

# The Hedgehog-induced Smoothed conformational switch assembles a signaling complex that activates Fused by promoting its dimerization and phosphorylation

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## SUMMARY

Hedgehog (Hh) transduces signal by regulating the subcellular localization and conformational state of the GPCR-like protein Smoothed (Smo) but how Smo relays the signal to cytoplasmic signaling components remains poorly understood. Here, we show that Hh-induced Smo conformational change recruits Costal2 (Cos2)/Fused (Fu) and promotes Fu kinase domain dimerization. We find that induced dimerization through the Fu kinase domain activates Fu by inducing multi-site phosphorylation of its activation loop (AL) and phospho-mimetic mutations of AL activate the Hh pathway. Interestingly, we observe that graded Hh signals progressively increase Fu kinase domain dimerization and AL phosphorylation, suggesting that Hh activates Fu in a dose-dependent manner. Moreover, we find that activated Fu regulates Cubitus interruptus (Ci) by both promoting its transcriptional activator activity and inhibiting its proteolysis into a repressor form. We provide evidence that activated Fu exerts these regulations by interfering with the formation of Ci-Sufu and Ci-Cos2-kinase complexes that normally inhibit Ci activity and promote its processing. Taken together, our results suggest that Hh-induced Smo conformational change facilitates the assembly of active Smo-Cos2-Fu signaling complexes that promote Fu kinase domain dimerization, phosphorylation and activation, and that Fu regulates both the activator and repressor forms of Ci.

**KEY WORDS:** Cos2, Fu, Ci, Hedgehog, Smo, Sufu, *Drosophila*

## INTRODUCTION

The Hedgehog (Hh) signaling pathway controls a myriad of key development processes and its malfunction causes many human disorders, including birth defects and cancer (Ingham and McMahon, 2001; Jiang and Hui, 2008; Varjosalo and Taipale, 2008). Hh exerts its biological influence through a conserved signaling cascade that culminates at the regulation of the latent transcription factor Ci/Gli (Jiang and Hui, 2008; Wilson and Chuang, 2010). In *Drosophila* wing development, the absence of Hh allows Ci to be proteolytically processed into a truncated form (Ci<sup>R</sup>) that blocks the expression of a subset of Hh target genes, including *decapentaplegic* (*dpp*). Hh signaling inhibits Ci processing into Ci<sup>R</sup> and converts the accumulated full-length Ci (Ci<sup>F</sup>) into an active but labile form (Ci<sup>A</sup>) that activates *dpp*, *patched* (*ptc*) and *engrailed* (*en*) (Aza-Blanc et al., 1997; Methot and Basler, 1999).

Multiple intracellular signaling complexes regulate the formation of Ci<sup>R</sup> and Ci<sup>A</sup>. The kinesin-like protein Costal2 (Cos2) and the Ser/Thr protein kinase Fused (Fu) form a complex with Ci to impede its nuclear import (Robbins et al., 1997; Methot and Basler, 2000; Wang et al., 2000; Wang and Holmgren, 2000; Wang and Jiang, 2004). Furthermore, Cos2 recruits multiple kinases, including PKA, GSK3 and CK1 to phosphorylate Ci, which targets Ci for SCF<sup>Slimb</sup>-mediated proteolytic processing to generate Ci<sup>R</sup>

(Jia et al., 2002; Price and Kalderon, 2002; Jia et al., 2005; Zhang et al., 2005; Smelkinson et al., 2007). Sufu forms a complex with Ci to block the conversion of Ci<sup>F</sup> to Ci<sup>A</sup> by impeding its nuclear import and probably by recruiting a co-repressor(s) (Monnier et al., 1998; Ohlmeyer and Kalderon, 1998; Methot and Basler, 2000; Wang et al., 2000; Cheng and Bishop, 2002). Interestingly, Cos2 also plays a positive role in the Hh pathway and is required for full pathway activation (Wang et al., 2000; Wang and Holmgren, 2000).

The Fu kinase is a crucial component of the *Drosophila* Hh pathway as *fu* mutations affect Hh target gene expression (Alves et al., 1998). Genetic studies suggest that Fu is required for high levels of Hh to convert Ci<sup>F</sup> to Ci<sup>A</sup> by antagonizing Sufu (Ohlmeyer and Kalderon, 1998). Fu consists of an N-terminal Ser/Thr kinase domain and a C-terminal regulatory domain that binds Cos2 and Sufu (Robbins et al., 1997; Monnier et al., 1998). Fu is required for Hh-induced phosphorylation of Cos2 and Sufu (Nybakken et al., 2002; Lum et al., 2003b; Dussillol-Godar et al., 2006; Ruel et al., 2007). In addition, Fu itself undergoes phosphorylation in response to Hh (Therond et al., 1996; Lum et al., 2003b); however, the biological relevance of these phosphorylation events and the mechanism by which Fu is activated remain an enigma.

In response to Hh, the seven-transmembrane protein and Hh signal transducer Smoothed (Smo) is hyperphosphorylated by multiple kinases, including PKA, CK1 and Gprk2 (Deneff et al., 2000; Jia et al., 2004; Zhang et al., 2004; Apionishev et al., 2005; Chen et al., 2010; Cheng et al., 2010). Phosphorylation promotes Smo cell surface accumulation and active conformation in a dose-dependent manner, leading to dimerization/oligomerization of its C tails (Jia et al., 2004; Zhao et al., 2007; Chen et al., 2010). Smo interacts with Cos2-Fu complex through its C tail as well as activates G $\alpha$ i to transduce signal (Hooper, 2003; Jia et al., 2003; Lum et al., 2003b; Ogden et al., 2003; Ruel et al., 2003; Ogden et al., 2008). Interaction

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between Smo and Cos2/Fu is mediated by at least two regions in the Smo C tail: a membrane proximal domain (amino acids 651-686) and a C-terminal domain (amino acids 818-1035) (Jia et al., 2003; Lum et al., 2003b). Although the C-terminal Cos2/Fu binding domain is essential for Smo activity (Jia et al., 2003), the membrane proximal Cos2-binding domain mediates an inhibition of Smo phosphorylation by Cos2/PP4, which is alleviated by Fu kinase activity (Claret et al., 2007; Liu et al., 2007; Jia et al., 2009). Because the association between Smo and Cos2/Fu is observed even in the absence of Hh, complex formation per se is insufficient for pathway activation. It is possible that Smo and Cos2/Fu form distinct complexes depending on the status of Hh signaling, and pathway activation may rely on changes in the location, composition and conformational state of the complexes.

In this study, we investigate how Hh signaling alters the state of Smo-Cos2-Fu complex to activate Fu and how activated Fu leads to a change in Ci activity. We provide evidence that Hh-induced Smo conformational switch assembles active Smo-Cos2-Fu signaling complexes that promote Fu kinase domain dimerization and activation loop phosphorylation, leading to Fu activation, and that activated Fu regulates both Ci<sup>A</sup> and Ci<sup>R</sup> by interfering with Ci-Sufu and Ci-Cos2-kinase complex formation.

## MATERIALS AND METHODS

### Mutations and transgenes

*smo<sup>2</sup>* and *cos2<sup>2</sup>* are null or strong allele and have been described previously (Grau and Simpson, 1987; Chen and Struhl, 1998). *UAS-Ci<sup>U</sup>*, *UAS-Ci*, *UAS-Sufu*, *dpp-lacZ* and *hh-lacZ* have been described previously (Methot and Basler, 1999; Wang et al., 2000). *MS1096*, *C765* and *ap-Gal4* drivers have been described previously (Wang et al., 1999; Chen et al., 2010). Smo SA, SD and RA mutants have been described previously (Jia et al., 2004; Zhao et al., 2007). Cos2-YFP and CFP/YFP-Fu contain CFP or YFP fused to the C or N terminus of Cos2 and Fu, respectively. To generate CC-Fu and CCm-Fu, peptides corresponding to the wild-type or mutant leucine zipper of the yeast GCN4 followed by a flexible linker (GSSG) were generated by multi-step PCR and subcloned between *Bgl*III and *Not*I sites of *pUAST-Myc* and *pUAST-HA* (Tong and Jiang, 2007). For generating flies with transgenes inserted at the 75B1 *attP* locus (Bischof et al., 2007), the coding regions for HA-tagged Fu variants were subcloned between *Eco*RI and *Xba*I sites of a modified *pUAST* vector with an *attB* sequence inserted upstream of the *UAS*-binding sites (Liu et al., 2007). CFP-Ci<sup>PKA</sup> and Sufu-YFP contain CFP or YFP fused to their N or C terminus, respectively.

### Cell culture, transfection, immunostaining, immunoprecipitation and western blot analysis

*Drosophila* S2 cells were cultured in *Drosophila* SFM (Invitrogen) with 10% fetal bovine serum, 100 U/ml of penicillin and 100 mg/ml of streptomycin at 24°C. Transfection was carried out by Calcium Phosphate Transfection Kit (Specialty Media). Hh-conditioned medium treatment was carried out as described previously (Lum et al., 2003a). Unless mentioned otherwise, Hh-conditioned medium was used at a 6:4 dilution ratio by fresh medium (referred to as 100% Hh). Immunostaining, immunoprecipitation and western blot analyses were carried out using standard protocols as previously described (Zhang et al., 2005). Images were captured by confocal microscopy and signals were quantified by ImageJ software. Phospho-Fu antibodies were generated by Genemed Synthesis (San Antonio, TX) with the following phospho-peptides as antigens: CDFGLARNMT(p)LGT(p)HVL (for pT151/pT154) and HVLT(p)S(p)IKGTPLYMAPE (for pT158/pS159). Phospho-antibodies were purified by positive and negative selections using the phosphopeptides and non-phosphopeptides affinity columns, respectively. Other antibodies used for this study are rat anti-Ci, 2A1 (Motzny and Holmgren, 1995), mouse anti-Ptc (DSHB), mouse anti-En (DSHB), mouse anti-HA (Santa Cruz), rabbit anti-*lacZ* (Affinity Bioreagents), mouse anti-*lacZ* (Sigma) and rabbit anti-GFP (Invitrogen).

### Luciferase assay and RNAi in *Drosophila* S2 cells

For *ptc-luc* reporter assays, S2 cells were transfected with 1 µg *ptc-luc* reporter construct and 50 ng RL-PolIII renilla construct in 12-well plates together with 0.5 µg Ci, 0.25 µg Sufu and 0.5 µg Fu constructs. After 48 hours incubation, the reporter assays were performed using the Dual-Luciferase reporter assay system (Promega). Dual-Luciferase measurements were performed in triplicate using FLUOstar OPTIMA (BMG LABTECH).

Double-stranded (ds) RNA was generated by MEGAscript High Yield Transcription Kit (Ambion). dsRNA targeting Fu or Cos2 was generated according to Lum et al. (Lum et al., 2003a). dsRNA targeting the Firefly Luciferase coding sequence was used as a control. For the RNAi knockdown experiments, S2 cells were cultured in serum-free medium containing the indicated dsRNA for 8 hours at 24°C. After adding fetal bovine serum to a final concentration of 10%, dsRNA-treated cells were cultured for 24 hours before transfection. Forty-eight hours after transfection, the cells were collected for analyses.

### FRET analysis

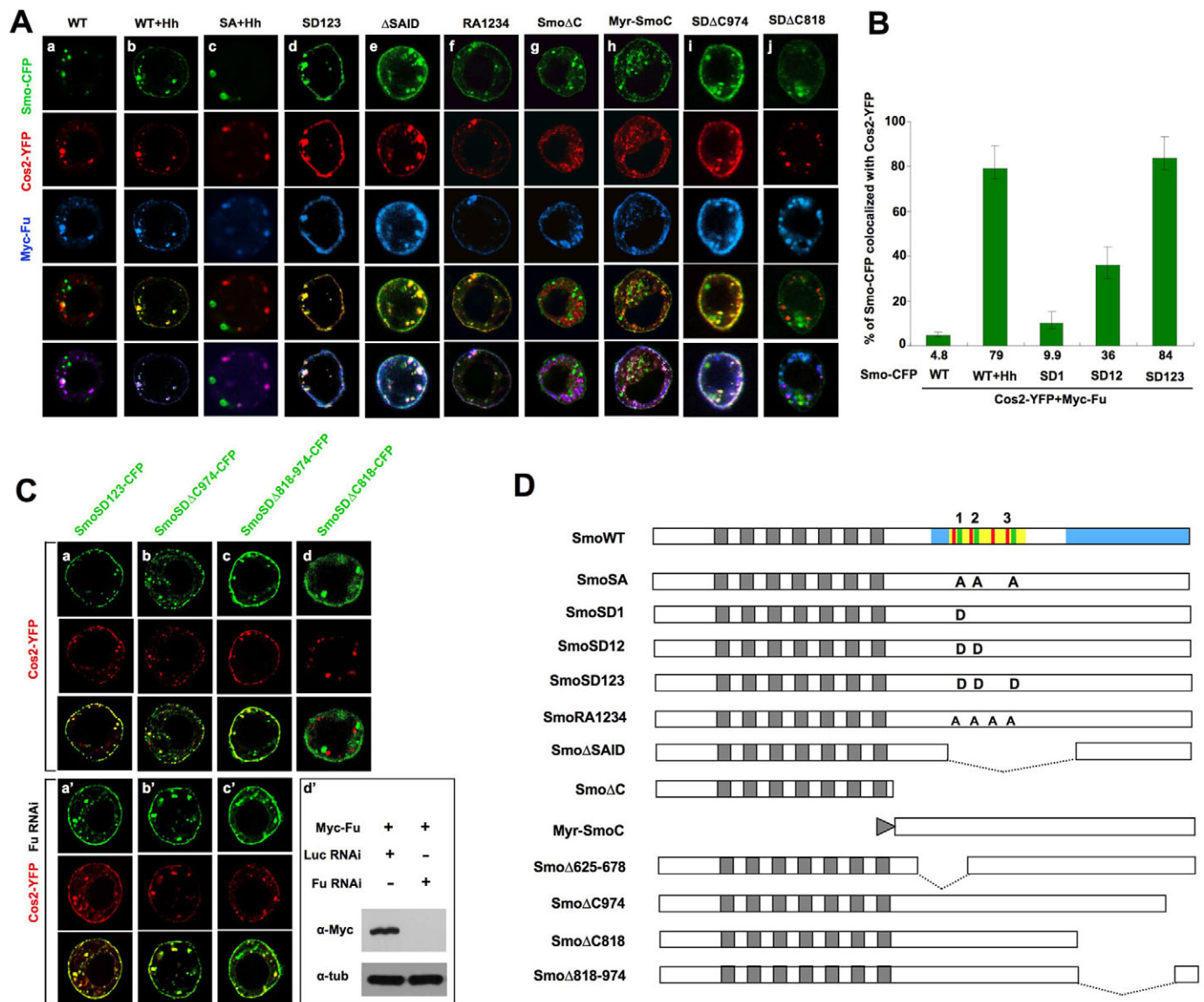
FRET analysis was carried out as previously described (Zhao et al., 2007). CFP/YFP-tagged constructs were transfected into S2 cells, together with an *ub-Gal4* expression vector. Cells were washed with PBS, fixed with 4% formaldehyde for 20 minutes and mounted on slides in 80% glycerol. CFP signals were acquired with 100× objective of Zeiss LSM510 confocal microscope before (BP) and after (AP) photobleaching YFP. Each data set was calculated using 10-20 individual cells. In each cell, four or five regions of interest in photobleached area were selected for analysis. The intensities of CFP signals were quantified by ImageJ software. The FRET efficiency was calculated using the formula: FRET% = [(CFP<sub>AP</sub> - CFP<sub>BP</sub>) / CFP<sub>AP</sub>] × 100. Of note, only CFP signals that colocalized with YFP signals (both membrane and intracellular) were selected for calculation. For FRET analyses in wing discs, CFP/YFP-tagged *UAS* transgenes were expressed using *MS1096*. CFP signals were acquired with 63× objective of Zeiss LSM510 confocal microscope before (BP) and after (AP) photobleaching YFP.

## RESULTS

### Hh-induced Smo conformational change facilitates its association with Cos2/Fu

To investigate how Hh-induced Smo phosphorylation affects its interaction with Cos2/Fu, we examined colocalization between Smo and Cos2/Fu in response to Hh stimulation. S2 cells were transfected with a Smo-CFP, Smo variants with three PKA sites mutated to Ala (SmoSA-CFP in Fig. 1D) or with one, two or three PKA/CKI phosphorylation clusters converted to Asp (SmoSD1-CFP, SmoSD12-CFP or SmoSD123-CFP in Fig. 1D), together with Cos2-YFP and Myc-Fu. In the absence of Hh, Smo-CFP exhibited limited colocalization with Cos2-YFP, whereas Cos2-YFP and Myc-Fu colocalized in puncta (Fig. 1A,B). Upon Hh stimulation, Smo-CFP colocalized well with Cos2-YFP, and a fraction of Cos2-YFP and Myc-Fu accumulated at the plasma membrane, probably through binding to Smo-CFP (Fig. 1Ab,B). The SA mutation blocked Hh-induced Smo/Cos2/Fu colocalization, whereas the SD123 mutation promoted Smo/Cos2/Fu colocalization even in the absence of Hh (Fig. 1Ac,d,B). Interestingly, increasing the number of phospho-mimetic mutations resulted in a progressive increase in Smo/Cos2/Fu colocalization (Fig. 1B; see Fig. S1A in the supplementary material).

To determine whether increased Smo/Cos2/Fu colocalization is due to increased Smo/Cos2 interaction, we examined FRET between Smo-CFP and Cos2-YFP in S2 cells. Hh markedly increased the FRET between Smo-CFP/Cos2-YFP (Fig. 2A; see Fig. S2A in the supplementary material). The SA mutation blocked the Hh-induced FRET, whereas the SD mutations increased the basal FRET in a dose-dependent manner (Fig. 2A,B). In wing discs

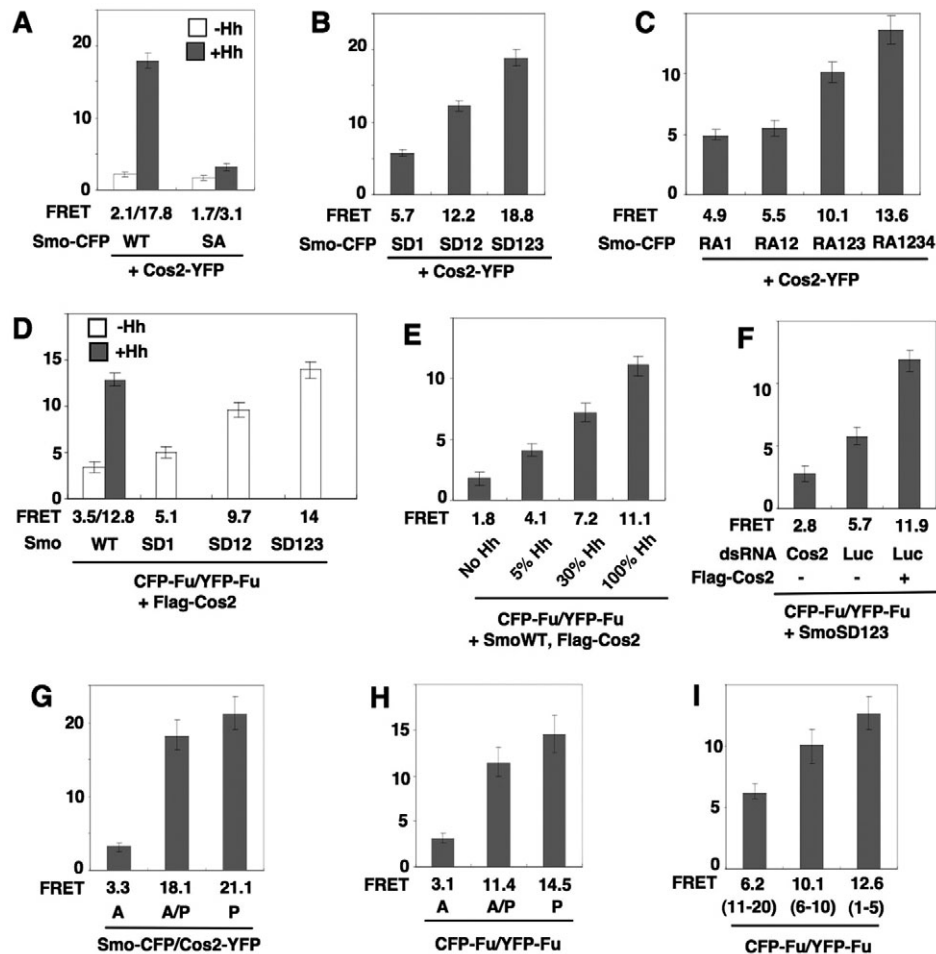


**Fig. 1. Hh-induced Smo phosphorylation and conformational change promote Smo-Cos2-Fu colocalization.** (A) S2 cells transfected with CFP-tagged wild-type (WT) or indicated Smo variants, Cos2-YFP and Myc-Fu, treated with or without Hh-conditioned medium, were immunostained to show the expression of different forms of Smo-CFP (green), Cos2-YFP (red) and Myc-Fu (blue). The fourth and fifth rows are the merged images of CFP/YFP or CFP/YFP/Myc signals, respectively. (B) Percentage of Smo variants colocalized with Cos2-YFP (mean $\pm$ s.d.,  $n \geq 20$ ). Only membrane and intracellular punctate signals were included for quantification. (C) S2 cells were transfected with indicated CFP-tagged Smo variants (green) and Cos2-YFP (red) in the absence (Ca-d) or presence of Fu RNAi (a'-c'). The efficiency of Fu RNAi was evaluated by western blot analysis of transfected Myc-Fu (d'). (D) Schematic drawings of wild-type Smo and Smo variants. Grey boxes indicate Smo transmembrane domains. Blue and yellow regions indicate Cos2/Fu-binding domains and the Smo autoinhibitory domain, respectively. Green and red bars represent the three PKA/CK1 phosphorylation clusters and multiple Arg motifs, respectively. Grey triangle indicates a myristoylation (Myr) signal.

expressing Smo-CFP and Cos2-YFP, FRET was low in anterior (A) compartment cells distant from the AP boundary but increased dramatically in A-compartment cells near the AP boundary or in posterior (P) compartment cells (Fig. 2G; see Fig. S2B,C in the supplementary material). FRET was not significantly affected by varying the levels of transgene expression (see Fig. S2C in the supplementary material).

As Cos2-binding domains in Smo were mapped outside the PKA/CK1 phosphorylation region (Jia et al., 2003; Lum et al., 2003b), phosphorylation may facilitate Smo/Cos2 association by antagonizing multiple Arg motifs located in the Smo autoinhibitory domain (SAID; Fig. 1D) (Zhao et al., 2007) to

promote unfolding of Smo C-tail, which could expose a Cos2/Fu-binding pocket(s). Indeed, deletion of SAID promoted Smo/Cos2/Fu colocalization (Fig. 1Ae). Furthermore, mutating the Arg clusters (RA mutations; Fig. 1D, see Fig. S1B in the supplementary material), which promotes unfolding of Smo C-tail (Zhao et al., 2007), increased Smo/Cos2/Fu colocalization (Fig. 1Af; see Fig. S1B in the supplementary material) as well as FRET between Smo-CFP/Cos2-YFP (Fig. 2C) in a dose-dependent manner similar to phospho-mimetic Smo mutations. Taken together, these results suggest that Hh-induced conformational change mediated by Smo phosphorylation promotes Smo/Cos2/Fu association.



**Fig. 2. FRET analysis of Smo/Cos2 interaction and Fu kinase domain dimerization.** (A-C) FRET efficiency between CFP-tagged wild-type (WT) or indicated Smo variants and Cos2-YFP in S2 cells co-transfected with Myc-Fu, and treated with or without Hh-conditioned medium (mean $\pm$ s.d.,  $n\geq 10$ ). (D) FRET efficiency between CFP-Fu and YFP-Fu in S2 cells co-transfected with Flag-Cos2 and indicated Smo variants, and treated with or without Hh-conditioned medium (mean $\pm$ s.d.,  $n\geq 10$ ). (E,F) FRET efficiency between CFP-Fu and YFP-Fu co-transfected with Flag-Cos2 into S2 cells stably expressing Myc-Smo and treated without or with 5%, 30% and 100% of the Hh-conditioned medium (E) or co-transfected with Flag-SmoSD123 into S2 cells with or without Flag-Cos2, and treated with dsRNA targeting Cos2 or Luciferase (Luc) as control (F) (mean $\pm$ s.d.,  $n\geq 10$ ). (G,H) FRET efficiency between Smo-CFP/Cos2-YFP (G) or CFP-Fu/YFP-Fu (H) expressed in wing discs using *MS1096* at 25°C (mean $\pm$ s.d.,  $n\geq 5$ ). A, A compartment (>30 cells from the AP boundary); P, P-compartment cells; A/P, A compartment (1-10 cells from the AP boundary). (I) FRET efficiency between CFP-Fu and YFP-Fu in A compartment 11-20, 6-10 or 1-5 cells from the AP boundary (mean $\pm$ s.d.,  $n\geq 5$ ).

### Cos2 recruits Fu to the C-terminal domain of activated Smo

A truncated Smo lacking its C-tail (Smo $\Delta$ C; Fig. 1D) failed to colocalize with Cos2/Fu, whereas a membrane-tethered Smo C-tail (Myr-SmoC; Fig. 1D) colocalized well with Cos2/Fu (Fig. 1Ag,h). Deleting the membrane proximal Cos2-binding domain (Smo $\Delta$ 625-678; Fig. 1D) did not affect Hh-induced Smo/Cos2/Fu colocalization (see Fig. S1C in the supplementary material). By contrast, deleting the C-terminal Cos2-binding domain (Smo $\Delta$ C818; Fig. 1D) abolished Smo/Cos2/Fu colocalization (see Fig. S1C in the supplementary material). Although the last 59 amino acids of Smo C-tails can directly bind Fu (Malpel et al., 2007), deleting this domain (Smo $\Delta$ C974; Fig. 1D) did not affect Hh-induced Smo/Cos2/Fu colocalization (see Fig. S1C in the supplementary material). Similar results were obtained by making deletions in SmoSD123 (SmoSD $\Delta$ C818 and SmoSD $\Delta$ C974; Fig. 1Ai,j), suggesting that amino acids 818-974 mediate the binding of Cos2/Fu to activated Smo.

We also found that SmoSD123-CFP and SmoSD $\Delta$ C974-CFP but not SmoSD $\Delta$ C818-CFP colocalized with Cos2-YFP without Fu co-transfected (Fig. 1Ca,b,d) and even when Fu was inhibited by RNAi (Fig. 1Ca',b',d'; see Fig. S1D in the supplementary material), indicating that Cos2 interacts with Smo C-terminal region independent of Fu. Moreover, SmoSD $\Delta$ C974-CFP did not colocalize with YFP-Fu in the absence of Cos2 co-transfection (data not shown). The observation that co-transfection of Cos2 allowed Fu to colocalize with SmoSD $\Delta$ C974 (Fig. 1Ai) suggests

that Cos2 can recruit Fu to SmoSD $\Delta$ C974. We also found that SmoSD $\Delta$ 818-974-CFP still colocalized with Cos2-YFP (Fig. 1Cc,d), which was not affected by Fu RNAi (Fig. 1Cc'). Thus, two non-overlapping domains in the Smo C-terminal region (amino acids 818-974 and 975-1035) may mediate interaction between Cos2 and activated Smo.

### Hh signaling induces Fu kinase domain dimerization

As clustering of Smo C-tails triggered Fu phosphorylation and Hh pathway activation (Zhao et al., 2007), we hypothesized that Hh-induced close proximity of Smo C-tails may facilitate the interaction among Smo-bound Cos2/Fu complexes, leading to Fu kinase domain dimerization and activation. We therefore measured FRET between N-terminally CFP- and YFP-tagged Fu (CFP-Fu/YFP-Fu) in S2 cells and found that Hh increased the FRET between them in a dose-dependent manner (Fig. 2D,E; see Fig. S2D in the supplementary material). Furthermore, co-expression of phospho-mimetic Smo variants increased the basal FRET between CFP-Fu/YFP-Fu in a manner depending on the number of SD mutations (Fig. 2D). These results suggest that Hh signaling induces Fu kinase domain dimerization/oligomerization. For simplicity, we used dimerization throughout the rest of the text.

To determine whether Hh signaling-induced Fu dimerization depends on Cos2, we co-transfected CFP/YFP-Fu and a Flag-tagged SmoSD123 into S2 cells with or without Flag-Cos2 or with endogenous Cos2 knocked down. As shown in Fig. 2F, FRET

between CFP-Fu/YFP-Fu was promoted by Flag-Cos2 but suppressed by Cos2 RNAi, suggesting that Cos2 is required for activated Smo to induce Fu kinase domain dimerization.

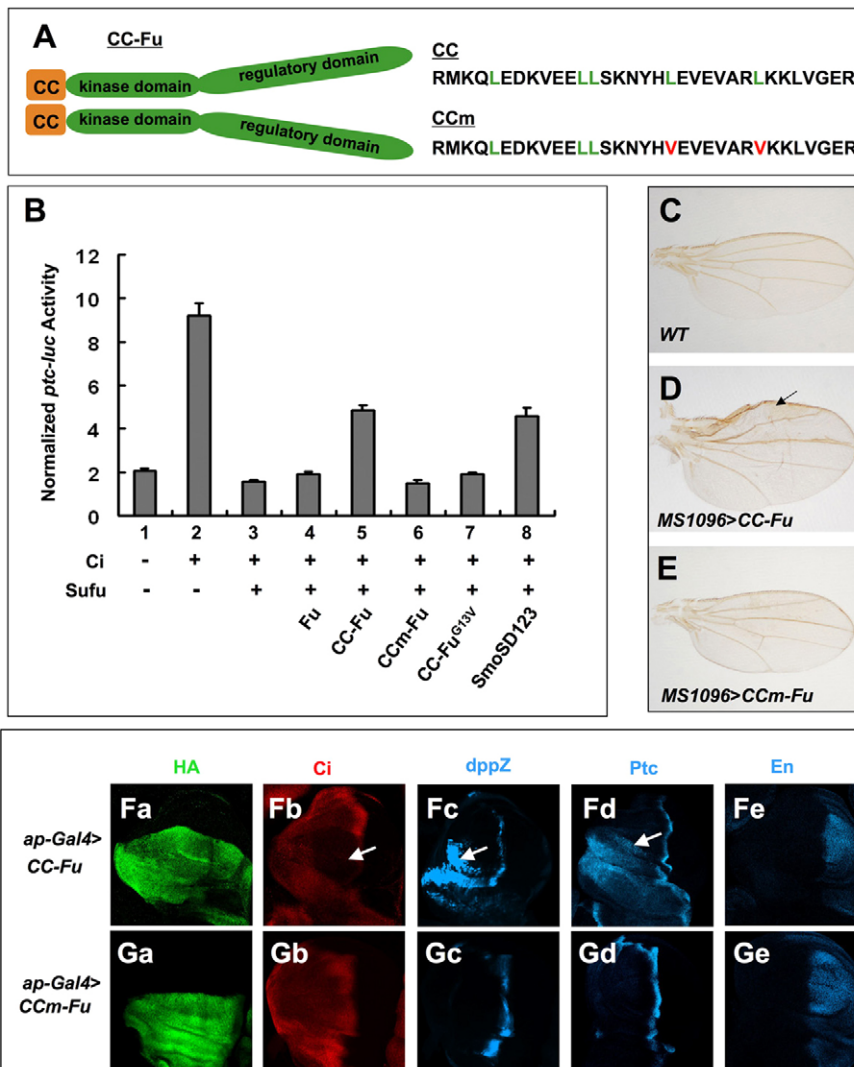
To confirm that Hh signaling induces Fu dimerization *in vivo*, CFP-Fu and YFP-Fu were expressed in wing discs using *MS1096*, and FRET was measured in different regions along the AP axis. We found that FRET between CFP-Fu/YFP-Fu was low in A-compartment cells distant from the AP boundary but increased markedly in A-compartment cells near the AP boundary or in P-compartment cells (Fig. 2H; see Fig. S2E in the supplementary material). Furthermore, FRET between CFP-Fu/YFP-Fu increased progressively in A-compartment cells located closer to the AP boundary (Fig. 2I), suggesting that graded Hh signals induce a progressive increase in the proximity between Fu kinase domains *in vivo*.

### Dimerization through Fu kinase domain activates Hh pathway

To determine whether Fu dimerization triggers its activation, we fused a coiled-coil dimerization motif from yeast GCN4 (referred to as CC) or its dimerization-deficient version (CCm) to the N terminus of Fu (Fig. 3A) (O'Shea et al., 1991). We established a *ptc-luciferase* (*ptc-luc*) reporter assay based on the premise that

activated Fu releases the inhibition of Ci by Sufu (Ohlmeyer and Kalderon, 1998). Overexpression of Ci in S2 cells activated the *ptc-luc* reporter gene, which was suppressed by co-expression of Sufu (Fig. 3B, columns 1-3). Co-expression of a wild-type Fu or CCm-Fu did not release the suppression (Fig. 3B, columns 4 and 6). By contrast, CC-Fu derepressed Ci in a similar way as SmoSD123 did (Fig. 3B, columns 5 and 8). Fusion of CC to a kinase dead Fu variant (Fu<sup>G13V</sup>) (Liu et al., 2007) failed to derepress Ci (Fig. 3B, column 7), indicating that dimerization activated Fu depending on its kinase activity.

To determine whether dimerization of Fu activates the Hh pathway *in vivo*, *UAS* transgenes expressing either CC-Fu or CCm-Fu were introduced into the 75B1 locus using the *phiC31* integration system to ensure similar levels of transgene expression (Bischof et al., 2007). Expression of CC-Fu but not CCm-Fu in wing resulted in anterior overgrowth indicative of Hh pathway activation (Fig. 3C-E). When expressed using a dorsal compartment-specific Gal4 driver *ap-Gal4*, CC-Fu but not CCm-Fu induced ectopic expression of *dpp* and *ptc* in anterodorsal cells (Fig. 3F,c,d and 3Gc,d). We also noticed that CC-Fu but not CCm-Fu dramatically reduced the level of Ci<sup>F</sup> (Fig. 3Fb and 3Gb), suggesting that CC-Fu may convert Ci<sup>F</sup> into labile Ci<sup>A</sup>. Consistent with activated Fu converting Ci<sup>F</sup> into Ci<sup>A</sup> by antagonizing Sufu



**Fig. 3. Fu kinase domain dimerization activates the Hh pathway.** (A) Left, a diagram of dimerized CC-Fu; right, amino acid sequence of CC and CCm, with green letters indicating the Leu residues essential for dimerization and red letters indicating Leu to Val substitutions that demolish the dimerization. (B) A *ptc-luc* reporter assay in S2 cells for the ability of Fu variants and SmoSD123 to release the inhibition of Ci by Sufu. (C-E) A wild-type male wing (C) and male wings expressing *UAS-HA-CC-Fu* (D) or *UAS-HA-CCm-Fu* (E) with *MS1096* at 25°C. Arrow indicates anterior overgrowth. (Fa-Ge) Wing discs expressing *UAS-HA-CC-Fu* (Fa-e) or *UAS-HA-CCm-Fu* (Ga-e) with *ap-Gal4* were immunostained to show the expression of HA, Ci, *dpp-LacZ*, Ptc and En. CC-Fu reduced the level of Ci, and induced ectopic expression of *dpp-LacZ* and Ptc (arrows in Fb-d).

(Ohlmeyer and Kalderon, 1998), co-expression of Sufu with CC-Fu restored Ci level and attenuated the ectopic *ptc* expression (see Fig. S3 in the supplementary material).

### CC-Fu can activate Ci independent of Smo and Cos2

Overexpression of CC-Fu failed to induce ectopic expression of *en* (Fig. 3Fe). One possibility is that CC-Fu may not effectively block Ci processing (see below) so that only a limited amount of Ci<sup>F</sup> is available for conversion into Ci<sup>A</sup>, which is insufficient for *en* activation. To test this hypothesis, we co-expressed a processing-deficient form of Ci (Ci<sup>U</sup>) with CC-Fu to boost the supply of Ci<sup>F</sup>. Misexpression of Ci<sup>U</sup> alone did not induce ectopic expression of Hh target genes in A-compartment cells (Fig. 4A-A'') (Methot and Basler, 1999; Wang et al., 1999). Co-expression of CC-Fu but not CCm-Fu with Ci<sup>U</sup> induced ectopic expression of both *ptc* and *en*, and promoted nuclear localization of Ci<sup>U</sup> in A-compartment cells (Fig. 4B-C''; see Fig. S4 in the supplementary material), indicating that Ci<sup>U</sup> was converted into Ci<sup>A</sup> by CC-Fu.

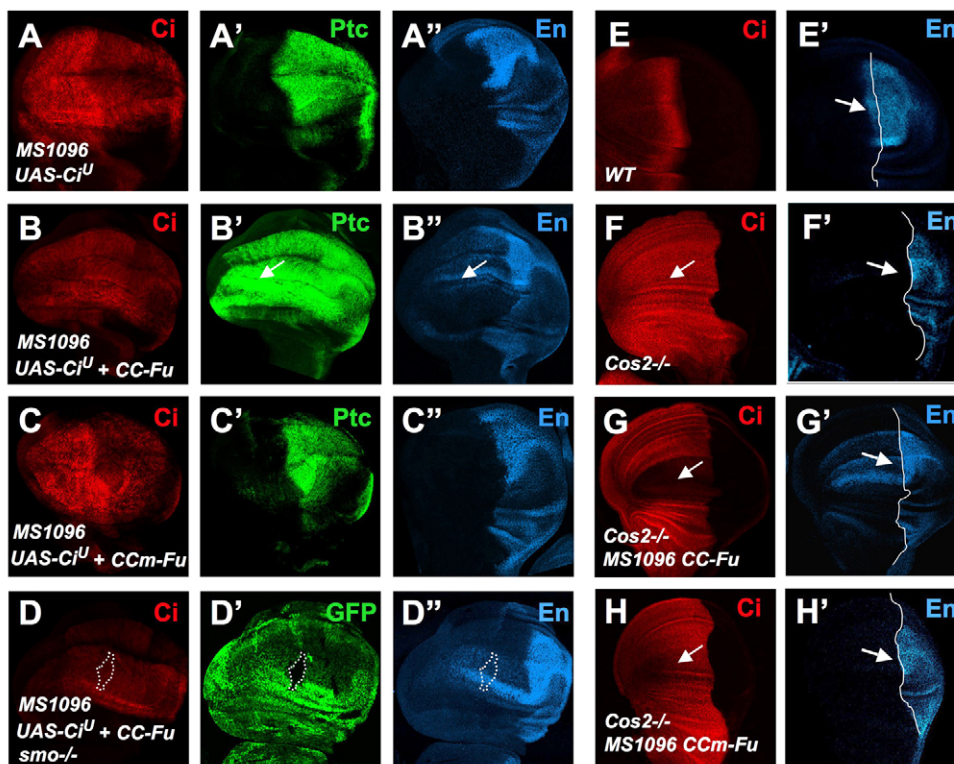
If Smo activates Fu by inducing its dimerization, one would predict that CC-Fu should activate Ci in the absence of Smo. Indeed, anteriorly situated *smo* mutant cells expressing CC-Fu and Ci<sup>U</sup> still activated *en* (Fig. 4D-D''). Cos2 is required for high levels of Hh signaling because Hh-dependent *en* expression is lost in *cos2* mutant discs (arrow in Fig. 4F') (Wang et al., 2000). If the positive role of Cos2 is due to its requirement for Hh-induced Fu dimerization, one would expect that dimerized Fu should activate Ci in the absence of Cos2. Indeed, expressing CC-Fu but not CCm-Fu in *cos2* mutant wing discs rescued the anterior *en* expression near the AP boundary (arrows in Fig. 4G',H') and induced ectopic *en* expression in distal A-compartment cells (Fig. 4G'). In addition, CC-Fu markedly reduced the level of Ci<sup>F</sup> normally accumulated in *cos2* mutant discs (arrows in Fig. 4F,G), consistent with CC-Fu converting Ci<sup>F</sup> into labile Ci<sup>A</sup>.

### Dimerization and Hh signaling induce Fu activation loop phosphorylation

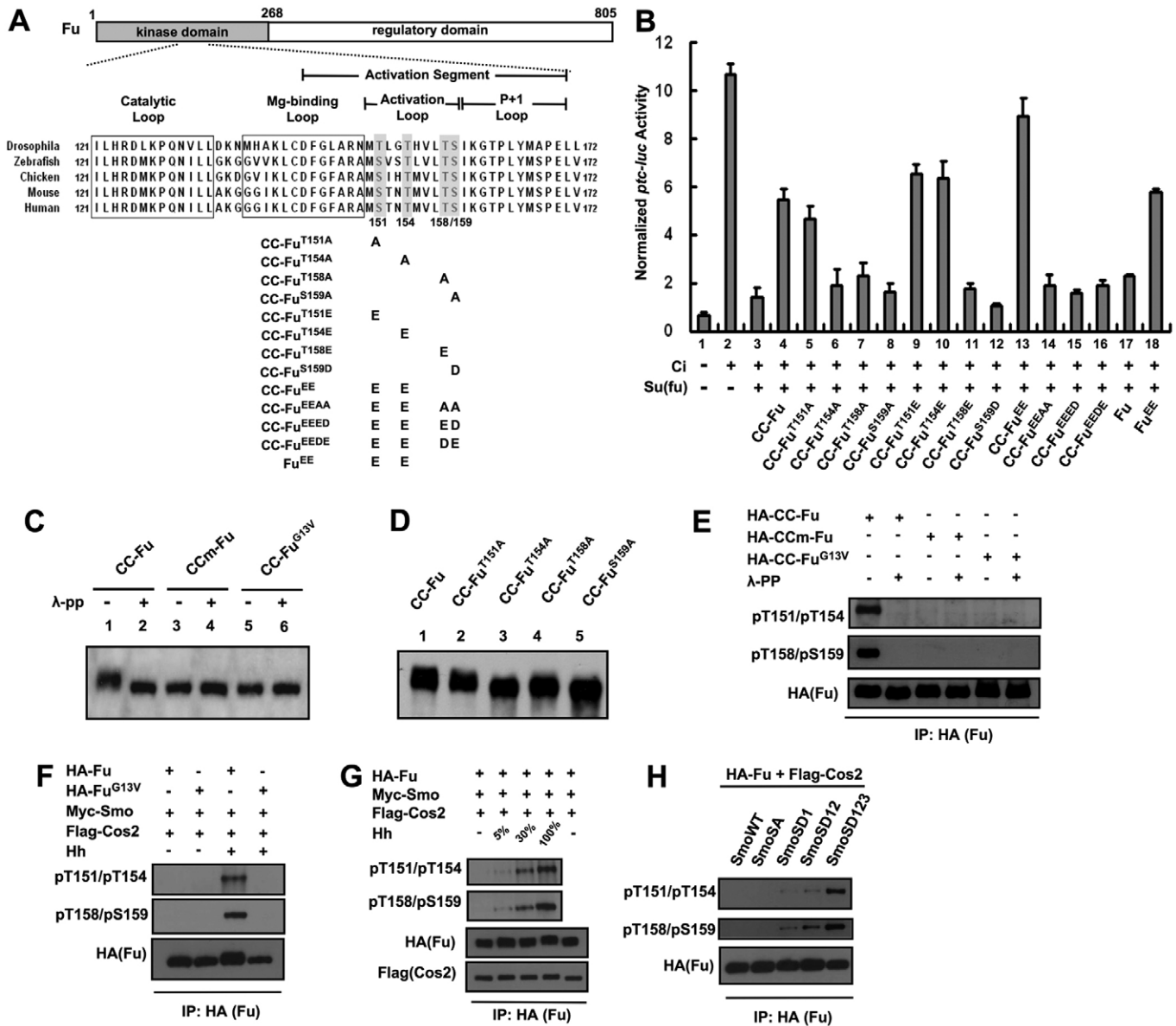
We next determined whether CC-mediated dimerization could induce Fu phosphorylation. When expressed in S2 cells, CC-Fu but not CCm-Fu or CC-Fu<sup>G13V</sup> exhibited a mobility shift that was abolished by phosphatase treatment (Fig. 5C), suggesting that dimerization induced Fu phosphorylation depending on Fu kinase activity. Furthermore, Hh induced mobility shift of HA-Fu but not kinase-dead HA-Fu<sup>G13V</sup> and HA-Fu<sup>K33R</sup> (see Fig. S5A in the supplementary material), suggesting that Hh-induced Fu phosphorylation also depends on Fu kinase activity.

Many kinases are activated by dimerization-induced autophosphorylation of their activation loop residues (Nolen et al., 2004; Pike et al., 2008). The Fu activation loop contains four Ser/Thr residues (T151, T154, T158 and S159) that are conserved among different species (Fig. 5A). To determine whether phosphorylation of any of these residues is required for dimerization-induced Fu activation, we mutated individual Ser/Thr residue to Ala (A) in CC-Fu (Fig. 5A). We found that T154A, T158A and S159A mutations nearly abolished, whereas T151A slightly reduced, CC-Fu activity (Fig. 5B, columns 5-8). Consistently, T154A, T158A and S159A mutations nearly abolished, whereas T151A slightly reduced, CC-Fu mobility shift (Fig. 5D), suggesting that T154, T158 and S159 are crucial for dimerization-induced Fu phosphorylation and activation.

We also substituted the activation loop Ser/Thr residues individually or in combination to acid residues Glu (E) or Asp (D) to mimic phosphorylation. T151E or T154E slightly enhanced the activity of CC-Fu, whereas T151E/T154E (CC-Fu<sup>EE</sup>) resulted in a more dramatic enhancement (Fig. 5B, columns 9, 10 and 13), suggesting that phosphorylation at T151 and T154 promotes Fu activation. However, T158E or S159D abolished the activity of CC-Fu (Fig. 5B, columns 11 and 12). Moreover, mutating these two residues either to A or D/E in the context of CC-Fu<sup>EE</sup> also



**Fig. 4. CC-Fu activates Ci independently of Smo and Cos2.** (A-C'') Wing discs expressing UAS-Ci<sup>U</sup> alone (A-A'') or together with UAS-HA-CC-Fu (B-B'') or UAS-HA-CCm-Fu (C-C'') using MS1096 were immunostained to show the expression of Ci, Ptc and En. (D-D'') A wing disc carrying anteriorly situated *smo*<sup>2</sup> mutant clones (marked by the lack of GFP and outlined by broken lines) and expressing Ci<sup>U</sup> and CC-Fu with MS1096 was immunostained to show the expression of Ci, GFP and En. (E-H') A wild-type wing disc (E,E') and *cos2*<sup>2</sup> mutant wing discs without (F,F') UAS-HA-CC-Fu, or with UAS-HA-CC-Fu (G,G') or UAS-HA-CCm-Fu (H,H') expressed by MS1096 were immunostained to show the expression of Ci and En. Arrows indicate Ci accumulation (F-H) and anterior En expression (E'-H').



**Fig. 5. Dimerization and Hh signaling induce Fu activation loop phosphorylation.** (A) A schematic drawing of Fu with a structure-based sequence alignment of the activation segments of Fu proteins from different species. Grey bars indicate the conserved Ser/Thr residues in the activation loop. Amino acid substitutions for CC-Fu and Fu variants are listed. (B) A *ptc-luc* reporter assay in S2 cells for the ability of indicated Fu variants to derepress Ci. Data are mean $\pm$ s.d. (C,D) Western blot analyses of Myc-CC-Fu and its derivatives expressed in S2 cells. Cell extracts were treated without or with  $\lambda$ -phosphatase ( $\lambda$ -pp). (E) HA-tagged CC-Fu, CCm-Fu and CC-Fu<sup>G13V</sup> were transfected into S2 cells. Cell lysates were immunoprecipitated with an anti-HA antibody, treated without or with  $\lambda$ -pp and followed by western blot using antibodies against pT151/pT154, pT158/pS159 or HA. (F-H) S2 cells were transfected with the indicated constructs and treated without or with increasing levels of Hh-conditioned medium. Cell extracts were immunoprecipitated with an anti-HA antibody, followed by western blot with the indicated antibodies.

abolished Fu activity (Fig. 5B, columns 14-16), suggesting that the function of T158 and S159 cannot be fulfilled by substitution with acidic residues.

To monitor Fu activation loop phosphorylation, we generated antibodies that specifically recognize phosphorylated T151/T154 (referred to as pT151/pT154) and T158/S159 (referred to as pT158/pS159). Both pT151/pT154 and pT158/pS159 antibodies detected CC-Fu but not CCm-Fu expressed in S2 cells and both signals were abolished by phosphatase treatment (Fig. 5E). pT151/pT154 was not detected with CC-Fu<sup>T151A</sup> and CC-Fu<sup>T154A</sup>,

whereas pT158/pS159 was not detected with CC-Fu<sup>T158A</sup> and CC-Fu<sup>S159A</sup> (see Fig. S5B-C in the supplementary material), confirming the specificity of these antibodies. Furthermore, dimerization of the kinase-dead Fu (CC-Fu<sup>G13V</sup>) failed to induce pT151/pT154 or pT158/pS159 signal (Fig. 5E), suggesting that dimerization-induced Fu activation loop phosphorylation depends on Fu kinase activity. In addition, we found that activated Fu trans-phosphorylated a kinase-dead Fu, as well as Fu C-terminal regulatory domain (see Fig. S5D,E in the supplementary material), suggesting that Fu may auto-phosphorylate both its kinase and regulatory domains.

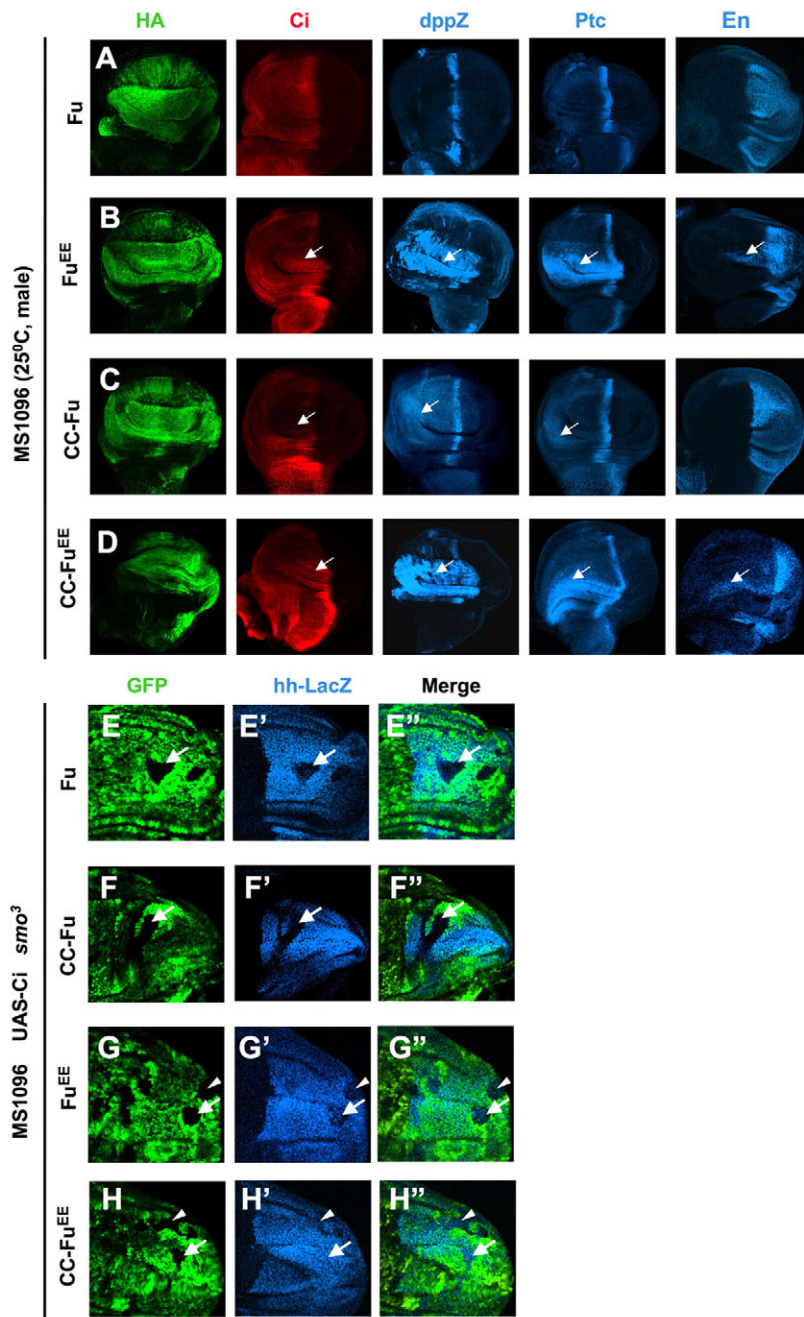
Hh stimulation also induced phosphorylation at T151/T154 and T158/S159 of HA-Fu but not HA-Fu<sup>G13V</sup> (Fig. 5F), suggesting that Hh stimulates phosphorylation of Fu activation loop depending on Fu kinase activity. Interestingly, treatment with increasing levels of Hh or transfection of different phospho-mimetic forms of Smo resulted in a progressive increase in the levels of pT151/pT154 and pT158/pS159 signals (Fig. 5G,H), suggesting that Hh signaling induces Fu activation loop phosphorylation in a dose-dependent manner.

### Fu activation loop phosphorylation triggers Hh pathway activation

To determine whether activation loop phosphorylation triggers Fu activation, we substituted T151 and T154 with E in the context of HA-Fu (HA-Fu<sup>EE</sup>). In the *ptc-luc* assay, HA-Fu<sup>EE</sup> released the

inhibition of Ci by Sufu (Fig. 5B, lane 18), albeit less effectively than HA-CC-Fu<sup>EE</sup>. When expressed in wing discs, HA-Fu did not induce ectopic expression of Hh target genes (Fig. 6A), whereas HA-CC-Fu induced weak ectopic expression of *dpp-lacZ* and *ptc* but not *en* (Fig. 6C). By contrast, both HA-Fu<sup>EE</sup> and HA-CC-Fu<sup>EE</sup> induced strong ectopic expression of *dpp-lacZ* and *ptc*, as well as ectopic *en* expression, which is more evident in the dorsal compartment where *MS1096* exhibits higher levels of expression (Fig. 6B,D), demonstrating that the T151E/T154E mutation activated Fu and Hh pathway.

Although their activities were nearly indistinguishable when expressed at high levels, HA-CC-Fu<sup>EE</sup> appeared to be more active than HA-Fu<sup>EE</sup> when expressed at lower levels (see Fig. S6 in the supplementary material). Furthermore, CC-Fu<sup>EE</sup> exhibited more robust phosphorylation at T158/S159 than did Fu<sup>EE</sup> (see



### Fig. 6. Fu activation loop phosphorylation activates Hh pathway.

(A-D) Wing discs from males expressing *UAS-HA-Fu* (A), *UAS-HA-Fu<sup>EE</sup>* (B), *UAS-HA-CC-Fu* (C) and *UAS-HA-CC-Fu<sup>EE</sup>* (D) with *MS1096* at 25°C were immunostained to show the expression of HA, Ci, *dpp-LacZ*, Ptc or En. Arrows indicate the effect of active forms of Fu on the expression of Ci and Hh target genes. (E-H'') Wing discs carrying *smo<sup>3</sup>* mutant clones (marked by the lack of GFP) and expressing *UAS-Ci* and *hh-lacZ* together with *UAS-HA-Fu* (E-E''), *UAS-HA-CC-Fu* (F-F''), *UAS-HA-Fu<sup>EE</sup>* (G-G'') or *UAS-HA-CC-Fu<sup>EE</sup>* (H-H'') using *MS1096* at 25°C were immunostained to show the expression of GFP and *hh-lacZ*. Arrows indicate dorsal clones; arrowheads indicate ventral clones.



Fig. S6 in the supplementary material), indicating that the level of Fu activity correlates with the level of T158/S159 phosphorylation.

### Activated Fu inhibits Ci<sup>R</sup> production

We noticed that wing discs expressing *MS1096>Fu<sup>EE</sup>* or *MS1096>CC-Fu<sup>EE</sup>* exhibited higher levels of Ci staining than wing discs expressing *MS1096>CC-Fu* (arrows in Fig. 6B-D, column 2). A likely explanation is that Fu<sup>EE</sup> and CC-Fu<sup>EE</sup> but not CC-Fu could effectively block Ci processing. To test this possibility, we applied an in vivo assay for Ci processing. When *UAS-Ci* was misexpressed in wing discs that carry *smo* mutant clones and an *hh-lacZ* reporter gene, the expression of *hh-lacZ* in P-compartment *smo* mutant cells was blocked due to Ci being processed into Ci<sup>R</sup> in these cells (Methot and Basler, 1999; Jia et al., 2005). Co-expression of Fu or CC-Fu did not significantly alleviate the blockage of *hh-lacZ* expression in *smo* mutant cells (arrows in Fig. 6E-F''), indicating that neither Fu nor CC-Fu was able to efficiently block Ci processing. By contrast, co-expression of Fu<sup>EE</sup> partially, whereas CC-Fu<sup>EE</sup> more completely, derepressed *hh-lacZ* expression in posterior *smo* mutant cells (arrows in Fig. 6G-H''). Of note, Fu<sup>EE</sup> or CC-Fu<sup>EE</sup> derepressed *hh-lacZ* expression less effectively in ventrally situated *smo* mutant clones (arrowheads in Fig. 6G-H''), probably owing to lower levels of transgene expression in this region. On the other hand, increasing the expression level of CC-Fu by growing larvae at 30°C rendered partial inhibition of Ci processing (arrows in Fig. S7B-B'' in the supplementary material). These results demonstrate that activated forms of Fu inhibit Ci processing with Fu<sup>EE</sup> and CC-Fu<sup>EE</sup> being more effective than CC-Fu.

### Activated Fu regulates Ci-Sufu and Ci-Cos2-kinase complex formation

When co-expressed in S2 cells, HA-CC-Fu, HA-CC-Fu<sup>EE</sup> or HA-Fu<sup>EE</sup>, but not HA-CC-Fu<sup>G13V</sup> or HA-Fu, induced a mobility shift of Flag-Sufu that was abolished by phosphatase treatment (Fig. 7A), suggesting that Fu activation promotes Sufu phosphorylation. To determine whether activated Fu converts Ci<sup>F</sup> to Ci<sup>A</sup> through attenuating the formation of Ci-Sufu complex, we examined the effect of different forms of Fu on the interaction between Sufu and Ci<sup>-PKA</sup>, a Ci variant with three PKA sites mutated to Ala and thus no longer processed (Wang et al., 1999). Using co-immunoprecipitation assay, we found that HA-CC-Fu, HA-CC-Fu<sup>EE</sup> and HA-Fu<sup>EE</sup> markedly decreased the amount of Flag-Sufu pulled down by Myc-Ci<sup>-PKA</sup> (Fig. 7B, compare lanes 4, 5 and 7 with lane 3), whereas neither HA-CC-Fu<sup>G13V</sup> nor HA-Fu altered the association between Myc-Ci<sup>-PKA</sup> and Flag-Sufu (Fig. 7B, lanes 6 and 8).

We next examined whether Fu activation affects Ci/Sufu association in intact cells. When nuclear export was inhibited by LMB, singly transfected CFP-Ci<sup>-PKA</sup> and Sufu-YFP exhibited nuclear and cytoplasmic staining, respectively (see Fig. S8 in the supplementary material). When co-transfected, CFP-Ci<sup>-PKA</sup> and Sufu-YFP colocalized in cytoplasmic puncta (Fig. 7C) and exhibited a significant FRET (Fig. 7D). CC-Fu, Fu<sup>EE</sup> and CC-Fu<sup>EE</sup> but not CC-Fu<sup>G13V</sup> or Fu disrupted the formation of cytoplasmic Ci/Sufu puncta, induced nuclear translocation of both CFP-Ci<sup>-PKA</sup> and Sufu-YFP (Fig. 7C), and markedly reduced the FRET between them (Fig. 7D), suggesting that activated Fu interferes with Ci-Sufu complex formation. Activated Fu promoted Sufu-YFP nuclear localization in the absence of

CFP-Ci<sup>-PKA</sup> (see Fig. S8 in the supplementary material), suggesting that Fu-induced Sufu-YFP nuclear localization is Ci independent.

Finally, we investigated whether activated Fu regulates the formation of a Ci-Cos2-kinase complex that is essential for Ci phosphorylation and processing (Zhang et al., 2005). We found that CC-Fu, CC-Fu<sup>EE</sup> but not CC-Fu<sup>G13V</sup> decreased the association between Flag-Cos2 and Myc-Ci<sup>-PKA</sup> (Fig. 7E), as well as their association with kinases involved in Ci phosphorylation, including PKA, CK1 and GSK3 (Fig. 7F). In line with CC-Fu being less effective in blocking Ci processing, CC-Fu affected Ci-Cos2-kinase complex formation less effectively than CC-Fu<sup>EE</sup> (Fig. 7E,F). Of note, CC-Fu and CC-Fu<sup>EE</sup>, but not CC-Fu<sup>G13V</sup>, induced mobility shift of Flag-Cos2 (Fig. 7E, second panel from bottom), suggesting that activated Fu promotes Cos2 phosphorylation.

### DISCUSSION

How Hh signal is transduced from the GPCR-like receptor Smo to the transcription factor Ci/Gli is still poorly understood. A major unsolved issue is how a change in the Smo activation state is translated into a change in the activity of intracellular signaling complexes, which ultimately changes the balance between Ci<sup>R</sup>/Gli<sup>R</sup> and Ci<sup>A</sup>/Gli<sup>A</sup>. Our current study suggests that Hh-induced conformational change of Smo exposes a Cos2 docking site(s) near the Smo C terminus that facilitates the assembly of an active Smo-Cos2-Fu complex, and that Smo activates Fu by promoting its kinase domain dimerization and phosphorylation (Fig. 7G). We also provide evidence that graded Hh signals progressively increase Fu kinase domain dimerization and phosphorylation, which may generate a Fu activity gradient, and that activated Fu regulates both Ci<sup>R</sup> and Ci<sup>A</sup> by controlling Ci-Sufu and Ci-Cos2-kinase complex formation.

### Hh induces the formation of active Smo-Cos2-Fu complexes

Previous immunoprecipitation studies have revealed that Smo pulled down Cos2/Fu in both quiescent cells and Hh-stimulated cells (Jia et al., 2003; Lum et al., 2003b; Ogden et al., 2003; Ruel et al., 2003), suggesting that Smo can form a complex with Cos2/Fu even in the absence of Hh. Furthermore, deletion analyses have indicated that both a membrane proximal domain and a C-terminal region of Smo C-tail can mediate the interaction between Smo and Cos2/Fu (Jia et al., 2003; Lum et al., 2003b). Intriguingly, deleting the C-terminal region impaired, whereas deleting the membrane proximal domain potentiated, Smo activity in vivo (Jia et al., 2003; Jia et al., 2009). Further study suggested that the membrane proximal domain recruits Cos2/PP4 to inhibit Smo phosphorylation and cell-surface accumulation, which is released by Fu-mediated phosphorylation of Cos2 Ser572 in response to Hh (Liu et al., 2007; Jia et al., 2009). These observations suggest that Smo-Cos2-Fu interaction is likely to be dynamic and that distinct complexes may exist depending on the Hh signaling status. For example, Cos2 may associate with the membrane proximal region of Smo to inhibit Smo phosphorylation in quiescent cells. Upon Hh stimulation, Cos2/Fu may interact with the C-terminal region of Smo to transduce the Hh signal. In support of this model, we found that Hh stimulated the recruitment of Cos2/Fu to the C-terminal region rather than the membrane proximal region of the Smo C tail (Fig. 1; see Fig. S1 in the supplementary material). The increased binding depends on phosphorylation-induced conformational change of Smo C-tail that may expose the C-terminal Cos2 binding pocket(s).

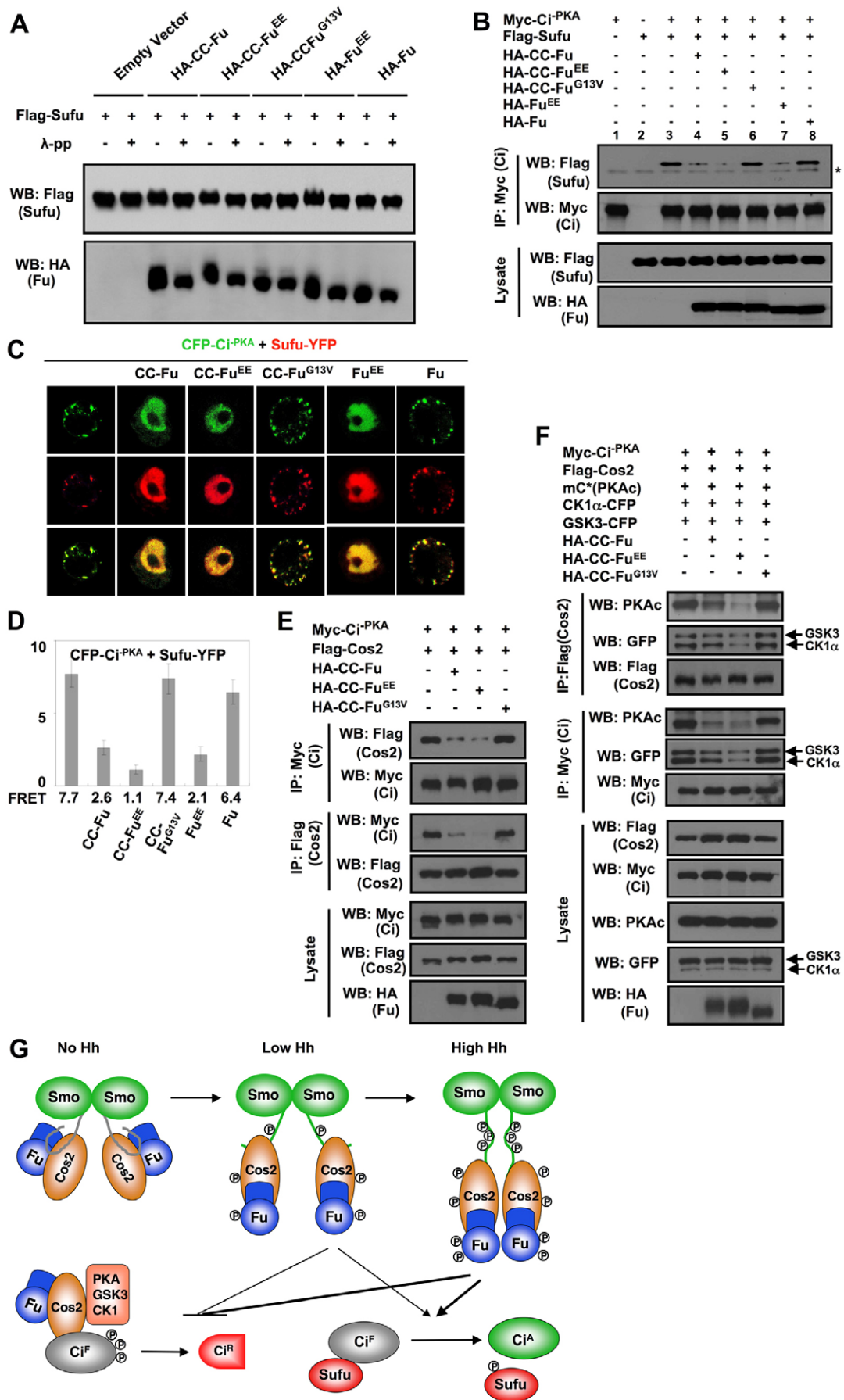


Fig. 7. See next page for legend.

**Fig. 7. Active forms of Fu regulate Ci-Sufu and Ci-Cos2-kinase complex formation.** (A) S2 cells were transfected with Flag-Sufu and indicated Fu variants. Cell extracts were treated without or with  $\lambda$ -phosphatase, followed by western blot with the indicated antibodies. (B) Western blots of immunoprecipitates (top two panels) and lysates (bottom two panels) from S2 cells transfected with indicated constructs and treated with 50  $\mu$ M MG132 for 4 hours before harvesting. The asterisk indicates IgG. (C) S2 cells were transfected with CFP-Ci<sup>PKA</sup> and Sufu-YFP without or with indicated Fu variants, and treated with 5 nM LMB for 2 hours before microscopy. (D) FRET efficiency between CFP-Ci<sup>PKA</sup> and Sufu-YFP expressed in S2 cells in the absence or presence of indicated Fu variants (mean  $\pm$  s.d.,  $n \geq 10$ ). (E,F) S2 cells were transfected with the indicated Ci, Cos2, Fu and kinase constructs. Cell lysates (bottom) or immunoprecipitates (top) were subjected to western blot analysis with the indicated antibodies. (G) A model for Fu activation through Hh-induced Smo conformational switch. In the absence of Hh, Cos2/Fu forms an inactive complex with Smo to inhibit Smo phosphorylation. Cos2 recruits multiple kinases to phosphorylate Ci<sup>F</sup>, leading to Ci processing to Ci<sup>R</sup>. Hh signaling triggers Smo phosphorylation, cell-surface accumulation and conformational change, leading to the assembly of active Smo-Cos2-Fu complexes. Hh-induced clustering of Smo C tails promotes Fu kinase domain dimerization, phosphorylation and activation. Graded Hh signals progressively increase Fu dimerization and phosphorylation, leading to a gradual increase in Fu activity. Activated Fu inhibits Ci processing to Ci<sup>R</sup> and converts Ci<sup>F</sup> to Ci<sup>A</sup> by regulating Ci-Cos2-kinase and Ci-Sufu complex formation. Low levels of Hh may initiate Fu activation by a dimerization-independent mechanism. See text for details.

### Fu activation by kinase domain dimerization and activation loop phosphorylation

We found that Hh signaling induces Fu kinase domain dimerization in a dose-dependent manner, most probably as a consequence of phosphorylation-induced conformational change and dimerization of Smo C tails. In addition, Hh-induced Fu dimerization depends on Cos2. Importantly, dimerization through the Fu kinase domain (CC-Fu) triggers Fu activation both in vitro and in vivo. Furthermore, CC-Fu can activate Ci in *smo* mutant clones and restore high levels of Hh signaling activity in *cos2* mutant discs. Taken together, these results support a model in which Hh-induced Fu dimerization via Smo/Cos2 leads to Fu activation (Fig. 7G).

Both Fu dimerization and Hh stimulation induce phosphorylation of multiple Thr/Ser residues in the Fu activation loop that are important for Fu activation. Fu phosphorylation depends on its kinase activity and Fu can trans-phosphorylate itself, suggesting that Hh and dimerization may induce Fu autophosphorylation, although our results do not exclude the involvement of additional kinase(s). CC-induced dimerization does not fully activate Fu, suggesting that Smo may promote Fu activation through additional mechanisms. Activated Fu can promote phosphorylation of its C-terminal regulatory fragment (see Fig. S5E in the supplementary material), raising a possibility that Fu activation may also involve phosphorylation of its regulatory domain. Indeed, while our manuscript was under review, Zhou and Kalderon provided evidence that phosphorylation of several Ser/Thr residues in the Fu regulatory domain, likely by CK1, modulates the activity of an activated form of Fu (Zhou and Kalderon, 2011).

The involvement of multiple phosphorylation events in Fu activation may provide a mechanism for fine-tuning Fu activity in response to different levels of Hh. Indeed, the efficiency of Fu dimerization and the level of activation loop phosphorylation correlate with the level of Hh signaling. Furthermore, the level of Fu activity correlates with the level of its activation loop phosphorylation (see Fig. S6 in the supplementary material). Thus, graded Hh signals may generate a Fu activity gradient by progressively increasing its dimerization and phosphorylation in response to a gradual increase in Smo phosphorylation and C-tail dimerization (Fig. 7G).

### Regulation of Ci<sup>R</sup> and Ci<sup>A</sup> by activated Fu

The conventional view is that Fu is required for high levels of Hh signaling by converting Ci<sup>F</sup> into Ci<sup>A</sup>. In support of this notion, *fu* mutations only affect the high, but not low, threshold Hh responsive genes (Alves et al., 1998). However, Fu function could have been underestimated because none of the *fu* mutations examined so far represents a null mutation. In addition, the existence of paralleled mechanisms, such as G $\alpha$ i activation (Ogden et al., 2008), could mask the contribution of Fu to low levels of Hh signaling. Nevertheless, a recent study using the phospho-specific antibody against Cos2 Ser572 revealed that Fu kinase activity could be induced by low levels of Hh, raising an interesting possibility that Fu may contribute to all levels of Hh signaling (Raisin et al., 2010). However, the lack of a *fu*-null mutation and the involvement of Fu in promoting Ci processing, probably through a structural role (Lefers et al., 2001), make it difficult to directly demonstrate a role of Fu in blocking Ci processing. Using an in vivo assay for Ci processing, we demonstrated that activated forms of Fu block Ci processing into Ci<sup>R</sup>. In addition, we found that activated Fu attenuates the association between Cos2 and Ci, as well as their association with PKA/CK1/GSK3, probably by phosphorylating Cos2, suggesting that activated Fu may block Ci processing by impeding the formation of the kinase complex required for efficient Ci phosphorylation.

We also provide evidence that activated Fu attenuates Ci/Sufu interaction. Because Sufu impedes Ci nuclear localization and may recruit a co-repressor(s) to further inhibit Ci activity in the nucleus (Methot and Basler, 2000; Wang et al., 2000; Cheng and Bishop, 2002), dissociation of Ci from Sufu may lead to the conversion of Ci<sup>F</sup> to Ci<sup>A</sup>. Interestingly, recent studies using mammalian cultured cells revealed that Shh signaling induces dissociation of full-length Gli proteins from Sufu (Humke et al., 2010; Tukachinsky et al., 2010), suggesting that inhibition of Sufu-Ci/Gli complex formation could be a conserved mechanism for Ci/Gli activation. Although activated forms of Fu promote Sufu phosphorylation, phospho-deficient and phospho-mimetic forms of Sufu behaved in a similar manner to wild-type Sufu in functional assays (Zhou and Kalderon, 2011), implying that phosphorylation of Sufu might not be a major mechanism through which Fu activates Ci. It has been shown that Shh also induces phosphorylation of full-length Gli3 that correlates with its nuclear localization (Humke et al., 2010). Furthermore, a Fu-related kinase Ulk3 can phosphorylate Gli proteins and promote their transcriptional activities (Maloverjan et al., 2010). Thus, Fu may activate Ci by promoting its phosphorylation, an interesting possibility that awaits further investigation.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.067959/-DC1>

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