualitatively different, (2) are mediated by distinct configurations of the regulatory complex and (3) are initiated by distinct activities of Smoothened. We present a model where low signaling is initiated when a Costal inhibitory site on the Smoothened cytoplasmic tail shifts the regulatory complex to its low state. High signaling is initiated when cooperating Smoothened cytoplasmic tails activate Costal and Fused, driving the regulatory complex to its high state. Thus, two activities of Smoothened translate different levels of Hedgehog into distinct intracellular responses.

Key words: Hedgehog, Smoothened, Frizzled, Morphogen, *Drosophila*

INTRODUCTION

Members of the Hedgehog (Hh) family of secreted proteins mediate many of the short-range cell interactions that underlie development and tissue maintenance in metazoans (Ingham and McMahon, 2001). In several contexts, Hh acts as a morphogen, such that different levels of signaling activity specify distinct responses. This is well illustrated in the vertebrate neural tube where Sonic Hedgehog (Shh) forms a concentration gradient, highest at the floorplate and lower in more dorsal regions. At least four different neural fates are dictated by different thresholds of Shh (Ericson et al., 1997; Hynes et al., 2000; Briscoe et al., 2001). Thus, a single signal establishes diverse cell types, in appropriate spatial relationship to one another. More recently it has become apparent that anomalous activation of the Hh signaling pathway in neonates and adults underlies a variety of cancers including basal cell carcinoma and medulloblastoma (Berman et al., 2002; Ruiz i Altaba et al., 2002). Hh was first identified in genetic screens for genes involved in segmentation of *Drosophila* (Nusslein-Volhard and Wieschaus, 1980). In this context, it maintains segment and parasegment borders, acts in a combinatorial fashion with Wingless (Wg) and Egf signaling to pattern ventral cuticle, and acts as a morphogen to pattern dorsal cuticle and imaginal discs.

Transcriptional responses to Hh are mediated by the Ci/Gli family of transcription factors, which can act both as repressors and activators of transcription (Dominguez et al., 1996; Aza-Blanc et al., 1997; Ruiz i Altaba, 1997; Methot and Basler, 1999; Wang et al., 2000a). The choice between these activities is regulated by Hh and implemented by a cytoplasmic complex that includes full length Ci (Ci155), the serine-threonine kinase Fused (Fu), the kinesin-like Costal (Cos) and Suppressor of Fused (Sufu) (Robbins et al., 1997; Sisson et al., 1997; Monnier et al., 1998; Stegman et al., 2000). Ci155 appears to be a latent precursor form. In the absence of Hh, limited proteolysis takes Ci155 to its repressor form, CiR (Aza-Blanc et al., 1997; Robbins et al., 1997; Methot and Basler, 2000). This processing involves Cos, Fu regulatory domain (FuReg), phosphorylation of Ci by PKA, GSK3 and CKI, and a ubiquitin

E3 ligase activity mediated by Slimb and Cul1 (Jiang, 2002). Absence of Hh also prevents Ci155 from entering the nucleus by a redundant mechanism that requires either Cos and a Cos-binding site on Ci, or FuReg with Sufu and a Sufu-binding site on Ci (Methot and Basler, 2000; Stegman et al., 2000; Wang et al., 2000b; Wang and Holmgren, 2000; Lefers et al., 2001). In the presence of Hh, the complex dissociates from microtubules, recruits Sufu, and hyperphosphorylates Fu and Cos (Therond et al., 1996; Robbins et al., 1997; Stegman et al., 2000; Nybakken et al., 2002). This curtails processing to CiR, allows nuclear access of Ci155, promotes depletion of Ci155 and generates the transcriptional activator, CiA (Ohlmeyer and Kalderon, 1998; Chen et al., 1999; Wang and Holmgren, 1999). Whether CiA differs from Ci155 by post-translational modification, by associated factors, and/or by subcellular localization has not been determined. Full activation of Ci requires activity of Fu and Cos (Ohlmeyer and Kalderon, 1998; Wang et al., 2000b; Lefers et al., 2001). Extensive analysis has failed to delineate simple roles for any of these components in regulation of Ci, or which components are the primary targets of Hh regulation.

Hh influences the Ci regulatory complex through two transmembrane proteins, Patched (Ptc) and Smoothened (Smo). Ptc binds Hh with nanomolar affinity (Chen and Struhl, 1996; Marigo et al., 1996; Stone et al., 1996; Fuse et al., 1999). Ptc is then internalized and traffics Hh to endosomal compartments where both are degraded (Capdevila et al., 1994; Tabata and Kornberg, 1994; Alcedo et al., 2000; Denef et al., 2000; Incardona et al., 2000; Incardona et al., 2002). In the process, Hh signaling is activated through Smo, a member of the serpentine receptor superfamily (Ingham et al., 1991; Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Ptc might regulate Smo through direct physical association, but the bulk of the two proteins is not co-localized, does not co-immunoprecipitate, and a 45:1 ratio of Smo:Ptc results in 80% reduction in Smo activity (Stone et al., 1996; Murone et al., 1999; Denef et al., 2000; Johnson et al., 2000; Taipale et al., 2002). This suggests a catalytic mechanism for inhibition of Smo by Ptc.

How Smo activates downstream signaling is unknown. Smo activity correlates with its phosphorylation and accumulation at the cell surface (Alcedo et al., 2000; Denef et al., 2000; Ingham et al., 2000). This phosphorylation and cell-surface accumulation may be a consequence of signaling, rather than being necessary for signaling (Kalderon, 2000; Incardona, 2002; Taipale, 2002). Smo has a large N-terminal extracellular domain that is evolutionarily conserved (Stone et al., 1996). Analogous to other serpentine receptors, this should be a ligand-binding domain that regulates Smo activity. However, there is no evidence that Smo has an extracellular ligand, nor any regulator other than Ptc. Structure function studies of rat Smoothened suggested that the extracellular and first two to four transmembrane domains are necessary for its association with and regulation by Ptc, while its third intracellular loop and seventh transmembrane domain activate downstream signaling (Xie et al., 1998; Murone et al., 1999). Serpentine receptors generally couple to heterotrimeric G proteins through these latter regions, suggesting that G proteins are involved in relaying the Smo signal. Pertussis toxin, which interferes with Gαi and Gαo, interferes with Hh-directed morphogenesis in zebrafish embryos, but not in primary myoblasts (Hammerschmidt and McMahon, 1998; Norris et al., 2000). Gαo can be activated when Hh, Ptc and Smo are co-transfected into melanophores, but the slow kinetics suggest that this effect may be indirect (DeCamp et al., 2000). Given the broad range of cellular processes modulated by G proteins and many potential mechanisms for across-regulation between pathways, these data are equally consistent with an indirect role for G proteins in Hh signaling. Smo has a long cytoplasmic tail, which is uncharacteristic of G-protein-coupled receptors. This suggests that something other than G proteins may be involved in transducing the Hh signal.

To understand how Smoothened transduces the Hh signal, we built a series of truncated and chimeric versions of Smo. Fz1, which is structurally related to Smo but has no genetic interactions with the Hh pathway, was used to generate chimeric Smo/Fz proteins. Their activity was measured through their effects on Ci, their regulation of Hh target genes, and their effects on wing patterning. We find that low and high Hh responses are independently affected by various transgenes. This leads to a model where Smo adopts three distinct states in response to zero, low and high levels of Hh. The OFF state exerts no influence on Ci or the regulatory complex, the low state binds to and inactivates Cos, while the high state involves Smo oligomers that activate Fu and Cos. In addition we find that the cytoplasmic tail of Smo attached to Fz can activate the full range of Hh responses, but in response to Wg rather than Hh. This suggests that Fz also responds to different ligand levels with distinct signaling states. Distinct signaling states of a receptor is a novel mechanism by which a morphogen could generate multiple responses to a single ligand.

MATERIALS AND METHODS

Fly strains and culture

Flies were grown on cornmeal molasses media at 25°C unless otherwise specified. The genetic background was *Df(1)w672c, y*. Gal 4 drivers and lines used for transgene expression were obtained from the Bloomington stock center unless otherwise specified: *Bx[MS1096]* (Dominguez et al., 1996) from K. Basler (Zurich), *C765* (Nellen et al., 1996), *ptcGal4[599.1]* (Hinz et al., 1994) from E. Knust (U Cologne), *prdGal4, UasWg* (Hays et al., 1997), *ptc[Ep941]* from the Szeged stock center, *UasFu* (Ascano et al., 2002) from David Robbins (U Cincinnati) and *Pcos+* (a genomic fragment which rescues *cos* mutants) (Sisson et al., 1997) from Matthew Scott (Stanford U). Mosaic expression of transgenes was achieved using the Flp-out Gal4 strategy (Pignoni and Zipursky, 1997), and 20 minute heatshock at 37°C of cultures 24-48 hours after egg laying (e.g. *y w hsFLP122/w; AYG, UasGFP/+; UasSmo/+* for Fig. 6A). The FLP-FRT system (Xu and Rubin, 1993) and 2 hour heatshock at 37°C of cultures 24-48 hours after egg laying was used to generate smo^{-} wing clones in a background where transgene expression was driven by *MS109*6 (e.g. *w MS1096/y w FLP122; smo^{3} Frt40A/Ubi:GFP Frt40A; UasSmoC/+* for Fig. 8B).

Staining procedures

Wings were wet with isopropanol, mounted in 1:1 Canada balsam:methyl salicylate, viewed under Kohler illumination and images captured with a Zeiss Axiocam. In situ hybridization with digoxigenin-labeled antisense RNA probes (Tautz and Pfeifle, 1989) was used to detect *col*, *ptc* and *dpp* expression. Immunofluorescent or immunohistochemical analysis of imaginal discs followed standard procedures (van den Heuvel et al., 1989). For LeptomycinB treatment, larval heads were removed and inverted in cl-8 media, cultured for 2

hours at room temperature in 50 ng/ml LMB (Sigma) in cl-8 media, then fixed and handled as above. The rat monoclonal antibody 2A1, which detects Ci155 but not CiR, was used at 1:3 (courtesy of R. Holmgren, Northwestern University); Iro was detected using rat *araucan* antibody at 1:1000 (Diez del Corral et al., 1999); Ptc was detected using a rat antibody raised against bacterially expressed Ptc fragment (I314-S542); Myc epitope by 9E10 (UCHSC Cancer Center); the HA epitope by 12CA5 (Boehringer Mannheim); the Fz CRD by 1C11 (Krasnow and Adler, 1994); and the Smo CRD by affinity purified rabbit antibody (Alcedo et al., 2000). Antibody detection used FITC conjugated anti-mouse or anti-rabbit (Jackson Laboratories), Cy5-conjugated anti-rat (Jackson Laboratories) for Ci155, biotinylated anti-rat followed by ABC reagent (Vector Labs) and Cy3-TSA (NEN) or horseradish peroxidase for Ptc or Iro. Discs were dissected in 50% glycerol and mounted in PermaFluor (ThermoShandon, Pittsburg, PA), or 50% glycerol for HRP detection. Histochemical images were captured using a Zeiss Axiocam; confocal images were captured using a Zeiss Pascal5 LSM.

Transgene construction and characterization

Smo full-length cDNA was truncated using PCR to introduce a *Sal*I site at nucleotide 174 in its 5′UTR and a *Xho*I site immediately after the termination codon. A double stranded oligonucleotide with compatible 3′ overhangs and encoding a Myc epitope was inserted in frame immediately following the putative signal sequence at the *Sfi*I site at nucleotide 332 (CGATGCAGCAAAAGCTCATTTCTGAAGAGGACTTGAATAGTT). An *Aat*I site was introduced at the end of the seventh transmembrane domain, changing TGGACACCTTCT to TGGACGTCTTCT and resulting in T554S, P555S. The endogenous *Nde*I site at I265 in the first transmembrane domain was used for domain swaps with Fz. SmoC was generated using PCR to insert an ATG codon embedded in a Kozak initiation consensus context, and a Myc epitope (±a myristoylation sequence derived from Src) immediately before T554; MycSmoC (TTAGATCTAACCAACATGGAGCAAAAGCTCATTTCTGAATATTACTTGAATACACCTTCTTCAATTGAG), MycMyr SmoC (inserting ATGGGCTCCTCCAAGTCCAAGCCCAAG before the first ATG of MycSmoC). MycSmoN was generated using PCR to introduce a termination codon into MycSmo after I255 (CCCCAAGCTTACTCGGCATGCTCATC). MycSmoT1 was generated using PCR to introduce a termination codon into MycSmo after the first transmembrane domain at P288 (CTACGGATACTTGTTTGGCATTC). Fz full-length cDNA (a gift from P. Adler) was modified immediately before the stop codon by in frame insertion of a double stranded oligonucleotide with compatible 5′ overhangs and encoding an HA epitope into the *Bsi*W1 site at nucleotide 1751 (GTACCCATACGACGTTCCAGACTACGCGTAGTCGAC). An *Aat*I site was introduced at the end of the seventh transmembrane domain by changing CTGTATTCCAGCAAG to CTGTGGACGTCCAAG and resulting in Y553W. An *Nde*I site was inserted in the first transmembrane domain by modifying GCACGGGTCTGT to GCACGCATATGT, and resulting in V256I. Nucleotide substitutions were all accomplished using the Altered Sites mutagenesis kit (Promega) and confirmed by sequencing.

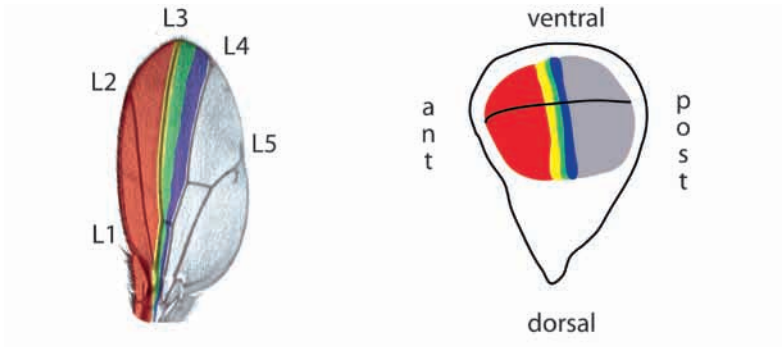
Chimeric transgenes were constructed by swapping Fz and Smo domains at the *Aat*I and *Nde*I sites. The chimeric, mutagenized and truncated transgenes (Fig. 3) were subcloned into pUAST (Brand and Perrimon, 1993) and introduced into the germline of flies by standard methods (Rubin and Spradling, 1982). Multiple independent lines were established for each transgene. Activity was scored by effects on wing vein patterning and wing hair polarity, following expression driven by *MS1096*. About one in six lines had unusually potent phenotypic effects, which correlated with unusually high levels of protein accumulation, as assayed by immunofluorescence. These unusual lines were judged high expressers rather than aberrant products if similar phenotypic effects were generated by high dosage (e.g. 4×) of more typical transgenes. Protein product of the expected size was confirmed by western blotting of embryo extracts where transgene expression was driven by *hsGal4*. Expression dosages are expressed as the product of the Gal4 driver dosage and Uas transgene dosage. Thus, heterozygous *C765*, *71B* or *MS1096* driving a single copy of *UasSmo* is 1×; hemizygous *MS1096* driving a single copy of *UasSmo* is 2×; hemizygous *MS1096* driving two copies of *UasSmo* is 4×; and transgenes with unusually high expression levels are High.

RESULTS

Dosage-dependent activation of Hh targets by Smoothened

Very high levels of Smo overexpression in transfected cells will drive Hh-independent activation of various Hh responses (Stone et al., 1996; Murone et al., 1999; Taipale et al., 2002). To determine whether Smo overexpression can activate the full range of Hh responses, we used the *Gal4-Uas* system to generate graded levels of Smo overexpression in the *Drosophila* wing. Different expression levels were achieved by varying the dosage of *Gal4* and *Uas* transgenes; dosages are expressed as the product of *Gal4* and *Uas* copy number, or as 'high' for insertions with unusually high levels of expression (see Materials and Methods). The dose-dependent responses to Hh that pattern the anterior-posterior axis of the wing are summarized in Fig. 1 (Ingham and Fietz, 1995; Mullor et al., 1997; Strigini and Cohen, 1997; Ingham and McMahon, 2001) and results are presented in Fig. 2. Consistent with previous reports, 1× *Smo* led to minor changes in wing morphology (Alcedo et al., 2000; Denef et al., 2000; Ingham et al., 2000). *Smo* 2× generated reproducible expansion of L3 into the L2/3 intervein territory (not shown). *Smo* 4× expanded L1 as well as L3, and generated mild hypertrophy of the anterior wing (Fig. 2F). High *Smo* generated severe overgrowth of the anterior compartment, which was generally pharate lethal. In rare adults that did eclose, the entire anterior compartment was transformed into vein, except for a small strip at the border (not shown). The changes in the adult morphology driven by *Smo* overexpression correlated with changes in expression of Hh target genes. Iro has a complex expression pattern in the wing imaginal disc, reflecting multiple layers of regulation (Gomez-Skarmeta et al., 1996; Gomez-Skarmeta and Modolell, 1996). Its expression in the center of the disc, the L3 region (brackets in all figures), is the most sensitive and specific reporter for low Hh responses. There it requires both Dpp and Hh signaling and is blocked by Wg signaling. Iro in the L3 region was expanded with both 4× and high *Smo* overexpression (compare Fig. 2G,L with 2B). *dpp* and *ptc* expression were unaffected by 4× *Smo* (compare Fig. 2H,I with 2C,D), but were strongly driven across the entire anterior compartment by high *Smo* (Fig. 2M,N). *col* and En expression were not affected by either 4× or high *Smo* (Fig. 2E,J,O; data not shown). Thus, increasing levels of *Smo* lead to activation of low and then intermediate Hh responses.

The failure of high *Smo* to activate the highest level of Hh signaling might be because of negative feedback resulting from high levels of induced Ptc (e.g. Fig. 2N). Ptc is a potent inhibitor of Smo (Taipale et al., 2002) and modest overexpression of *Ptc* is sufficient to eliminate high Hh responses (Johnson et al., 1995; Johnson et al., 2000). Wings co-expressing *Ptc* and high levels of *Smo* (*UasSmo*^{high}/*ptc[EP941]*) were indistinguishable from those expressing *Ptc*



Hh level	morphology	CiR	CiA	Ci155	*en*	*ptc*	*col*	*dpp*	*iro*
Off	L2 and anterior	++	-	cyto	-	+/-	-	-	-
Low	L3	+/-	?	?	-	+/-	-	+	++
Medium	L3/4 intervein	?	+/-	nuc	-	+	++	++	++
High	L3/4 intervein	-	++	nuc	++	++	++	+	-

Fig. 1. Hedgehog response zones and wing patterning. The dorsal surface of a wing is on the left and a cartoon of wing imaginal disc is on the right. Five longitudinal veins (L1-L5) punctuate the anteroposterior axis of the wing. The center of the disc, the wing pouch, is the primordium for the wing and is colored. The horizontal line across the center of the wing pouch indicates where dorsal and ventral compartments meet at the future wing margin. Hh is secreted by cells of the posterior compartment (towards the right in this and all other figures), shaded gray. The anterior compartment responds to the gradient of Hh that forms near its border because it produces the Hh receptor Patched (Ptc) (Chen and Struhl, 1996; Marigo et al., 1996; Stone et al., 1996). The first two or three cells have high levels of Hh (blue), which allows nuclear access of Ci155, depletes Ci155 to make CiA and blocks production of CiR. This activates transcription of *en*, *ptc*, *col* and *dpp* (Blair, 1992; Guillen et al., 1995; Ohlmeyer and Kalderon, 1998). The next four to six cells see intermediate or low levels of Hh (green and yellow, respectively), which allow nuclear access of Ci155, and make little or no CiA and little or no CiR so that Ci155 accumulates. *ptc*, *col*, *dpp* and *iro* are made in the intermediate zone, whereas only *dpp* and *iro* are made in the low zone (Mullor et al., 1997; Strigini and Cohen, 1997; Vervoort et al., 1999; Mullor and Guerrero, 2000). Far from Hh sources (red), Ci155 is depleted to produce CiR, and Hh target genes are repressed. En expression defines the high response zone where it prevents Iro expression and where Ptc sequesters Hh to limit the range of signaling (Hidalgo, 1994; Chen and Struhl, 1996; de Celis and Barrio, 2000; Crozatier et al., 2002). The intermediate zone is defined by the overlap of *iro* and *col*. Col in the high and intermediate zones downregulates Dpp responses; the result is the L3/4 intervein (Vervoort et al., 1999; de Celis and Barrio, 2000; Mohler et al., 2000; Crozatier et al., 2002). Col also activates transcription of the secreted EGFR ligand Vein, which signals to posterior adjacent cells to make vein L4 (Mohler et al., 2000; Crozatier et al., 2002). In the low zone, only *dpp* and *iro* are induced. They cooperate to specify vein L3 (Mullor and Guerrero, 2000). Dpp also promotes growth along the anteroposterior axis and acts as a morphogen to pattern deeper in the anterior and posterior compartments (Lawrence and Struhl, 1996). Finally, G1 cyclins are activated by Hh to promote growth and proliferation (Duman-Scheel et al., 2002).

alone (not shown), consistent with negative feedback by Ptc preventing high responses by overexpressed Smo.

The induction of Hh responses deep in the anterior compartment by *Smo* overexpression might be independent of Hh. Alternatively, Hh levels anterior to L3 might normally be below the response threshold but might elicit a response when the ratio of Smo to Ptc increases. To test for Hh dependence, Hh levels were severely reduced using a temperature sensitive allele of hh^{ts2} (not shown). After 24 hours at the restrictive temperature, *col* and Iro were not detected at the compartment border of hh^{ts2} homozygous discs, whereas expression of *ptc* and *dpp* was greatly reduced (Strigini and Cohen, 1997). *Smo* 2× restored Iro and *dpp* expression to hh^{ts2} homozygous discs, whereas high *Smo* restored Iro, *dpp* and *ptc* levels but not *col* expression. In all cases the restored expression was broader than its domain in wild-type discs. We conclude that the dosage-dependent responses to *Smo* overexpression are Hh independent.

FFS mediates all Hh responses but is regulated by Wg

To identify domains of Smoothened responsible for regulation of Hh signaling, we built truncated and chimeric versions of Smo. Smo is a divergent member of the Frizzled (Fz) family of receptors. Fzs bind their ligands, the Wnts, through a conserved extracellular N-terminal cysteine-rich domain (CRD), transduce the signal across the membrane via seven conserved transmembrane domains (TM), and initiate signaling with divergent cytoplasmic tails (CT) (Bhanot et al., 1996; Xu and Nusse, 1998; Dann et al., 2001). Fz1, which appears to have no genetic interactions with the Hh pathway, was used to generate chimeric Smo/Fz proteins. Chimeras swapped the extracellular CRDs, the TM domains and the CTs. Constructs and results are summarized in Fig. 3.

A chimera with Fz CRD, Fz TM and Smo CT (FFS) activated the full spectrum of Hh responses, but was regulated by Wg rather than Ptc and Hh (Fig. 4). FFS 4× gave some excess venation distally, between the second and third wing veins (not shown). High FFS gave ectopic venation near the wing margin and overgrowth of the costa (Fig. 4A). Iro and *dpp* expression changed little at the border but showed a distinct new focus at the anterior edge of the dorsal wing pouch and along the prospective wing margin (Fig. 4B,C). *ptc* and *col* expression were unaffected (Fig. 4D,E). Consistent with previous reports (Krasnow and Adler, 1994), *Fz* had no effect on overall wing morphology and affected only wing hair polarity (not shown). Thus, the effects of FFS on Hh responses must be through the Smo cytoplasmic tail. *FSS* failed to mount Hh responses, so either the chimeric junction in the first transmembrane domains cripples this construct or the TM domains must be compatible with the CRD for chimera activity.

As Fz is a Wg receptor, and Wg is strongly expressed at the wing margin, the margin-specific effects of FFS might be mediated by Wg. Ectopic expression of *Wg* in conjunction with FFS generated potent activation of *dpp*, *ptc*, *col* and En across the anterior compartment (Fig. 4H-J, not shown). As Wg can inhibit *col* expression if Hh signaling is only moderately activated (Glise et al., 2002), activation of *col* by FFS in the presence of Wg is indicative of high Hh signaling. Thus, FFS activates low Hh signaling (e.g. *dpp*) in response to modest levels of Wg and activates high Hh signaling (e.g. *col*) in response to high levels of Wg.

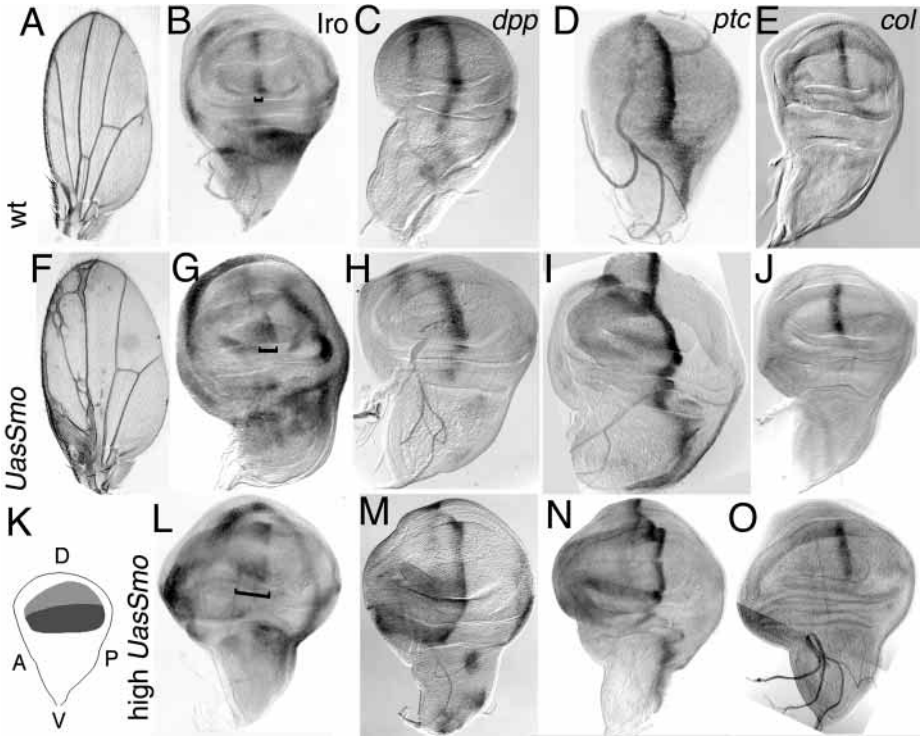


Fig. 2. Smo overexpression progressively activates Hh responses. (A-E) Wild type, (F-N) hemizygous *MS1096*, which express *Gal4* throughout the wing pouch, higher dorsally than ventrally and highest in the dorsal hinge (K). (F-J) *UasSmo* 4×, (L-O) *UasSmo*^{high}. (B,G,L) Iro, with bracket indicating the L3 region, (C,H,M) *dpp*, (D,I,N) *ptc*, (E,J,O) *col*. *UasSmo* 4× increased venation (F) and expanded Iro and *dpp* (G,H), but had little effect on *ptc* or *col* (I,J). High *UasSmo* expanded the wing pouch and caused dorsal anterior misexpression of Iro, *dpp* and *ptc* (L-N), but not *col* (O). In this, and all other figures, anterior is towards the left and (for imaginal discs) ventral is upwards.

The extracellular and TM regions of Smo are thought to mediate its regulation by Ptc, so FFS should not be regulated by Ptc. Coexpression of *Ptc* did not suppress the ectopic venation and overgrowth mediated by FFS, and instead allowed ectopic venation in a broad zone around the wing margin (Fig. 4G). Apparently repression of endogenous Smo (e.g. Fig. 4F) uncovers an FFS activity where Wg should be low. We did not test this interpretation by asking whether FFS can activate Hh responses without endogenous Smo. With that caveat, we conclude that FFS activates different levels of Hh signaling in response to different levels of Wg. Therefore, the cytoplasmic tail of Smo is sufficient to activate all Hh responses. The regulated activity of FFS also argues that similar structural transitions underlie signaling by Fz and by Smo.

SSF is dominant negative for high signaling

The converse chimera, with Smo CRD, Smo TM and Fz CT (SSF) had no effect on Fz responses, and instead interfered with high but not low Hh responses (Fig. 5). SSF 2× generated a subtle narrowing of the L3/4 interval (not shown). Higher SSF in a background with only one dose of wild-type Smo blocked high Hh responses; the spacing between the third and fourth wing vein was reduced (Fig. 5A), in concert with lost expression of *col* (Fig. 5E) and reduced expression of *ptc* (Fig. 5D). SSF also interfered with two aspects of Ci155 regulation that normally accompany CiA, depletion immediately adjacent to the border (Fig. 5M,N) and nuclear access (Fig. 5N). Lower levels of Ptc allow Hh to penetrate deeper into the anterior compartment (Chen and Struhl, 1996), so that *dpp* and Iro expression and Ci155 accumulation were expanded (Fig. 5B,C,M). Increasing dosages of SSF beyond this had no further effect, and under no conditions did expression of SSF

Construct	Expression	Signaling high	Signaling low	smo⁻ rescue
Smo	++	+/-	++	++
Fz	++	-	-	-
SSF	++	DN	-	-
SFS	+	-	-	nd
FSS	++	-	-	nd
SFF	++	-	-	nd
FSF	+	-	-	-
FFS	++	++	++	-
SmoN	+	-	-	-
SmoC	++*	DN	++	-

Fig. 3. Deletion and chimeric forms of Smoothened. Green indicates Smo sequences, pink indicates Fz sequences. The chimeric and deleted forms are schematized as blocks representing extracellular CRD (leftmost), TM (center) and cytoplasmic tails (right). The transmembrane domain and myristoylate that should anchor SmoN and SmoC to membranes are indicated with zig-zags. Expression scored levels (+ versus ++) relative to endogenous Fz and/or Smo by immunofluorescence in embryos where transgene expression was driven by *ptcGal4*. All constructs except SmoC (indicated by asterisk) showed ratios of cell surface and internal localization similar to those of Smo and Fz at physiological levels; SmoC alone failed to outline cells. Signaling scored the ability of the transgenes to change L3/4 spacing (high) or to change L3 and A/P growth (low) in the presence of endogenous Smo. DN indicates narrowing of L3/4 spacing. FFS signaling was regulated by Wg rather than Ptc and Hh. Only Fz affected wing hair polarity. '*smo*⁻ rescue' scored ability of *prdGal4* driven transgenes to restore *wg* and *ptc* expression, and segmentation in alternate segments of *smo*^{1}/*smo*^{3} embryos. nd, not determined.

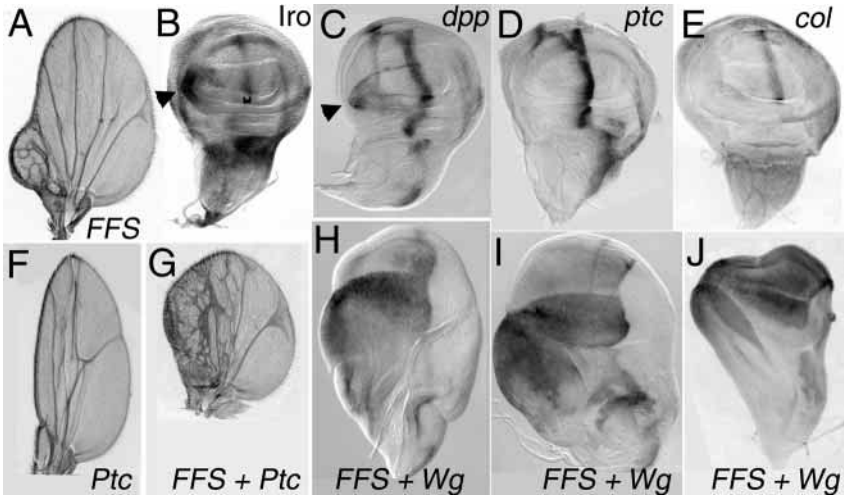


Fig. 4. FFS activates low signaling and is regulated by Wg. All tissue is from *MS1096* hemizygotes. (A-E) Heterozygous *UasFFS*^{high}, (F) heterozygous *EP941* that drives endogenous *ptc*, (G) *EP941/UasFFS*^{high}, (H-J) *UasWg/UasFFS*^{high}*h*. *FFS* generated mild overgrowth of the anteroposterior axis of the wing and expansion of the costa, with no disturbance of wing hair polarity (A). It also expanded expression of Iro and *dpp* at the anterior margin of the wing pouch (arrowheads in B,C), and *dpp* in a line corresponding to the presumptive wing margin. *ptc* (D) and *col* (E) expression remained essentially normal, though there was a small gap in *ptc* expression at the presumptive wing margin. *ptc* overexpression reduced the size of the AP axis, abolished the L3/4 territory and much of L3 (F). *ptc* overexpression had no effect on the anterior overgrowth driven by FFS, though it transformed the L3/4 intervein to excess venation (G). *UasWg* in combination with *UasFFS* caused massive activation of Hh targets including *dpp* (H), *ptc* (I) and *col* (J).

compromise L3. This is distinct from overexpression of Ptc (Fig. 4F), where the 'fused' phenotype is often accompanied by interruption or elimination of L3 and loss of *dpp* expression (Johnson et al., 1995). The Fz tail of SSF might contribute to its dominant-negative activity, though the lack of dominant negative activity by FSF nor SFF render this less likely. Instead the extracellular CRD and the TM domains both appear to be necessary for the dominant negative activity of SSF.

This raised the possibility that SSF might sequester an extracellular ligand. In this case the effects of SSF would not be cell autonomous. Induction of Ptc by *Smo* is cell autonomous; Ptc was elevated in all cells of mosaic wing

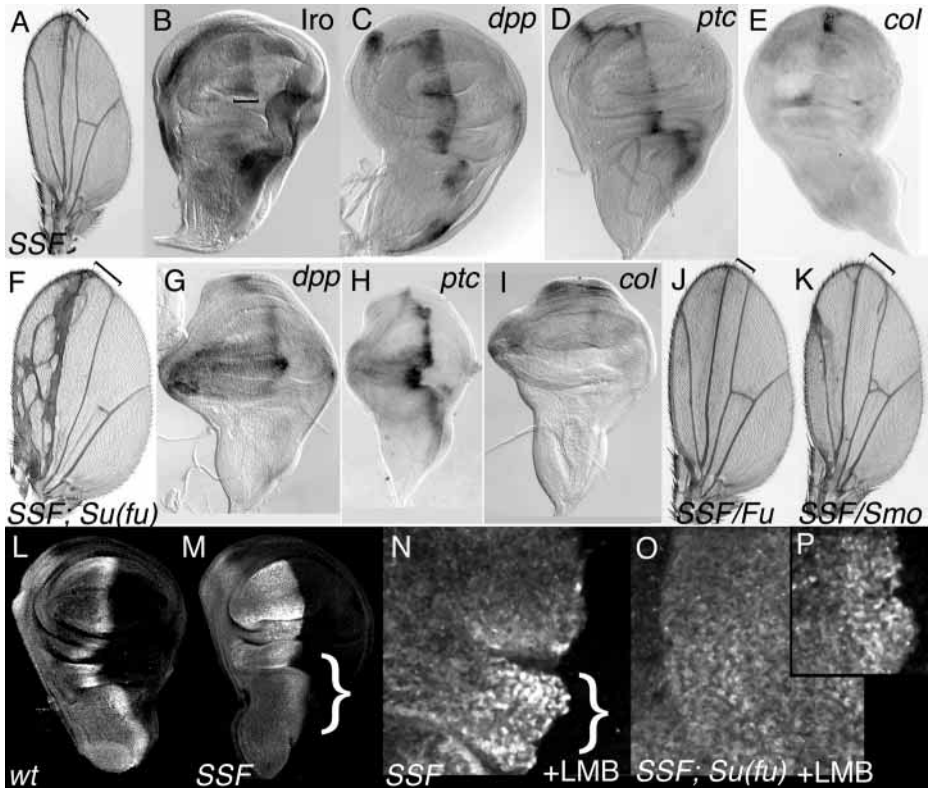


Fig. 5. SSF is dominant negative for high signaling. All tissue (except L) is from *MS1096* hemizygotes. (A-E,M,N) Heterozygous *smo3 UasSSF*^{high}, (F-I,O,P) heterozygous *smo3 UasSSF*^{high}; *Su(fu)*^{LP}, (J) *smo*^{3} *UasSSF*^{high}*/UasFu*, (K) *smo*^{3} *UasSSF*^{high}*/UasSmo*. (C,G) Expression of *dpp*, (D,H) *ptc*, (E,I) *col* and (L-P) Ci C-terminal epitope. SSF gave strong narrowing of L3/4 (bracket in A). This reflected loss of *col* (E) and *ptc* (D), and expansion of *dpp* (C) and Iro (bracket in B). In the notum primordium, where *MS1096* does not drive transgene expression (bracket in M,N), and in wild-type imaginal discs (L), Ci155 accumulated in a zone some four or five cells wide. The apparent fading of Ci155 in the posterior-most two or three cells of its domain (immediately adjacent to the compartment border) is due to Hh-dependent depletion (e.g. Ohlmeyer and Kalderon, 1998). With SSF, Ci155 accumulated in a broad domain with no depletion at the compartment border (M,N). If Ci155 is free to enter nuclei, it is trapped there by the nuclear export blocker, LMB (N-P). In wild type, Ci155 was trapped in nuclei of six or seven cells adjacent to the border, but not deeper in the anterior compartment (bracket in N). SSF blocked nuclear accumulation of Ci155 at the border (remainder of N). SSF did not affect the L3/4 intervein in a *Su(fu)* mutant background (bracket in F), and instead gave anterior expansion of L3 and overgrowth along the AP axis. *ptc* (H) and *col* (I) were partly rescued at the compartment border, while *dpp* (G) and *ptc* expanded across the overgrown dorsal anterior compartment. Ci155 accumulated in nuclei of LMB-treated *Sufu* mutant discs, both at the border (P) and deep in the anterior compartment (O). The L3/4 narrowing of SSF was mildly suppressed by simultaneous expression of *Fu* (J) and was more strongly suppressed by simultaneous overexpression of *Smo* (K).

imaginal discs expressing high levels of *Smo*, both near the compartment border and deep within the anterior compartment (Fig. 6A). Ptc levels were reduced in all border cells expressing SSF, even when small groups were surrounded by wild-type cells (Fig. 6B). In addition, large clones expressing SSF failed to interfere with Ptc accumulation in neighboring cells (Fig. 6B). Thus the effect of SSF on Hh responses is cell-autonomous and is inconsistent with sequestration an extracellular ligand. SSF might interfere with the ability of Ptc to bind Hh or it might interfere with signal transduction. As SSF suppresses the Hh-independent ectopic venation of *Smo* 4× (Fig. 5K) we conclude that SSF interferes with signal transduction rather than signal reception.

The phenotypes generated by SSF overexpression are similar to those generated by loss of *fu*. *fu* is required for transduction of high but not low Hh responses (Mariol et al., 1987; Preat et al., 1990; Sanchez-Herrero et al., 1996; Mullor et al., 1997; Alves et al., 1998; Ohlmeyer and Kalderon, 1998; Lefers et al., 2001; Glise et al., 2002; Nybakken et al., 2002). Fu has a kinase domain that is necessary only for high Hh responses, and a regulatory domain that is instrumental in assembly of the Ci regulatory complex (Robbins et al., 1997; Ascano et al., 2002; Monnier et al., 2002). SSF might interfere with response to high Hh by blocking activation of Fused kinase, thus mimicking class I *fu* alleles, or by preventing its assembly of Fu into the regulatory complex, thus mimicking class II *fu* alleles. A genetic test for these alternatives is offered by *Sufu*, the removal of which restores properly regulated Hh signaling to class I *fu* alleles but constitutively activates signaling in class II *fu* alleles. Removal of *Sufu* from wings expressing SSF fits the latter profile (Fig. 5F-I,O,P). Although high Hh responses were rescued (L3-4 spacing and *ptc* expression), low Hh responses were enhanced (ectopic venation, expansion of the anteroposterior axis and of *dpp* expression). Finally, *Sufu* discs expressing SSF allowed Ci155 to enter nuclei even deep in the anterior compartment where Hh should be absent (Fig. 5O). Thus, the spectrum of phenotypes generated by SSF expression is very similar to that of class II *fu* alleles (Lefers et al., 2001). If SSF were interfering with high Hh responses by decreasing the levels of Fu available for signaling (e.g. like classII *fu* alleles), then increasing levels of Fu should restore high signaling. Overexpression of *Fu* along with SSF only weakly suppressed the 'fused' phenotype (Fig. 5J), suggesting that SSF is acting on a regulator of Fu rather than on Fu itself.

The structure of SSF, retaining all of the transmembrane and extracellular sequences of Smo, suggests that SSF acts on a membrane protein rather than a cytoplasmic protein. If SSF were interfering with endogenous Smo, then increasing levels of wild-type Smo should restore high signaling. Indeed, the SSF phenotype was suppressed by co-expression of 2× *Smo* (Fig. 5K). Thus, the ratio of Smo to SSF is crucial for blockade of high signaling by SSF. SSF is unlikely to interfere with Smo through triggering its degradation, because reducing levels of Smo should affect low responses as well as high responses. Instead, we suggest that SSF titrates out Smo by direct binding, and that these Smo/SSF heterodimers cannot activate high signaling. It follows that normal high signaling may involve a dimeric (or oligomeric) form of Smo.

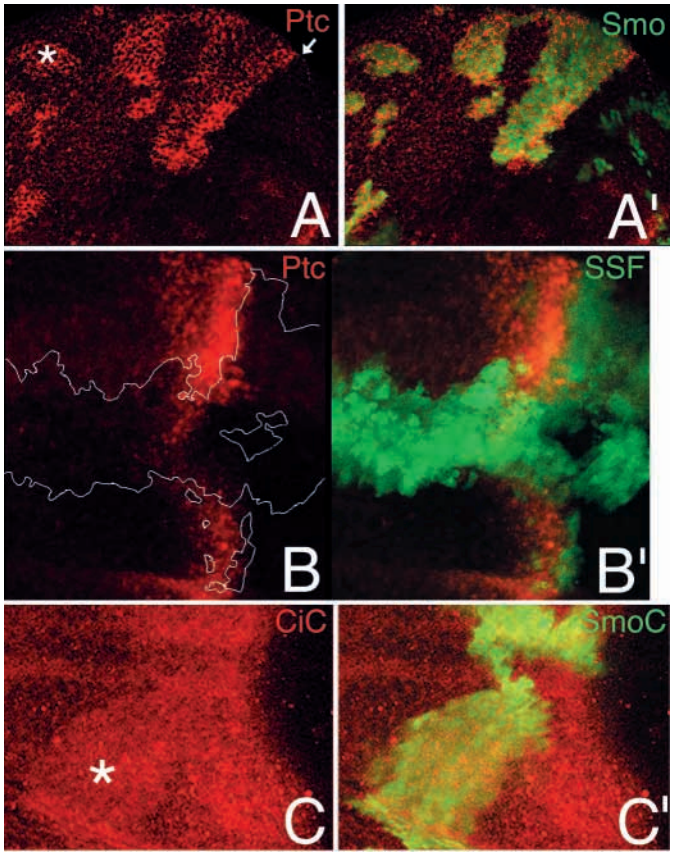


Fig. 6. Smo transgenes act cell autonomously to affect Hh responses. *UasSmo^{high}* (A), *UasSSF* (B) and *UasSmoC* (C) were clonally expressed using the FLP-out Gal4 system and marked by *UasGFP* (green in all panels). Ptc protein (red in A and B) is normally expressed at high levels just anterior to the compartment border (arrow indicates the border). Overexpression of *Smo* (green in A′) induced high levels of Ptc protein, both near the compartment border and near the anterior edge of the disc. Levels of Ptc protein (red) were reduced when cells along the compartment border expressed SSF (green in B′, outlined in white in B). While the levels of Ptc in wild type cells are somewhat variable on a cell-by-cell basis, Ptc levels were distinctly reduced in cells expressing SSF, even in the small clones at the bottom of B, demonstrating a cell-autonomous response to SSF. Ci155 (red in C) accumulated in clones away from the compartment border that expressed *SmoC* (asterisk in C), as well as in its normal domain paralleling the compartment border.

SmoC activates low Hh responses through endogenous Smo

For most receptors, the cytoplasmic domain without its transmembrane and extracellular regulatory domains is constitutively active. The Smo cytoplasmic tail (SmoC) was not constitutively active in the expected sense. Its strongest activity was ectopic activation of low Hh responses. *SmoC* 2× gave ectopic venation with no effect on L3/4 intervein (not shown). *SmoC* 4× gave strong ectopic venation and variable costal overgrowth (Fig. 7A,F,H). Ectopic venation was accompanied by expansion of Iro and *dpp* (Fig. 7B,C). Ci155 accumulated ectopically in the costal primordium, at levels equivalent to or higher than those normally seen within a few cell diameters of the compartment border (Fig. 7P). Thus,

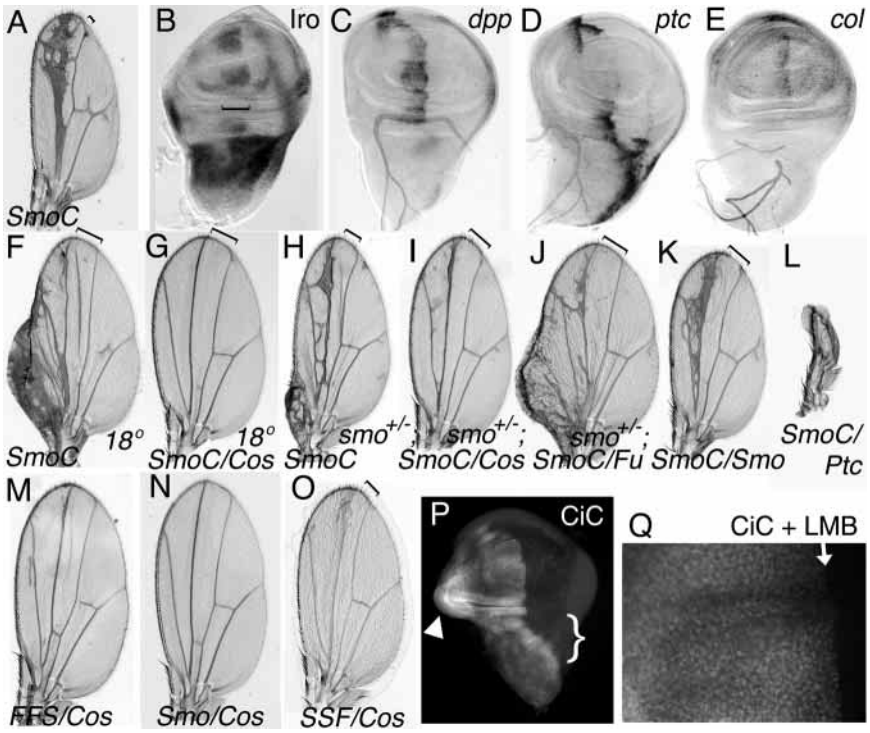


Fig. 7. *SmoC* activates low signaling and interferes with high signaling. All tissue is from *MS1096* hemizygotes grown at 25°C, except for F and G which were grown at 18°C. (A-E,P,Q) Homozygous *UasSmoC*. (F) *UasSmoC/+; UasSmoC/+* and (G) *UasSmoC/+; UasSmoC/Pcos*$^{+}$ siblings. (H-J) Doubly heterozygous *smo*^{3} *SmoC2, SmoC3* with *Pcos*$^{+}$ (I) or *UasFu* (J). (K,L) homozygous *UasSmoC* with heterozygous *UasSmo* (K) or *EP941* (K). *SmoC* 4× expanded L3 anteriorly to fill the L2/3 intervein (square brackets), reduced the L3/4 intervein, and expanded the costa or reduced the size of the anterior compartment (A,F,H), though the extent was sensitive to genetic background and growth conditions. *SmoC* expanded expression of Iro (bracket in B) and *dpp*(C), as well as the reduced expression of *ptc* (D) and *col* (E). At 18°C *SmoC* consistently produced costal overgrowth (F) that was eliminated in siblings carrying a third copy of wild-type *cos* (G). *Pcos*$^{+}$ reduced the L3/4 narrowing and ectopic venation caused by *SmoC*, relative to siblings (compare H with I). *Pcos*$^{+}$ failed to suppress the L3/4 narrowing of high SSF (O). *Pcos*$^{+}$ also suppressed the costal overgrowth of 4× FFS (M) and the ectopic venation of 4× *Smo* (N). *Fu* enhanced the ectopic venation and costal overgrowth of *SmoC*, as well as suppressing the L3/4 narrowing (J). The L3/4 narrowing and ectopic venation of 4× *Sm°C* was suppressed in siblings carrying 2× *Smo* (compare K with A). *SmoC* enhanced the growth reduction caused by *Ptc*, so that the wing is virtually eliminated (compare L with Fig. 4F). *SmoC* reduced accumulation of Ci155 near the compartment border (P) in the wing pouch where *MS1096* is expressed relative to the notum where *MS1096* is not expressed (bracket). It also caused abnormal accumulation of Ci155 in the costal primordium (arrowhead). *SmoC* promoted nuclear access of Ci155 far from compartment border (arrow) in LMB-treated imaginal discs (Q).

SmoC can curtail CiR production in the absence of Hh. *SmoC* also allowed Ci155 to enter nuclei, even deep within the anterior compartment (Fig. 7Q) where it is normally excluded. Small clones overexpressing *SmoC* also permitted Iro expression (not shown) and Ci155 accumulation (Fig. 6C), indicating cell autonomy for *SmoC* activity. We conclude that *SmoC* constitutively activates low signaling.

As SmoC lacks the transmembrane and extracellular domains that have been implicated in regulation of Smo by Ptc (Murone et al., 1999), Ptc should not be able to repress SmoC. However *Ptc* co-expression completely suppressed the ectopic venation and costal overgrowth caused by *SmoC* and instead reduced the wing to a tiny scrap (Fig. 7L, compared with Fig. 4F). This suggested that SmoC activates low signaling through de-repression of endogenous Smo. To test this, we examined Iro expression, as a reporter for low Hh responses, in *smo*$^{-}$ cells. Expression of *SmoC* did not rescue Iro expression in clones of *smo* null cells (Fig. 8), demonstrating that SmoC acts through endogenous Smo to activate low Hh responses.

This unexpected result was confirmed in embryos lacking all endogenous *smo* activity (data not shown). *smo*^{3} germline clone (*smoGLC)* embryos lose expression of *wg*, *en* and *hh* during stage 10 and fail to upregulate *ptc* at the segment and parasegment borders during and after stage 11 (van den Heuvel and Ingham, 1996). *UasSmo* expressed under control of *prdGal4* or *Krgal4* rescued *wg*, *en* and *ptc* expression in *smoGLC* embryos. No other transgene, including *SmoC* and FFS, had any rescuing activity in *smoGLC* embryos. To test whether the potent inhibitory effects of Ptc might mask a weak activity of SmoC, we expressed our transgenes in embryos lacking *ptc* and with near-threshold levels of *smo*. In *ptc*^{W}, *smo*^{3} embryos grown at 25°C, the maternal contribution of *smo*$^{+}$ is sufficient to sustain Hh target gene expression until stage 11. *ptc* expression at the segment border, which is *fu*-independent (Therond et al., 1999) and should be the equivalent of low signaling in wings, was not rescued by *SmoC* nor FFS in *ptc*^{W}, *smo*^{3} embryos. *wg* expression, which requires *fu* and should be equivalent to high signaling in the wing, was prematurely eliminated during stage 10 when *SmoC*, SSF or *Ptc* were driven by *prdGal4*. This recapitulated the dominant negative effects of SSF and *SmoC* on a *fu*-dependent response, and demonstrated that the transgenes were effectively expressed. The failure to rescue *wg* or *ptc* expression, even with near-threshold endogenous Smo and without Ptc, confirms that SmoC has no ability to activate Hh target genes in the absence of endogenous Smo.

SmoC may act by interfering with costal

In addition to activating low Hh responses, *SmoC* interfered with high Hh responses. *SmoC* 4× reduced the L3/4 spacing as well as *ptc* and *col* expression (Fig. 7A,D,E). It interfered with the depletion of Ci155 immediately adjacent to the compartment border that normally accompanies CiA (Fig. 7P,Q). It also prevented Ci155 accumulation to normal levels in the zone three to eight cells from the compartment border (Fig. 7P). The net effect of *SmoC* expression, including both ectopic low responses and curtailed high responses, was to drive all cells towards the low response state, regardless of Hh.

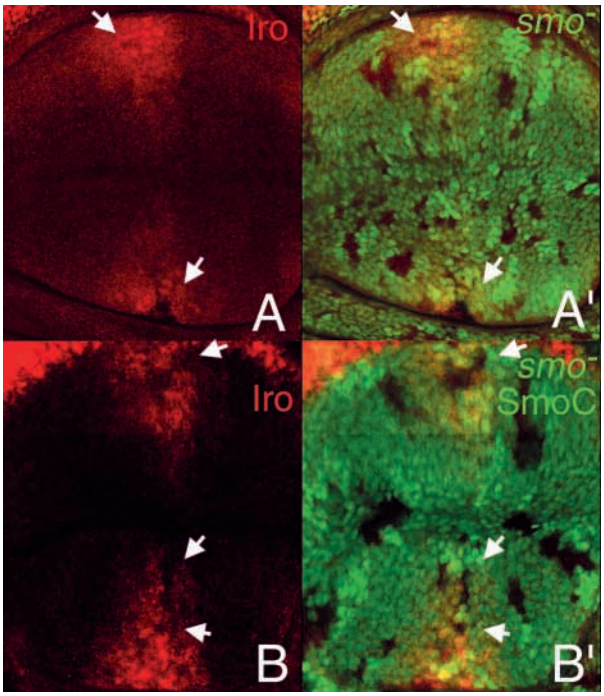


Fig. 8. *SmoC* requires endogenous Smo to activate signaling. Clones of wing cells lacking endogenous *smo* activity were generated using the *FLP;FRT* system and marked by loss of GFP. Iro (red) was lost in the L3 region (arrows) when *smo* was removed (A). Expression of *UasSmoC/+* under control of hemizygous *MS1096* (e.g. 2× *SmoC*) did not rescue Iro expression in clones of cells lacking endogenous *smo* (arrows in B). Adult brothers of the larvae used in B had significant ectopic venation, demonstrating that the expression level of *SmoC* was sufficient for significant activity in the presence of endogenous Smo.

This might be through two activities of *SmoC*, one mediating inhibition of high signaling and the other mediating activation of low signaling. A simpler alternative is provided by the striking similarities between the phenotypes of *SmoC* overexpression and *cos* loss. Like *SmoC* overexpression, insufficient *cos* compromises both full activation and full inhibition of Hh signaling (Forbes et al., 1993; Wang et al., 2000b; Wang and Holmgren, 2000). In the presence of Hh, *cos*-null clones fail to activate high Hh responses; En is not turned on and Ptc accumulates only to moderate levels (Wang et al., 2000b). *cos* null clones also activate low Hh responses without Hh; clones deep in the anterior compartment express *dpp* (Wang et al., 2000b) and Iro (data not shown), and allow Ci155 accumulation and nuclear entry (Sisson et al., 1997; Wang and Holmgren, 1999; Wang et al., 2000b). Hypomorphic *cos* alleles give overgrowth of the costa (Grau and Simpson, 1987) similar to that driven by *SmoC* (Fig. 7F). Published data do not address whether low signaling in cos^{-} cells requires endogenous Smo activity (Methot and Basler, 2000). The similarity between the phenotypes of *SmoC* overexpression and insufficient Costal suggests that *SmoC* may act through interfering with Cos.

If SmoC were acting by interfering with Cos then increasing levels of Cos should suppress the effects of *SmoC*. An extra copy of cos^{+} suppressed all *SmoC* effects, including the ectopic venation and the costal overgrowth indicative of low signaling, and the L3/4 narrowing indicative of high signaling (Fig. 7F-I). Thus the dual activities of *SmoC* could occur through a single mechanism, inactivating or sequestering Costal. The restoration of high signaling by cos^{+} is specific to *SmoC* as cos^{+} failed to suppress the L3/4 narrowing of SSF (Fig. 7O) and had no effect in a wild-type background (not shown). This indicates that *SmoC* and SSF are interfering with high signaling through different mechanisms. Fu might be a target for misregulation by *SmoC* as Class II *fu* alleles affect both CiR production and high responses (Lefers et al., 2001). Co-expression of *Fu* with *SmoC* rescued high responses, the L3/4 narrowing, but enhanced low responses, the ectopic venation and costal overgrowth (Fig. 7J). Thus, Fu is unlikely to be the primary target through which *SmoC* exerts its effects. Finally, co-expression of *Smo* effectively suppressed both the ectopic venation and L3/4 narrowing of *SmoC*, while 50% reduction of *smo* (in smo^{3} heterozygotes) enhanced both *SmoC* activities (not shown). These data suggest that SmoC constitutively drives Smo into a state that inactivates Cos, thereby permitting activation of low Hh responses. As *cos* mutants are constitutively in the low state, whereas excess cos^{+} restores the OFF state to *Smo* and FFS (Fig. 7M-N), it follows that normal low signaling results from inactivation of Cos by Smo.

DISCUSSION

Analyses of the activities of truncated and chimeric forms of Smo in a variety of genetic backgrounds yielded four principal observations. First, the FFS chimera activated the full spectrum of Hh responses, but was regulated by Wg rather than Hh. From this, we conclude that the Smo cytoplasmic tail initiates all intracellular responses to Hh, while the remainder of Smo regulates activity of the tail. Second, the SSF chimera interfered with high signaling but had no effect on low signaling. SSF mimicked Class II *fu* mutants and was suppressed by increasing smo^{+} but not fu^{+} or cos^{+}. From this, we conclude that high Hh instructs Smo to activate Fu by a mechanism that is likely to involve dimeric/oligomeric Smo. Third, the cytoplasmic tail of Smo (SmoC) derepressed endogenous Smo activity in the absence of Hh and repressed endogenous Smo activity in the presence of high Hh. That is, SmoC drove cells to the low response regardless of Hh levels. This mimicked *cos* mutants and was suppressed by 50% increase in cos^{+}. From this, we conclude that low Hh instructs Smo to inactivate Cos, by a mechanism that may involve stoichiometric interaction between Cos and the Smo cytoplasmic tail. Fourth, chimeras where the extracellular CRD and TM domains were mismatched failed to exhibit any activity. From this, we conclude that these two domains act as an integrated functional unit. This leads us to a model for signaling where Fz or Smo can adopt three distinct states, regulating two distinct activities and translating different levels of ligand into distinct responses. Many physical models are consistent with these genetic analyses. Those presented here are favored because they are the simplest.

Three states of Smoothened signaling may dictate three states of the Ci regulatory complex

We have identified two mutant forms of Smo that regulate downstream signaling through different activities. These

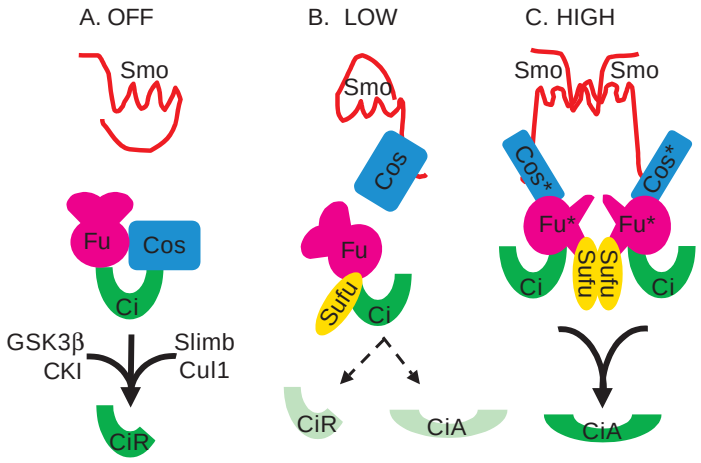


Fig. 9. The model: three states of Smoothened translate Hh levels into distinct responses. Smo can adopt three states, a decision normally dictated by Hh, via Ptc. The Ci regulatory complex, which includes full-length Ci, Cos and Fu, likewise can adopt three states (Ingham and McMahon, 2001). In the absence of Hh (A) Smo is OFF. Its cytoplasmic aspect is unavailable for signaling. The Cos/Fu/Ci regulatory complex is anchored to microtubules and promotes efficient processing of Ci155 to CiR. Low levels of Hh (B) expose Cos inhibitory sites in the cytoplasmic tail of Smo. Cos interaction with these sites drives the Ci regulatory complex into the low state, which recruits Sufu and makes little CiR or CiA. high levels of Hh (C) drive a major change in Smo, possibly dimerization. This allows the cytoplasmic tails of Smo to cooperatively activate Fu and Cos. Fu* and Cos* then cooperate to inactivate Sufu, to block CiR production, and to produce CiA at the expense of Ci155.

mutant forms of Smo mimic phenotypes of mutants in other components of the Hh pathway, as well as normal responses to different levels of Hh (Mullor and Guerrero, 2000; Wang and Holmgren, 2000). These data suggest a model where Smo can adopt three distinct states that instruct three distinct states of the Ci regulatory complex (Fig. 9). The model further suggests that Smo regulates Ci through direct interactions between Fu, Cos and the cytoplasmic tail of Smo. This is consistent with the failure of numerous genetic screens to identify additional signaling intermediates, and with the exquisite sensitivity of low signaling to Cos dosage.

The OFF state is normally found deep in the anterior compartment where cells express no Hh target genes (except basal levels of Ptc). In this state, the Ci regulatory complex consists of Fu/Cos/Ci155 (Robbins et al., 1997; Sisson et al., 1997; Stegman et al., 2000). Cos and Fu contribute to efficient processing of Ci155 to the repressor form, CiR (Wang and Holmgren, 1999; Wang et al., 2000b; Lefers et al., 2001), presumably because the complex promotes access of PKA and the processing machinery to Ci155 (Kiger and O'Shea, 2001). Cos also prevents nuclear entry of Ci155 (Wang and Holmgren, 2000), correlating with microtubule binding of the complex. This state is universal in *hh* or *smo* mutants, indicating that intracellular responses to Hh cannot be activated with out Smo (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Therefore Smo can adopt an OFF state where it exerts no influence on downstream signaling components and the OFF state of the Ci regulatory complex is its default state.

The low state is normally found approximately five to seven cells from the compartment border, where cells are exposed to lower levels of Hh. These cells express Iro, moderate levels of *dpp*, no Col and basal levels of Ptc. They accumulate Ci155, indicating that little CiA or CiR is made. That Ci155 can enter nuclei but is insufficient to activate high responses. The physical state of the Ci regulatory complex in the low state has not been investigated. Cells take on the low state regardless of Hh levels when Ci is absent (Methot and Basler, 1999), when Cos is absent (Wang et al., 2000b) or when SmoC is expressed, and are strongly biased towards that state in *fu(classII); Su(fu)* double mutants (Methot and Basler, 2000; Lefers et al., 2001). This state normally requires input from Smo, which becomes constitutive in the presence of SmoC. As SmoC drives only low responses and cannot activate high responses, this identifies a low state of Smo that is distinct from both OFF and high. We propose that the low state is normally achieved when Smo inactivates Cos, perhaps by direct binding of Cos to Smo and dissociation of Cos from Ci155. Neither CiR nor CiA is made efficiently, and target gene expression is similar to that of *ci* null mutants.

The high state is normally found in the two or three cells immediately adjacent to the compartment border where there are high levels of Hh. These cells express En, Col, high levels of Ptc and moderate levels of Dpp. They make CiA rather than CiR (Ohlmeyer and Kalderon, 1998), and Ci155 can enter nuclei. In this state a cytoplasmic Ci regulatory complex consists of phosphorylated Cos, phosphorylated Fu, Ci155 and Sufu (Therond et al., 1996; Robbins et al., 1997; Sisson et al., 1997; Monnier et al., 1998; Stegman et al., 2000). Dissociation of Ci from the complex may not precede nuclear entry, as Cos, Fu, and Sufu are all detected in nuclei along with Ci155 (Methot and Basler, 2000). Sufu favors the low state, whereas Cos and Fu cooperate to allow the high state by repressing Sufu, and also by a process independent of Sufu (Methot and Basler, 2000; Wang et al., 2000b; Lefers et al., 2001). This high state is the universal state in *ptc* mutants and requires input from Smo. As this state is specifically lost in *fu* mutants, Fu may be a primary target through which Smo activates the high state. SSF specifically interferes with the high state by a mechanism that is most sensitive to dosage of Smo. This suggests SSF interferes with the high activity of Smo itself. We suggest that dimeric/oligomeric Smo is necessary for the high state, and that Smo:SSF dimers are non-productive. Cooperation between Smo cytoplasmic tails activates Fu and thence Cos. The activities of the resulting Fu* and Cos* are entirely different from their activities in the OFF state, and mediate downstream effects on Sufu and Ci.

Regulation of Smo

We find that the cytoplasmic tail of Smo is sufficient to activate all Hh responses, and that its activity is regulated through the extracellular and TM domains. This is exemplified by the FFS chimera, which retains the full range of Smo activities, but is regulated by Wg rather than Hh. The extracellular and transmembrane domains act as an integrated unit to activate the cytoplasmic tail, as all chimeras interrupting this unit failed to activate any Hh responses, despite expression levels and

subcellular localization similar to those of active SSF or FFS. As is true of other serpentine receptors, a global rearrangement of the TM helices is likely to expose 'active' (Cos regulatory?) sites on the cytoplasmic face of Smo. The extracellular domain of Smo must stabilize this conformation and Ptc must destabilize it. But how? Ptc may regulate Smo through export of a small molecule, which inhibits Smo when presented at its extracellular face (Chen et al., 2002; Taipale et al., 2002). Hh binding to Ptc stimulates its endocytosis and degredation, leaving Smo behind at the cell surface (Denef et al., 2000; Incardona et al., 2002). Thus, Hh would separate the source of the inhibitor (Ptc) from Smo, allowing Smo to adopt the low state. Transition from low to high might require Smo hyperphosphorylation (see below). The high state, which is likely to involve Smo oligomers, might be favored by cell surface accumulation if aggregation begins at some threshold concentration of low Smo. Alternatively, these biochemical changes may all be unnecessary for either the low or high states of Smo.

There is no suggestion that Ptc has multiple states in response to different levels of Hh. Ptc mutants that fail to derepress signaling (Mullor and Guerrero, 2000), or that constitutively derepress signaling (Johnson et al., 2000; Martin et al., 2001; Strutt et al., 2001; Johnson et al., 2002) coordinately affect both high (e.g. En) and low (e.g. Iro) responses. Thus, we suggest that Smo and not Ptc is the first step in which the Hh pathway adopts three distinct states.

Fz signaling

Both Hhs and Wnts act as morphogens, with different levels of ligand dictating different intracellular responses (Zecca et al., 1996; Neumann and Cohen, 1997; Ingham and McMahon, 2001). Those intracellular responses are respectively initiated by Smo and Fz. Fz and Smo have a high degree of sequence similarity in their extracellular and transmembrane domains (Alcedo et al., 1996). The similarity must extend to function, as graded levels of Wg acting through the FFS chimera drive low and then high signaling by the Smo cytoplasmic tail. This suggests unanticipated complexity in Fz function, where low levels of Wnts 'low-activate' Fz while higher levels trigger oligomerization-dependent 'high activation'. Fz8 CRD crystallizes as a dimer, suggesting a physical basis for Fz family oligomerization (Dann et al., 2001).

Multiple signaling states for serpentine receptors

There is precedent within the serpentine receptor superfamily for dimerization/oligomerization and for multiple signaling states. β2-adrenergic receptor (β2AR), the archetypical serpentine receptor has at least three states (reviewed by Pitcher et al., 1998; Brzostowski and Kimmel, 2001; Pierce et al., 2002). In the absence of ligand, β2AR is OFF. The agonist-occupied state favors a global conformational change which allows the cytoplasmic loops and tail to activate heterotrimeric G proteins as well as the receptor kinase, GRK2. GRK2 then phosphorylates the cytoplasmic tail of β2AR. In the phosphorylated state, β2AR binds β-arrestin. β2AR + β-arrestin1 then assemble novel trafficking and signaling complexes which mediate endocytosis, Src binding and ERK activation. Complementation between two inactive β2AR mutant forms demonstrates that adjacent molecules can exchange helices to reconstitute a functional receptor; that is, β2AR can homodimerize. Moreover, a peptide derived from the sixth TM domain simultaneously blocks dimerization and activation (Hebert et al., 1996). There is substantial parallel between this model of β2AR signaling and our model of Smo signaling. Each recruits and activates a kinase when the receptor is stimulated. Each stimulated receptor then becomes a substrate for assembly of a new signaling complex. We suggest that multiple signaling states could be a general mechanism by which serpentine receptors translate different levels and/or kinetics of ligand exposure into distinct responses.

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