Fused–Costal2 protein complex regulates Hedgehog-induced Smo phosphorylation and cell-surface accumulation

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The seven-transmembrane protein Smoothened (Smo) acts as a signal transducer in the Hedgehog (Hh) pathway that mediates many key developmental processes. In Drosophila, Hh-induced phosphorylation promotes Smo cell-surface accumulation and signaling activity; however, the mechanisms controlling Smo phosphorylation and cell-surface accumulation are still unknown. The intracellular signaling complex containing Fused (Fu) and Costal2 (Cos2) is thought to transduce the Hh signal downstream from Smo. Here, we identify a novel feedback mechanism that regulates Smo through the Fu–Cos2 complex. We found that Hh-induced Smo accumulation is inhibited in fu mutant clones or by expressing a dominant-negative form of Fu, and such inhibition is alleviated by removal of Cos2. Conversely, overexpressing Cos2 blocks Smo accumulation, which is reversed by coexpressing Fu. Cos2 blocks Smo accumulation through its C-terminal Smo-interacting domain, and Fu antagonizes Cos2 by phosphorylating Cos2 at Ser572. Furthermore, we found that Ser572 phosphorylation attenuates the Cos2–Smo interaction and promotes Cos2 instability. Finally, we provided evidence that Fu and Cos2 control Smo cell-surface accumulation by regulating Smo phosphorylation. Our data suggest that Cos2–Smo interaction blocks Hh-induced Smo phosphorylation, and that Fu promotes Smo phosphorylation by antagonizing Cos2.

[Keywords: Smo; Fu; Cos2; Hh; phosphorylation; signal transduction]

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The Hedgehog [Hh] pathway is responsible for a variety of developmental processes, including cell proliferation, embryonic patterning, and organ development [Ingham and McMahon 2001; Lum and Beachy 2004; Jia and Jiang 2006]. Dysregulation of Hh signaling is associated with a wide variety of human disorders, including cancer [Taipale and Beachy 2001; Pasca di Magliano and Hebrok 2003]. In the Drosophila wing disc, Hh is secreted by posterior (P) compartment cells (P-cells), and induces the anterior (A) compartment cells (A-cells) adjacent to the A/P boundary to express decapentaplegic (dpp), which encodes a member of the TGFβ/BMP family of secreted proteins [Ingham and McMahon 2001]. The Dpp protein then diffuses into both the A- and P-compartments and functions as a long-range morphogen to control the growth and patterning of cells in the entire wing [Ingham and McMahon 2001]. Hh also specifies cell patterning near the A/P boundary by functioning as a local morphogen. Low levels of Hh are able to induce the expression of dpp, whereas higher levels of Hh are required to activate ptc. The induction of en requires peak levels of Hh signaling activities [Strigini and Cohen 1997]. Thus, the levels of Hh signaling activity can be monitored by the expression of different responsive genes.

The Hh signal is transduced through a reception system that includes the 12-span transmembrane protein Patched (Ptc) and seven-span transmembrane Smoothened (Smo). In the absence of Hh, Ptc inhibits Smo. Binding of Hh to Ptc alleviates such inhibition, allowing Smo to regulate the Cubitus interruptus (Cib/Gli) family of Zn-finger transcription factors [Ingham and McMahon 2001].

How Smo signaling activity is regulated remains poorly understood. In Drosophila, Hh induces Smo phosphorylation and cell-surface accumulation [Denef et al. 2000]. We and others have identified PKA and CKI as two of the kinases that phosphorylate Smo and increase its cell-surface localization and signaling activity [Jia et al. 2004; Zhang et al. 2004; Apionishev et al. 2005]. Block-
ing PKA or CKI activity prevents Hh-induced Smo accumulation on the plasma membrane and attenuates its pathway activity, whereas increasing PKA activity promotes Smo accumulation and pathway activation [Jia et al. 2004]. It appears that phosphorylation is sufficient to induce Smo cell-surface accumulation and activation, as phosphorylation-mimicking Smo variants show constitutive signaling activity and cell-surface accumulation [Jia et al. 2004; Zhang et al. 2004]. Despite the identification of Smo kinases, how Smo phosphorylation is regulated still remains a mystery.

The Ser/Thr kinase Fused (Fu) and the kinesin-related protein Costal2 (Cos2) exist in a large protein complex with the full-length Ci [Ci155] [Robbins et al. 1997; Sisson et al. 1997]. Cos2 mainly acts as a negative regulator in the Hh signaling pathway [Sisson et al. 1997; Wang and Holmgren 2000, Wang et al. 2000; Ho et al. 2005] and inhibits the transcriptional activator activity of Ci155 by inhibiting its nuclear translocation [Chen et al. 1999; Stegman et al. 2000; Wang et al. 2000; Monnier et al. 2002]. Cos2 also acts as a scaffold protein to bring PKA, CKI, and GSK3 kinases to Ci155 [Zhang et al. 2005], promoting sequential phosphorylation of Ci155 by these kinases [Price 2006], leading to proteasome-mediated Ci155 processing to generate its repressor form, C15T [Chen et al. 1999; Wang and Holmgren 1999; Tian et al. 2005; Wang and Li 2006]. Ci15T binds DNA and represses the transcription of Hh target genes such as dpp [Azablan et al. 1997; Methot and Basler 1999].

Mutations in fu affect high-threshold Hh responses [Preat 1992; Alves et al. 1998]. It has also been shown that Fu converts Ci into a hyperactive and labile form by antagonizing Su(fu) [Ohmley and Kalderon 1998]. The effects of Fu and Cos2 appear to be more complicated than simply acting as a pathway activator and repressor, respectively. Fu is required for Ci processing in certain genetic backgrounds [Lefers et al. 2001]. On the other hand, Cos2 has a positive role in Hh-responding cells, and this correlates to its ability to form a complex with the C-terminal intracellular tail of Smo [Wang et al. 2000; Jia et al. 2003; Lum et al. 2003; Ogden et al. 2003]. In addition, Fu is diminished in cells lacking Cos2 [Lum et al. 2003; Ruel et al. 2003], which might explain, at least in part, why Hh signaling activity is attenuated in cos2 mutant cells adjacent to the A/P boundary [Ho et al. 2005]. Another unexpected observation is that overexpression of Cos2 blocks Smo accumulation in P-compartments of wing discs [Ruel et al. 2003], implying that the Fu-Cos2 complex may play additional roles in regulating Hh signal transduction.

The mechanisms by which Fu and Cos2 are regulated by Hh also remain elusive. Both are phosphorylated in response to Hh, but the biological function of these phosphorylation events is largely unknown. Although the physiological substrate(s) for the Fu kinase is still not known, it has been shown that Su(fu) is phosphorylated in response to Hh, and this phosphorylation depends on Fu [Lum et al. 2003]. However, it is not clear whether Su(fu) is a direct substrate for Fu. Another candidate for a physiological Fu substrate is Cos2. It has been shown that Fu can phosphorylate Cos2 when they are coexpressed in insect cells using the baculovirus system, and the Ser572 of Cos2 is the major site of Fu-mediated phosphorylation [Nybakken et al. 2002]. Additional evidence suggests that Cos2 Ser572 phosphorylation is stimulated by Hh in S2 cells [Nybakken et al. 2002], however, the biological function of Cos2 phosphorylation by Fu remains to be determined.

In this study, we further investigated the function of Fu and Cos2 in the Hh pathway and identified a novel feedback loop in which Fu promotes Smo phosphorylation and cell-surface accumulation by antagonizing Cos2. We found that Cos2 inhibits Smo phosphorylation and cell-surface accumulation through its C-terminal Smo interaction domain, and that Fu alleviates Cos2 inhibition of Smo by phosphorylating Cos2 at Ser572.

**Results**

**FuG13V is a dominant inhibitor of endogenous Fu**

To investigate the mechanism by which the kinase Fu is involved in Hh signaling, we generated an HA-tagged Fu mutant that changes Gly13 into Val [FuG13V]. This mutation occurs naturally and has been characterized as a strong allele of Class I mutations [Therond et al. 1996]. Consistent with this, we found that FuG13V did not rescue the fuA mutant phenotype when expressed under the control of a weak Gal4 driver, armadillo-Gal4, whereas expressing HA-tagged wild-type Fu (HA-FuWT) rescued fuA [data not shown]. To determine if FuG13V has a dominant-negative effect over the endogenous Fu, we expressed FuG13V using a strong, wing-specific driver, MS1096 Gal4. We found that overexpression of FuG13V induced a “fused” wing phenotype (Fig. 1C) similar to that caused by the fuA mutation [Fig. 1B]. We also generated marked clones expressing FuG13V in wing discs using act > CD2 > Gal4 driver and found that FuG13V blocked ptc-lacZ expression [Fig. 1D,D'], a phenotype similar to that caused by strong fu mutations [Alves et al. 1998; Methot and Basler 2000]. As Fu forms a tight complex with Cos2, the nonfunctional FuG13V may compete with endogenous Fu for Cos2, leading to a fuA mutant phenotype. If this is the case, coexpression of FuWT with FuG13V may attenuate the dominant-negative effect of FuG13V and restore Hh signaling. We thus used the wing-specific ap-Gal4 driver [Jia et al. 2004], which expresses Gal4 in dorsal compartment cells, to overexpress FuWT together with FuG13V. Expressing HA-FuG13V along with ap-Gal4 inhibited the expression of endogenous en in anterior–dorsal cells near the A/P boundary [Fig. 1F–F'], Coexpressing Myc-FuWT with HA-FuG13V restored en expression [Fig. 1G–G'], and rescued the FuG13V wing phenotype [data not shown], suggesting that HA-FuG13V can interfere with the endogenous Fu in transducing high-level Hh signaling activity.

**Fu is essential for Hh-induced Smo accumulation**

Smo, Fu, and Cos2 form a large protein complex involved in Hh signal transduction [Jia et al. 2003; Lum et al.
To determine if Fu could regulate Smo, we examined Smo distribution in wing discs expressing HA-FuG13V with ap-Gal4. Surprisingly, overexpressing the dominant-negative form of Fu blocked Hh-induced elevation of Smo in P-cells, as well as in A-cells adjacent to the A/P boundary (Fig. 2B), suggesting that Fu activity is required for Hh-induced Smo accumulation.

To investigate whether the blockade of Smo accumulation by FuG13V is due to preventing Smo cell-surface localization, we turned to a cell-based assay we established earlier for studying Smo cell-surface accumulation (Jia et al. 2004). Consistent with our previous findings (Jia et al. 2004), Smo accumulated on the cell surface upon Hh stimulation (Fig. 2C,D). The Hh-induced CFP-Smo cell-surface accumulation was blocked by expressing FuG13V (Fig. 2H), but not by expressing FuWT (data not shown), suggesting that the attenuation of Smo levels by FuG13V is due to blocking Smo cell-surface accumulation.

Smo cell-surface accumulation is promoted by Hh-induced phosphorylation at three clusters of PKA and CKI sites (Jia et al. 2004; Zhang et al. 2004; Apionishev et al. 2005). We have previously shown that SmoSD123, in which the three clusters of PKA and CKI phosphorylation sites are replaced by Asp to mimic phosphorylation, has constitutive cell-surface expression and signaling activity (Jia et al. 2004). To determine if FuG13V could block the cell-surface localization of SmoSD123, S2 cells were transfected with SmoSD123 and FuG13V, then treated with Hh-conditioned or control medium. Strikingly, the constitutive cell-surface accumulation of SmoSD123 was not

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**Figure 1.** FuG13V has a dominant-negative effect in Hh pathway. (A–C) Adult wings from wild-type (A), fuA mutant (B), or flies expressing HA-FuG13V by MS1096 Gal4 [C]. Note the fusion between L3 and L4 in fuA- and FuG13V-expressing wings. (D,D') A wing disc expressing HA-FuG13V with act > CD2 > Gal4 was immunostained for HA (green) and ptc-lacZ (red). FuG13V blocked ptc-lacZ expression (arrows). (E,E') A wing disc expressing GFP (green) with ap-Gal4 was immunostained to show the expression of En (red). (F,F') A wing disc expressing GFP (green) and HA-FuG13V with ap-Gal4 was immunostained to show the expression of En (red) and HA (blue). Expressing HA-FuG13V inhibited the expression of en in anterior dorsal cells near the A/P boundary (arrow in F). (G-G'') A wing disc coexpressing Myc-FuWT with HA-FuG13V restored anterior dorsal En expression (arrow in G) that was inhibited by FuG13V. All wing discs shown in this study were oriented with anterior to the left and ventral on the top.
affected by FuG13V (Fig. 2J), even in the absence of Hh (Fig. 2I). Thus, the phosphorylation-mimicking form of Smo escaped regulation by Fu, suggesting that FuG13V may block Smo cell-surface accumulation by preventing Smo phosphorylation [see below].

The blockade of Smo accumulation by FuG13V is mediated by Cos2

Several lines of evidence suggest that Cos2 may be involved in the regulation of Smo by Fu. First, we and others have shown a physical interaction between Smo and the Fu/Cos2 complex (Jia et al. 2003; Lum et al. 2003; Ogden et al. 2003; Ruel et al. 2003), likely through a direct association with Cos2 (Lum et al. 2003; Ogden et al. 2003). Second, Cos2 can be phosphorylated by Fu (Nybakkken et al. 2002). Third, Cos2 is accumulated in P-cells in which Fu activity is compromised by FuG13V overexpression [see below]. Finally, overexpression of Cos2 blocked Smo accumulation in P-cells (Fig. 4A, below; Ruel et al. 2003), phenocopying loss of Fu activity. To test our hypothesis, FuG13V was expressed under the control of the wing-specific MS1096 Gal4 driver in cos2 mutant wing discs. As shown in Figure 3, FuG13V blocked Smo accumulation in the cos2 heterozygous disc [cos2−/+], but not in the cos2 homozygous disc [cos2−/−] (Fig. 3B–B′), suggesting that Cos2 is required for FuG13V to inhibit Smo.

To determine whether the effect of FuG13V on Smo cell-surface accumulation in S2 cells relies on Cos2, we used double-stranded RNA [dsRNA] to knock down endogenous Cos2. We found that Hh induced Smo cell-surface accumulation in the presence of FuG13V when Cos2 was knocked down by RNA interference (RNAi) (Fig. 3E), indicating that the effect of FuG13V on Smo accumulation was dependent on Cos2.

To confirm the physiological regulation of Smo by Fu and Cos2, we turned to fu and cos2 loss-of-function mutants. We examined Smo distribution in wing discs carrying mutant clones homozygous for a strong fu allele, fuH63 (a Class I allele), and found that Smo accumulation was blocked in fu mutant cells situated in A-cells near the A/P boundary [Fig. 3C], suggesting that Fu activity is essential for Hh-induced Smo accumulation. Significantly, the down-regulation of Smo by the fu mutation was alleviated by the loss of cos2, as Hh-induced

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**Figure 2.** Fu is required for Hh-induced Smo accumulation. [A] A wild-type wing disc was immunostained to show Hh-induced Smo accumulation in P-cells. [B–B′] Expression of FuG13V (red) with ap-Gal4 blocked Smo accumulation in the dorsal compartment cells (arrow in B). [C–F] S2 cells were transfected with CFP-tagged Smo (C,D) or SmoSD123 (E,F) and treated with Hh-conditioned medium (D,F) or control medium (C,E). [G–J] S2 cells were cotransfected with HA-FuG13V plus either CFP-Smo (G,H) or CFP-SmoSD123 (I,J), followed by treatment with Hh-conditioned medium (H,I) or control medium (G,J). FuG13V blocked Hh-induced CFP-Smo cell-surface accumulation (H) but did not block CFP-SmoSD123 accumulation (I,J). SmoN antibody staining indicates the cell-surface-localized Smo, and CFP signal indicates the total amount of expressed Smo.
Smo accumulation was restored in \textit{fu cos2} double-mutant clones (Fig. 3D). We also found that \textit{fu cos2} double clones show normal Smo levels in both A- and P-compartments [data not shown]. Taken together, these observations suggest that Cos2 is responsible for blocking Hh-induced Smo accumulation when Fu activity is compromised.

The C-terminal region of Cos2 mediates its inhibition of Smo accumulation

The above data suggest that Cos2 may prevent Smo cell-surface accumulation, whereas Fu activity is normally required to overcome this blockade. Consistent with this, overexpression of Cos2 down-regulates Smo in wing discs [Fig. 4A; Ruel et al. 2003]. To further test this hypothesis and determine whether down-regulation of Smo cell-surface expression by Cos2 depended on the Smo phosphorylation status, we cotransfected S2 cells with CFP-Smo or CFP-Smo\textsuperscript{SD123} together with Cos2, followed by treatment with Hh-conditioned medium. As shown in Figure 3F, Hh-induced CFP-Smo cell-surface accumulation was blocked by Cos2 coexpression. In contrast, coexpression of Cos2 had no effect on CFP-Smo\textsuperscript{SD123} cell-surface accumulation [Fig. 3G], as was the case with \textit{Fu G13V} coexpression, implying that Cos2 prevented Smo accumulation at a step prior to Smo phosphorylation.

To define the Cos2 domain[s] responsible for inhibiting Smo, we tested a set of Cos2 deletion mutants [Fig.
4G) in wing discs to assess their ability to down-regulate Smo. We found that a Cos2 mutant with its C-tail deleted (Cos2ΔC1) no longer blocked Smo accumulation (Fig. 4D). In contrast, deletion of the microtubule-binding domain alone (Cos2ΔN1) or together with the neck region (Cos2ΔN2) did not affect the ability of the resulting Cos2 mutants to block Smo accumulation (Fig. 4B,C, respectively). In addition, we found that the Cos2 C-tail (Cos2CT) alone was sufficient to block Smo (Fig. 4F). Consistent with a recent finding that expression of a Cos2 C-terminal domain disrupts Hh signaling (Ogden et al. 2006), we found that the overexpression of Cos2CT in imaginal discs diminished ptc expression in A-cells adjacent to the A/P boundary (data not shown). We further examined the Cos2 deletion mutants in S2 cells and found their ability to prevent Smo cell-surface accumulation in S2 cells [Supplementary Fig. S1] correlated with their ability to down-regulate Smo in wing discs.

**Cos2 blocks Smo phosphorylation**

Neither Cos2 nor FuC13V blocked SmoSD123 cell-surface accumulation, suggesting that the Fu–Cos2 complex may regulate Smo accumulation by regulating its phos-
Phosphorylation. To test this, we transfected S2 cells with Myc-tagged Smo plus HA-tagged full-length or truncated forms of Cos2 and treated the transfected cells with Hh-conditioned or control medium. Myc-Smo was immunoprecipitated with anti-Myc antibody followed by Western blot with anti-Myc antibody to detect the phosphorylation status of Smo. The arrow indicates hyperphosphorylated forms of Smo and the arrowhead indicates hypophosphorylated and unphosphorylated forms. (Bottom panel) The expression of individual Cos2 constructs was detected by direct Western blot with anti-HA antibody. Of note, Cos2CT ran out of the gel due to its small molecular weight (27 kDa). (WB) Western blot. (B, lane 4) S2 cells were cotransfected with Myc-Smo, HA-FuG13V, and Cos2 dsRNA followed by treatment with Hh-conditioned medium to detect the effect of Cos2 RNAi [cf. lane 3 without RNAi]. (Lanes 1,2) S2 cells transfected with Myc-Smo followed by treatment with Hh-conditioned or control medium served as control. The inhibitory efficiency of Cos2 RNAi is shown in the right panel. (C) A wing disc overexpressing HA-Cos2WT driven by ap-Ga4 was immunostained with anti-SmoN antibody to show the blockade of Smo accumulation in dorsal P-cells (arrow). (D, D’) A wing disc overexpressing a strong line of mC* was stained with anti-SmoN (blue) and anti-PKA (red) to show Smo expansion in A-cells near the A/P boundary [arrowhead]. (E, E’) A wing disc coexpressing HA-Cos2WT, mC*, and Fg-CKIα was immunostained with anti-SmoN (E) and anti-PKA (E’) antibodies. (F) A control disc indicates the coexpression of PKA (red) and Fg-CKIα (green) for the experiment in E and E’.

Figure 5. Cos2 blocks Smo phosphorylation. (A) S2 cells were transfected with the indicated constructs and treated with Hh-conditioned or control medium. (Top panel) Cell extracts were immunoprecipitated with anti-Myc antibody followed by Western blot with anti-Myc antibody to detect the phosphorylation status of Smo. The arrow indicates hyperphosphorylated forms of Smo and the arrowhead indicates hypophosphorylated and unphosphorylated forms. (Bottom panel) The expression of individual Cos2 constructs was detected by direct Western blot with anti-HA antibody. Of note, Cos2CT ran out of the gel due to its small molecular weight (27 kDa). (WB) Western blot. (B, lane 4) S2 cells were cotransfected with Myc-Smo, HA-FuG13V, and Cos2 dsRNA followed by treatment with Hh-conditioned medium to detect the effect of Cos2 RNAi [cf. lane 3 without RNAi]. (Lanes 1,2) S2 cells transfected with Myc-Smo followed by treatment with Hh-conditioned or control medium served as control. The inhibitory efficiency of Cos2 RNAi is shown in the right panel. (C) A wing disc overexpressing HA-Cos2WT driven by ap-Ga4 was immunostained with anti-SmoN antibody to show the blockade of Smo accumulation in dorsal P-cells (arrow). (D, D’) A wing disc overexpressing a strong line of mC* was stained with anti-SmoN (blue) and anti-PKA (red) to show Smo expansion in A-cells near the A/P boundary [arrowhead]. (E, E’) A wing disc coexpressing HA-Cos2WT, mC*, and Fg-CKIα was immunostained with anti-SmoN (E) and anti-PKA (E’) antibodies. (F) A control disc indicates the coexpression of PKA (red) and Fg-CKIα (green) for the experiment in E and E’.
planation is that an excess amount of Cos2 blocks Smo phosphorylation by titrating out PKA and CKI. If so, one would expect that supplying excess amounts of PKA and CKI would reverse the blockade of Smo phosphorylation by Cos2. To test this, we coexpressed PKA and CKI with Cos2. Overexpressing Cos2 depleted Smo in dorsal P-cells (Fig. 5C) that was not rescued by the coexpression of a strong line of a constitutively active form of PKA catalytic subunit (mC*) and Flag-tagged CKIα (FgCKIα) (Fig. 5E), even though the overexpression of mC* alone resulted in a dramatic anterior expansion of the domain that accumulated high levels of Smo (Fig. 5D). These results suggest that Cos2 does not simply block Smo phosphorylation by titrating out the kinases.

**Hh-induced Smo accumulation requires Cos2 phosphorylation by Fu**

The requirement of Cos2 in blocking Smo accumulation when Fu activity is compromised suggests that Fu promotes Smo phosphorylation and accumulation by antagonizing Cos2. A previous study suggested that Cos2 acts downstream from Fu, as the mu mutant phenotype was partially suppressed by a cos2 mutation (Preat et al. 1993). It has been also shown that Fu binds to the neck region of Cos2 (Monnier et al. 2002; Wang and Jiang 2004). FuWT but not FuG13V phosphorylates Cos2 Ser572 in the neck domain, and Ser 572 phosphorylation appears to be a critical phosphorylation event induced by Hh in S2 cells (Nybäken et al. 2002). To determine if Fu regulates Cos2 activity by phosphorylating Ser572, we generated Fu phosphorylation-mimicking (Cos2S572D) and phosphorylation-deficient (Cos2S572A) forms of Cos2. We analyzed Cos2 phosphorylation by using FuWT, FuG13V, or Fu RNAi. We confirmed that Ser572 was critical for Hh-induced Cos2 phosphorylation, as Cos2S572A exhibited a faster-migrating, unphosphorylated form whereas Cos2 exhibited a mobility shift in the presence of Hh (Fig. 6A, cf. lanes 2 and 6). Furthermore, we found that Fu was responsible for Cos2 phosphorylation, as perturbation of endogenous Fu kinase activity by either coexpression of FuG13V or Fu RNAi abolished Cos2 phosphorylation (Fig. 6A, lanes 7–10). Confirmation of effective knockdown of Fu by RNAi is shown in Figure 6A (right panel).

We next examined the in vivo effect of Ser572 phosphorylation on Smo accumulation by overexpressing HA-Cos2WT, Cos2S572A, or HA-Cos2S572D in wing discs via ap-Gal4. We found that Cos2S572A behaved like Cos2WT, as dorsal P-cells expressing Cos2S572A abolished Smo accumulation (Fig. 6C). In contrast, expressing Cos2S572D did not prevent Smo accumulation (Fig. 6D). We further found that the effects of the exogenously expressed Cos2WT and Cos2S572A on Smo accumulation were independent of endogenous Cos2 protein (Supplementary Fig. S3). Furthermore, we found that Cos2S572D had a dominant-negative effect over endogenous Cos2 activity because anterior-situated clones expressing HA-Cos2S572D induced ectopic Ptc expression (Supplementary Fig. S2D,D'), similar to the effect of Cos2S182N (Ho et al. 2005). We also found that Cos2S572D neutralized the FuG13V effect of inhibiting Smo accumulation (Fig. 6E–E'), likely by forming an inactive heterodimer with endogenous Cos2 and/or titrating out FuG13V.

Consistent with its failure to block Smo accumulation, Cos2S572D also failed to block Hh-induced Smo phosphorylation in S2 cells (Fig. 6F, lane 5, top panel). In contrast, Cos2S572A behaved like Cos2WT in preventing Smo phosphorylation (Fig. 6F, lane 4, top panel). We further found that Cos2ΔC1S572A and Cos2ΔC1S572D behaved like Cos2ΔC1 and did not block Smo phosphorylation (data not shown) and cell-surface accumulation (Supplementary Fig. S1), further strengthening the view that the Cos2ΔC1 is essential for Cos2 to block Smo phosphorylation and accumulation.

We found that the blockade of Hh-induced Smo cell-surface accumulation by Cos2 in S2 cells was alleviated by coexpressing FuWT [Fig. 6G], but not the kinase-dead Fu mutant FuG13V [Fig. 6H], suggesting that Fu antagonizes Cos2 to promote Smo cell-surface accumulation through its kinase activity. If phosphorylation of Ser572 by Fu is essential for preventing Cos2 from inhibiting Smo, we would expect that Cos2S572A should be resistant to FuWT coexpression. Indeed, Cos2S572A dominantly blocked Smo accumulation even in the presence of FuWT coexpression (Fig. 6I). Consistently, inhibition of Hh-induced Smo phosphorylation by Cos2WT was eliminated by FuWT [Supplementary Fig. S3C]. Taken together, these observations suggest that the negative effect of Cos2 on Hh-induced Smo phosphorylation and cell-surface expression is abolished by Fu phosphorylation of Cos2 at Ser572.

**Phosphorylation of Cos2 at Ser 572 attenuates Cos2–Smo interaction**

We next explored the mechanism by which phosphorylation of Cos2 antagonizes its ability to block Smo. Cos2 blocks Smo phosphorylation through its C-terminal cargo domain, which has been shown to interact with the Smo C-terminal intracellular tail (SmoCT), raising the possibility that binding of Cos2 to Smo could block the accessibility of PKA and CKI to Smo, whereas Fu phosphorylation of Cos2 at Ser572 could attenuate Cos2–Smo interaction, allowing PKA and CKI to phosphorylate Smo. To determine if Ser572 phosphorylation affects direct interaction between Cos2 and SmoCT, we carried out GST pull-down experiments with a purified, bacterially expressed GST-Smo557–686 (Fig. 7A, lane 5, top panel), suggesting that...
Ser572 phosphorylation prevents Cos2 from binding directly to the membrane-proximal region of SmoCT.

Cos2 Ser572 phosphorylation promotes Cos2 degradation

When we examined the effect of Cos2<sup>5572D</sup> on Smo phosphorylation we noticed that the expression levels of Cos2<sup>5572D</sup> were consistently low, suggesting that phosphorylation of Cos2 at Ser572 may trigger Cos2 degradation, which may represent an additional mechanism to alleviate Smo from Cos2 inhibition. We first examined the relative stability of Smo<sup>WT</sup>, Cos2<sup>5572A</sup>, and Cos2<sup>5572D</sup> proteins in S2 cells. S2 cells were transfected with constructs expressing each of these Cos2 variants, and Cos2 protein levels were monitored at different time points after the treatment with the protein synthesis inhibitor, cycloheximide. We found that little Cos2<sup>5572D</sup> was detectable after 4 h of incubation with cycloheximide [Fig. 7C, lane 9]. In contrast, the Ser572Ala mutation made Cos2 protein more stable [Fig. 7C, lanes 4–6] compared with Cos2<sup>WT</sup> (Fig. 7C, lanes 1–3). This was further demonstrated by a quantification analysis [Fig. 7D]. These data support the idea that Ser572 phosphorylation by Fu destabilizes Cos2.

To determine if Fu regulates Cos2 stability in response to Hh, we cotransfected S2 cells with Cos2-, Fu-, and Smo-expressing constructs, followed by treatment with Hh-conditioned or control medium. As shown in Figure 7E, Flag-tagged wild-type Fu [Fu-Fu<sup>WT</sup>] promoted HA-Cos2<sup>WT</sup> degradation in response to Hh [lane 1]. Hh-in-
duced Cos2 instability depended on Fu kinase activity, as the kinase-dead, dominant-negative FuG13V stabilized HA-Cos2WT in response to Hh [Fig. 7E, lane 7]. Hh-induced Cos2 instability also depended on Ser572 phosphorylation by Fu, as the stability of HA-Cos2S572A remained nearly unchanged regardless of Hh stimulation. On the other hand, HA-Cos2S572D was unstable under these conditions. To determine if Fu regulates Cos2 stability in vivo, we examined Cos2 distribution in wing discs expressing the dominant-negative FuG13V or FuWT. It has been shown that Hh induces Cos2 instability in both embryos and imaginal discs [Ruel et al. 2003] so that the cells in the P-compartment or the A-compartment abutting the A/P boundary exhibit low levels of Cos2. We found that expression of FuG13V stabilized Cos2 in the P-compartment [Fig. 7F, arrow], but expression of FuWT did not [Fig. 7G, arrow], suggesting that Hh-induce Cos2 instability is mediated by Fu in vivo. Furthermore, we found that expression of FuWT promoted Cos2 turnover in A-cells adjacent to the A/P boundary but not in A-cells distant from the A/P boundary [Supplementary Fig. S5B], suggesting that the ability of Fu to promote Cos2 instability depends on Hh.

Figure 7. Cos2 phosphorylation at Ser572 attenuates Cos2–Smo interaction and promotes Cos2 degradation. (A) Extracts from S2 cells expressing the indicated HA-tagged Cos2 constructs were incubated with GST-Smo557–686 fusion protein. [Top panel] The bound Cos2 proteins were analyzed by Western blot with anti-HA antibody. [Bottom panel] Equal amounts of GST-Smo557–686 fusion protein and equal amounts of input Cos2 protein were used. [Right panel] The interaction between GST and Cos2CT served as a negative control. (B) Quantification of Cos2 variants associated with GST-Smo557–686. The pull-down efficiency was determined by the amount of total input. Three independent experiments were performed. (C, top panel) The stability of Cos2WT, Cos2S572A, and Cos2S572D in transfected S2 cells was analyzed by direct Western blot with anti-HA antibody after incubation with cycloheximide for the indicated times. [Bottom panel] The β-tubulin levels served as loading control. (D) Quantification of Cos2WT, Cos2S572A, and Cos2S572D by Western analysis performed in C. [E] S2 cells were transfected with the indicated constructs and treated with Hh-conditioned or control medium. Cell lysates were subjected to direct Western blot with anti-HA antibody. (F) A model for regulating Smo phosphorylation and accumulation by a positive feedback loop. Fu is required for Hh-induced Smo phosphorylation and accumulation through antagonizing the inhibitory role of Cos2. By phosphorylating Cos2 at Ser572, Fu kinase prevents Cos2 from inhibiting Smo, which attenuates Cos2–Smo interaction and promotes Cos2 degradation.
Discussion

The seven-transmembrane protein Smo plays a central role in transducing the Hh signal. In this study, we demonstrate that Fu kinase is essential for Smo phosphorylation and cell-surface accumulation. We further show that the Fu kinase acts through phosphorylation of Cos2 at Ser572, which releases an inhibition of Smo phosphorylation imposed by Cos2. Thus, our study provides the first evidence for a physiologically relevant Fu substrate, and uncovers a feedback mechanism by which Fu promotes Smo hyperphosphorylation and cell-surface accumulation, which is essential for optimal Hh pathway activation (Fig. 7H). Mechanistically, we provide evidence that Cos2 blocks Smo phosphorylation through direct binding to Smo, and that Cos2 Ser572 phosphorylation by Fu attenuates Cos2–Smo interaction and induces Cos2 protein degradation, both of which may contribute to the alleviation of Cos2 inhibition on Smo. Our study thus unravels an unanticipated layer of Smo regulation by the downstream Hh signaling complex.

Opposing roles of Fu and Cos2 in regulating Smo

Fu is characterized as a positive regulator in Hh signal transduction (Ohlmeyer and Kalderon 1998; Wang et al. 2000; Levers et al. 2001). In the absence of Hh, Fu is required for Ci sequestration in the cytoplasm, whereas in the presence of Hh, it is required for Ci nuclear translocation (Wang and Holmgren 2000; Wang et al. 2000). The finding that Smo directly interacts with the Cos2–Fu protein complex even in the absence of the Hh signal prompted us to further dissect the role of this complex in regulating Hh signal transduction. Unexpectedly, we found that overexpression of a kinase-dead, dominant-negative form of Fu blocked Hh-induced Smo accumulation in both S2 cells and wing discs. Furthermore, we found Hh-induced Smo accumulation was blocked in fu mutant clones in wing discs. However, overexpressing Fu alone does not induce Smo accumulation in A-cells away from the A/P boundary (Supplementary Fig. S5A), and does not promote Smo phosphorylation in S2 cells (data not shown). These results suggest that Fu plays a positive role in regulating Smo cell-surface accumulation and this regulation depends on Hh.

The blockade of Smo accumulation in the absence of Fu activity appears to be mediated by Cos2, as removal of Cos2 from FuG13V-expressing cells by genetic mutation or RNAi restored Hh-induced Smo accumulation [Fig. 3B,E]. Furthermore, Hh-induced Smo accumulation is restored in fu cos2 double-mutant clones [Fig. 3D]. These observations suggest that Cos2 normally exerts a negative regulation on Smo, and that this inhibition needs to be alleviated through the Fu kinase activity in order for Hh to induce Smo accumulation and hyperactivation. This mechanism may explain why overexpression of Cos2 in wing discs and S2 cells can block Smo accumulation, as excess amounts of Cos2 can titrate out endogenous Fu, which leads to formation of Smo–Cos2 complexes devoid of Fu and keeps Smo from being activated by Hh.

We found that fu clones in P-cells do not show attenuated Smo levels [Fig. 3C’–C’’], which could be due to the highest level of Hh and/or lack of Ptc in these cells so that Smo exists in a hyperphosphorylated and active form and may no longer be subject to the feedback regulation in fu mutant clones.

Regulation of Smo phosphorylation by Fu and Cos2

Although FuG13V and Cos2 can block Hh-induced cell-surface accumulation of wild-type Smo, we found that the phosphorylation-mimicking Smo variant SmoSD123, which exhibits constitutive cell-surface accumulation and signaling activity (Jia et al. 2004), escaped the inhibition by FuG13V and Cos2 [Figs. 2L,J, 3G], suggesting that the regulation of Smo by Fu and Cos2 is upstream of Smo phosphorylation. Consistent with this idea, we found that overexpressing FuG13V or Cos2 blocked Hh-induced Smo phosphorylation in S2 cells [Fig. 5A].

We further demonstrated that Cos2 inhibits Hh-induced Smo phosphorylation through its C-terminal Smo-binding domain. We showed that the Cos2 cargo domain from amino acids 990–1201 is both necessary and sufficient for blocking Hh-induced Smo phosphorylation in S2 cells [Fig. 5A]. In addition, the Cos2 cargo domain is also necessary and sufficient for blocking Smo accumulation in both wing discs and S2 cells [Fig. 4F; Supplementary Fig. S1]. The Cos2 cargo domain has been shown to interact with PKA and CKI (Zhang et al. 2005), raising the possibility that it may inhibit Smo phosphorylation by titrating out these kinases. However, the observation that coexpression of PKA and CKI with Cos2 failed to restore Smo accumulation does not support this model.

It has also been shown that the Cos2 cargo domain mediates binding between Cos2 and Smo [Jia et al. 2003; Lum et al. 2003; Ruel et al. 2003]. GST pull-down assays suggest that the Cos2 cargo domain binds the Smo C-terminal region between amino acids 661 and 686 [Lum et al. 2003], which includes the first PKA and CKI phosphorylation cluster. It is possible that binding of Cos2 to this region blocks the access of PKA and/or CKI to their target sites in the Smo C-tail, leading to inhibition of Smo phosphorylation.

Although our data suggest that Cos2 plays an inhibitory role in regulating Smo phosphorylation, removal of Cos2 in the absence of Hh is not sufficient to promote Smo phosphorylation, as A-compartment cos2 mutant cells situated at a distance from the A/P boundary did not accumulate high levels of Smo [Supplementary Fig. S3A], suggesting that additional mechanism(s) must exist to block Smo phosphorylation in the absence of Hh. An obvious candidate is Ptc, since ptc mutant clones situated in the A-compartment of wing discs accumulate high levels of Smo, and RNAi knockdown of ptc results in Smo phosphorylation in S2 cells [Zhu et al. 2003; Nakano et al. 2004]. Furthermore, the inhibitory effect of Ptc on Smo is further strengthened in cos2 mutant cells as ptc expression is up-regulated in these cells [Zhang et al. 2005].
Fu regulates Smo by phosphorylating Cos2

It has been shown previously that Fu phosphorylates Cos2 at Ser572, which appears to be a major site for Cos2 phosphorylation in response to Hh [Nybakken et al. 2002]. We found that the phosphorylation-mimicking Cos2 variant (Cos25572D) no longer blocked Smo phosphorylation and cell-surface accumulation in response to Hh, whereas the phosphorylation-deficient Cos2 variant (Cos25572A) exhibited strong inhibitory activity on Smo phosphorylation [Fig. 6F, cf. lanes 3–5] and cell-surface accumulation [Fig. 6J]. In S2 cells, the blockade of Smo accumulation by wild-type Cos2 was alleviated by coexpression of Fu, in contrast, the activity of Cos25572A in blocking Smo was no longer inhibited by Fu coexpression [Fig. 6I]. Taken together, these observations suggest that Ser572 phosphorylation is a major regulatory event by which Fu antagonizes the inhibitory activity of Cos2 toward Smo in response to Hh. However, our data do not rule out the possibility that Fu may also exert a direct positive role on Smo, e.g., by phosphorylating Smo.

How does Ser572 phosphorylation of Cos2 alleviate its inhibition on Smo? We found that Ser572 phosphorylation appeared to decrease Cos2–Smo interaction, as our data suggested, then decreasing Cos2 phosphorylation in response to Hh, allowing Smo to be hyperphosphorylated and fully activated. Cos2 associated with the inhibited and fully activated forms of Smo might undergo other modification(s), such as additional phosphorylation, that could stabilize it. Alternatively, other factors present in the active or inactive Smo–Cos2–Fu complexes might influence Cos2 stability.

The complex relationship among Smo, Cos2, and Fu

The prevalent view for the role of Fu in the Hh pathway is that it acts downstream from Smo to transduce the Hh signal [Ingham and McMahon 2001; Lum and Beachy 2004; Jia and Jiang 2006]. Here we demonstrate that Fu is required to promote Smo phosphorylation and thus activation, therefore placing Fu upstream of Smo. However, activation of Smo is unlikely to account for all the Fu activity in the Hh pathway, as we found that Smo5D123, which is no longer regulated by Fu, still depends on Fu to activate Hh target genes. For example, coexpression of FuG13V with Smo5D123 attenuated Smo5D123-induced ptc-lacZ expression and abolished Smo5D123-induced En expression [Supplementary Fig. S4]. Hence, we propose that Fu plays a dual role in both activating Smo and transducing the Hh signal downstream from Smo [Fig. 7H]. One possible Fu substrate downstream from Smo is Su(fu). Genetic studies suggest that Fu activates Ci by antagonizing Su(fu) [Ohlmeyer and Kalderon 1998]. In addition, Hh-induced Su(fu) phosphorylation depends on Fu [Lum et al. 2003].

Cos2 phosphorylation by Fu at Ser572 may also inhibit its activity toward promoting Ci processing, as Cos2Ser572D failed to restore Ci processing in cos2 mutant wing discs [Supplementary Fig. S3D]. In addition, Cos2Ser572D has a dominant-negative effect over endogenous Cos2 and blocks Ci processing, leading to partial activation of the Hh pathway [Supplementary Fig. S2D; data not shown]. Thus, Fu phosphorylation of Cos2 at Ser572 may prevent Cos2 from inhibiting both Smo and Ci. Further studies are needed to investigate the mechanism by which Ser572 phosphorylation of Cos2 inhibits its activity in promoting Ci processing.

The Hh signal transduction pathway between Smo and Ci/Gli appears to diverge between insects and mammals [Huangfu and Anderson 2006]. Most notably, targeted disruption of the mammalian homolog of Fu (mFu) failed to reveal any defects in Hh signaling, although one cannot rule out the possibility that a distant relative of mFu
could compensate for the loss of mFu [Chen et al. 2005; Merchant et al. 2005]. Similarly, a study in cultured cells using RNAi failed to reveal a role of mammalian Cos2 homologs (Ki67 and Ki627) in Shh singaling [Varjosalo et al. 2006]. Thus, the feedback mechanism involving Fu and Cos2 in regulating Smo may not apply to the mammalian system. It has been shown that mammalian Smo (mSmo) translocates to the primary cilium in response to Hh, although the underlying regulatory mechanism remains poorly understood. It is not impossible that mSmo might be regulated by an analogous feedback mechanism.

Materials and methods

Mutants and transgenes

fu4 is a strong fu mutant allele with a deletion in the extracata-
lytic domain [Alves et al. 1998], and fu4mi63 is a strong hypomor-
phic allele [Preat 1992]. cos22 is a null allele [Sisson et al. 1997]. M51096, act > CD2 > Gal4, armadillo-Gal4, ap-Gal4, UAS-mc*, UAS-Fg-Klka, UAS-hh, and ptc-lacZ have been de-
scribed (Li et al. 1995; Jia et al. 2004, 2005; FlyBase at http://
FlyBase.bio.indiana.edu). A Fu mutation with substitution of Gly13 to Val and Cos2 mutations with substitutions at Ser572
have been described (Wang et al. 1997).

HA-Cos2S572A and HA-Cos2 S572D were generated using the endogenous HindIII
sites. Multiple independent transgenic lines were generated and ex-
amined for each construct.

Cell culture, transfection, immunoprecipitation, and Western
blot analysis

S2 cells were cultured in Schneider’s Drosophila Medium (In-
vitrogen) with 10% fetal bovine serum, 100 U/mL penicillin, and
100 µg/mL streptomycin. Transfection, immunoprecipitation,
and immunoblot analysis were performed with standard
protocols as described previously [Jia et al. 2004]. The cell-based
assay to detect Smo cell-surface accumulation was carried out by
immunostaining with anti-SmoN antibody before cell per-
meabilization [Jia et al. 2004]. Fu and Cos2 dsRNA were syn-
thesized against the regions described previously [Lum et al.
2003; Ruel et al. 2003] and used for both treatment and trans-
faction to get better interference with the endogenous protein
expression. Control GFP dsRNA was synthesized against EGFP
amino acids 2–201. Cycloheximide (Sigma) treatment was per-
formed at a final concentration of 100 µM for the indicated time
periods before harvesting S2 cells. Antibodies used in this study
were mouse anti-Myc, 9E10 [Santa Cruz Biotechnology], anti-
HA, F7 [Santa Cruz Biotechnology], anti-Flag, M2 [Sigma], anti-
Cos2 [gift from D. Robbins], anti-SmoN [Developmental Studies
Hybridoma Bank (DSHB)], anti-ribulbin [DSHB]; rabbit anti-
Fu [gift from D. Robbins], anti-PKA [Santa Cruz Biotechnology],
and anti-HA, Y-11 [Santa Cruz Biotechnology].

GST fusion protein pull-down assay

GST fusion proteins were produced from Escherichia coli lys-
ates. Equal amount of GST-Smo fusion protein or GST protein
were incubated with glutathione-Sepharose 4B beads and
washed in phosphate-buffered saline. GST-fusion protein
loaded beads were incubated with S2 cell lysates with equal
amounts of HA-tagged Cos2 proteins for 2 h at 4°C, followed by
ten 10-min washes with lysis buffer and then by Western blot
analysis to detect the bound Cos2 proteins. To normalize the
protein levels, GST-Smo or GST was stained with Commassie
blue and their intensity was calculated by Metamorph software.

Immunostaining of imaginal discs

Standard protocols for immunofluorescence staining of imagi-
nal discs were used [Jiang and Struhl 1995] with the antibodies
mouse anti-Myc, 9E10 [Santa Cruz Biotechnology], anti-HA, F7
[Santa Cruz Biotechnology], anti-Flag, M2 [Sigma], anti-Cos2,
5D6 [gift from D. Robbins], anti-En [DSHB], anti-SmoN [DSHB],
anti-Ptc [DSHB]; rabbit anti-Fu [gift from D. Robbins], anti-PKA
[Santa Cruz Biotechnology], anti-Flag (ABR), anti-HA, Y-11
[Santa Cruz Biotechnology], anti-βGal [Cappell], anti-GFP (Clon-
tech), and rat anti-Ci, 2A [gift from R. Holmgren].

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Fused–Costal2 protein complex regulates Hedgehog-induced Smo phosphorylation and cell-surface accumulation

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