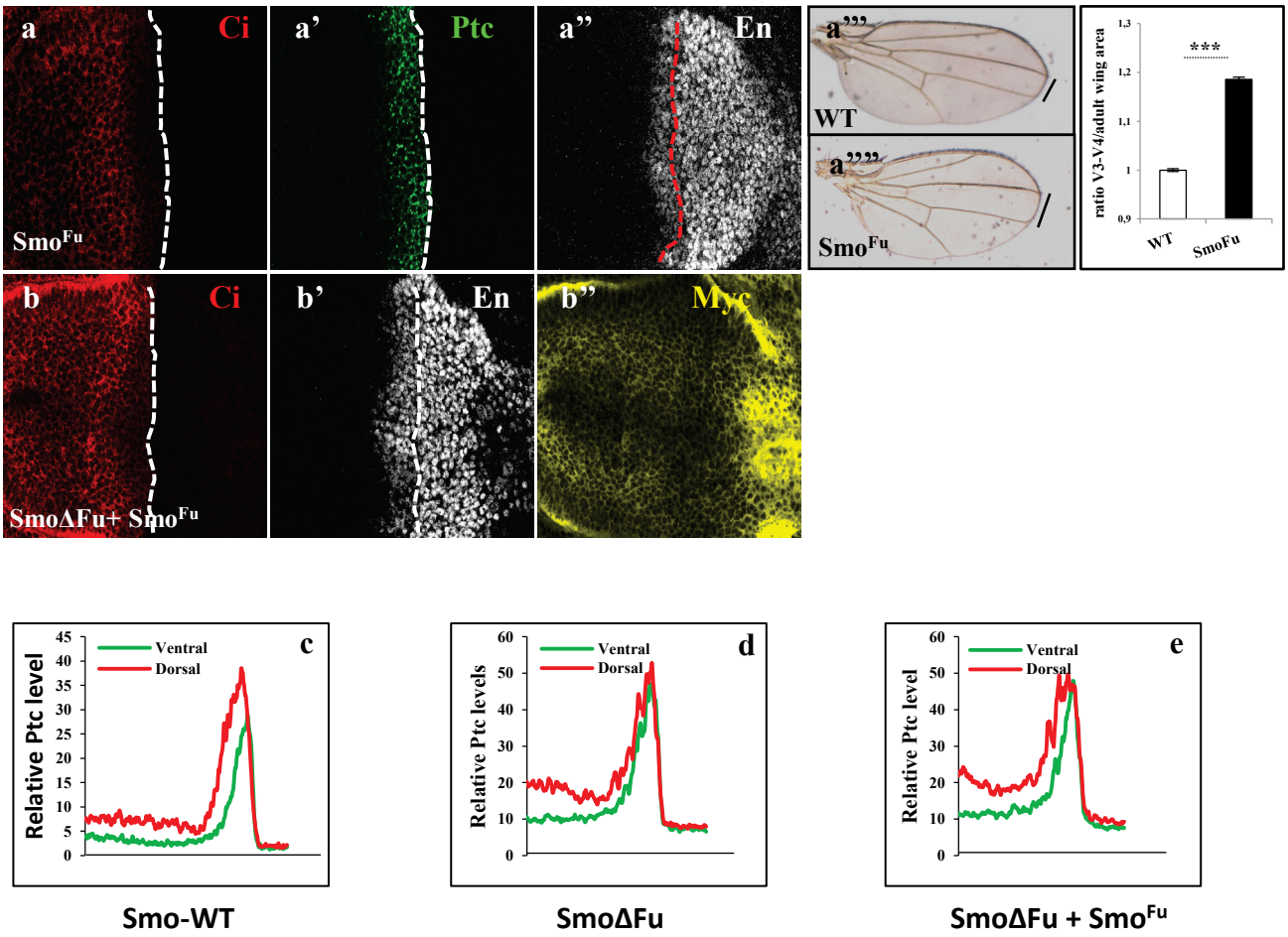
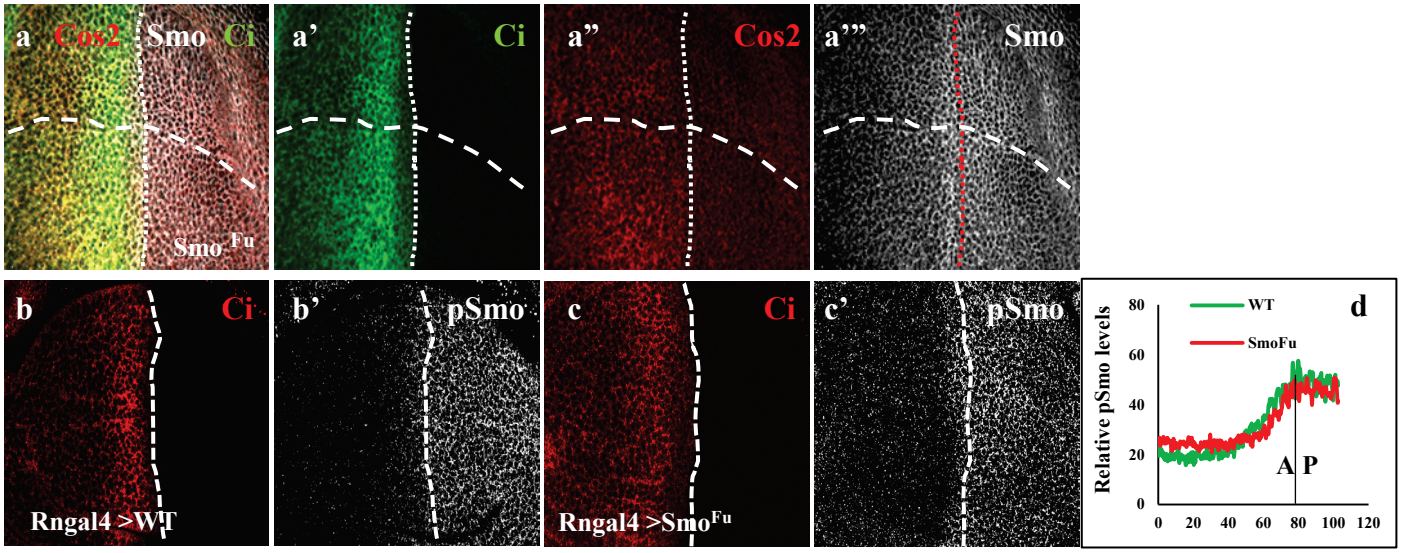
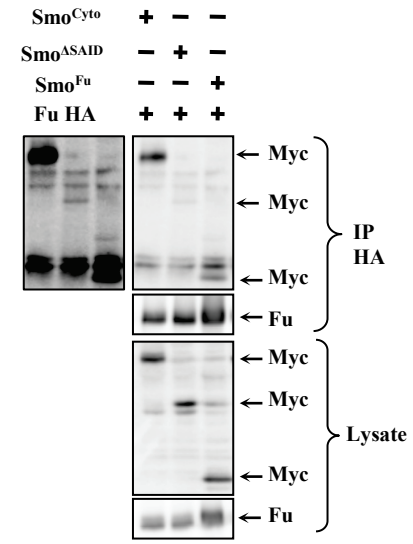


Figure S1. The last 52 amino acids of Smo activate the Hh pathway

(**a**) A wing disc expressing Smo^{Fu}-Myc driven by *rotund*-Gal4 (*rn*gal4) was stained for Ci155 (red, a), Ptc (green, a') and En (grey, a''). Representative examples of adult wings from controls (a''') or animals expressing Smo^{Fu} using *rn*-Gal4 driver (a'''') with quantification of the V3-V4 intervein space on the right panel (means -/+ s.d of 10 wings ***p<0,001). (**b**) Smo^{Fu} and SmoΔFu were expressed in the pouch of the disc by using *rn*gal4 driver. Ci (red, b), En (grey, b') and Myc (yellow, b'') were analysed by immunostaining. (**c-e**). From the figures 1j-l, related quantification graphs (n=4 discs) of Ptc in wing discs expressing Smo-WT (graph c, Fig. 1j), SmoΔFu (graph d, Fig. 1k) or SmoΔFu and Smo^{Fu} (graph e , Fig. 1l).





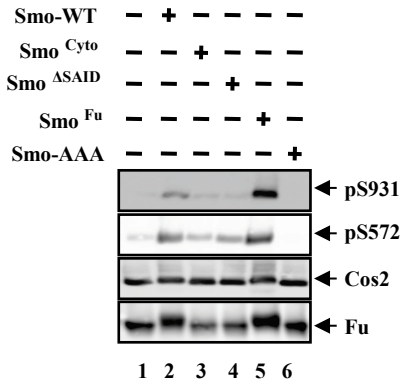


Figure S2. The very last amino acids of Smo promote Fu kinase activity *in vivo*

(**a**) Wing discs expressing Smo^{Fu} driven by *apt*-Gal4 were stained for Cos2 (red, a, a''), Ci (green, a) and Smo (grey, a"'). (**b-c**) Wild-type imaginal wing discs (b) or wing discs expressing Smo^{Fu}-Myc (c) driven by *rotund*-Gal4 (*rn*gal4) were stained for Ci155 (red, b, c) and phospho-Smo (pSmo) against the serine at position 687 (PKA phospho site), (grey, b', c'). (**d**) Related quantification graph (n=4 discs for each condition) of pSmo in WT (b') or Smo^{Fu} -expressing wing discs (c'). (**e**) S2R+ cells were transfected with Fu-WT-HA and variants of Smo. Cell lysates were subjected to immunoprecipitation with HA-antibody, and the presence of Smo was analyzed by Western blotting. Two exposures of IP HA are shown. (**f**) S2R+ cells were transfected with indicated Smo constructs in presence of Fu-WT and Cos2, and lysates were analyzed by Western blotting for the presence of pS572, pS931, Cos2 and Fu.

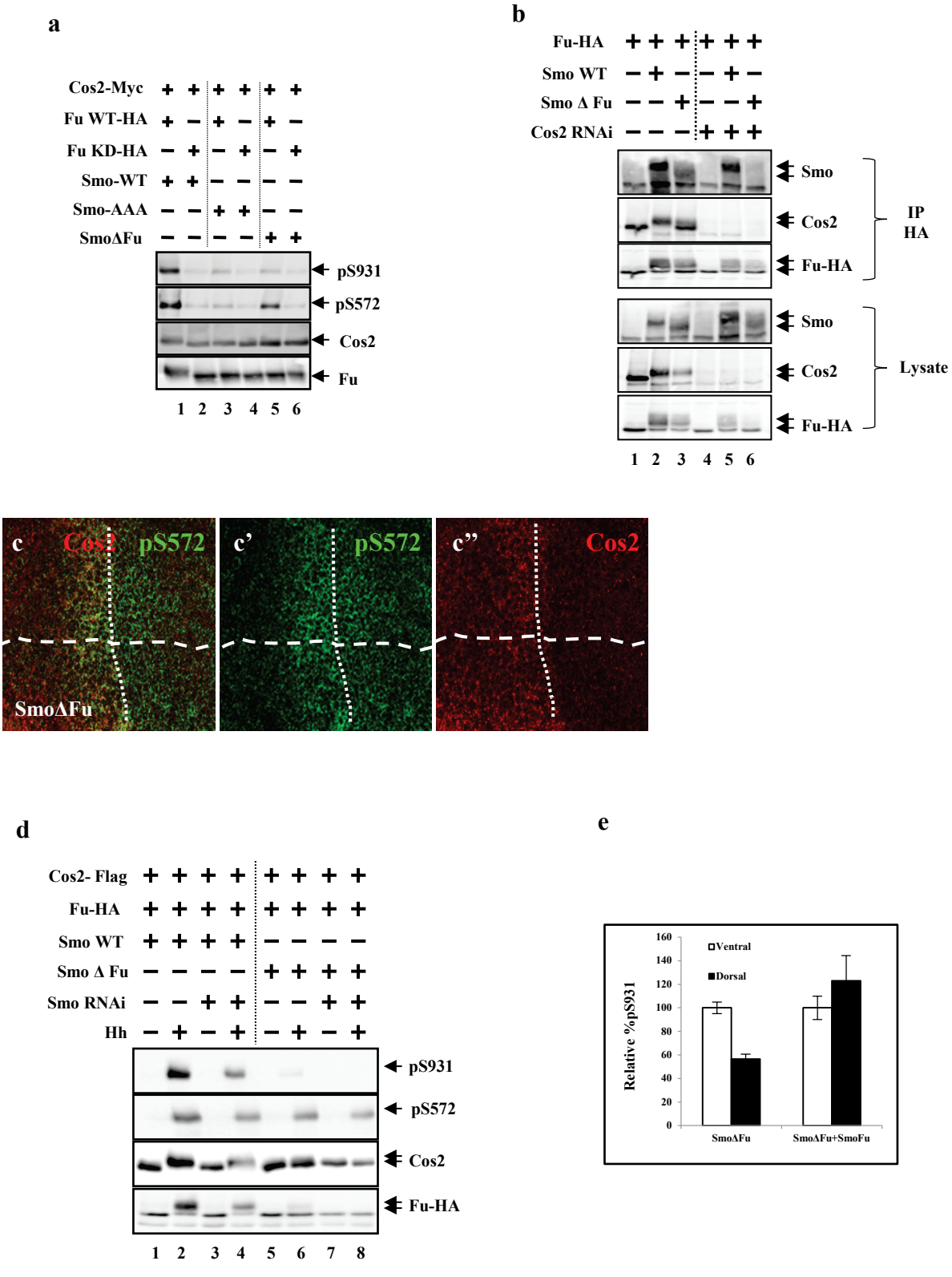
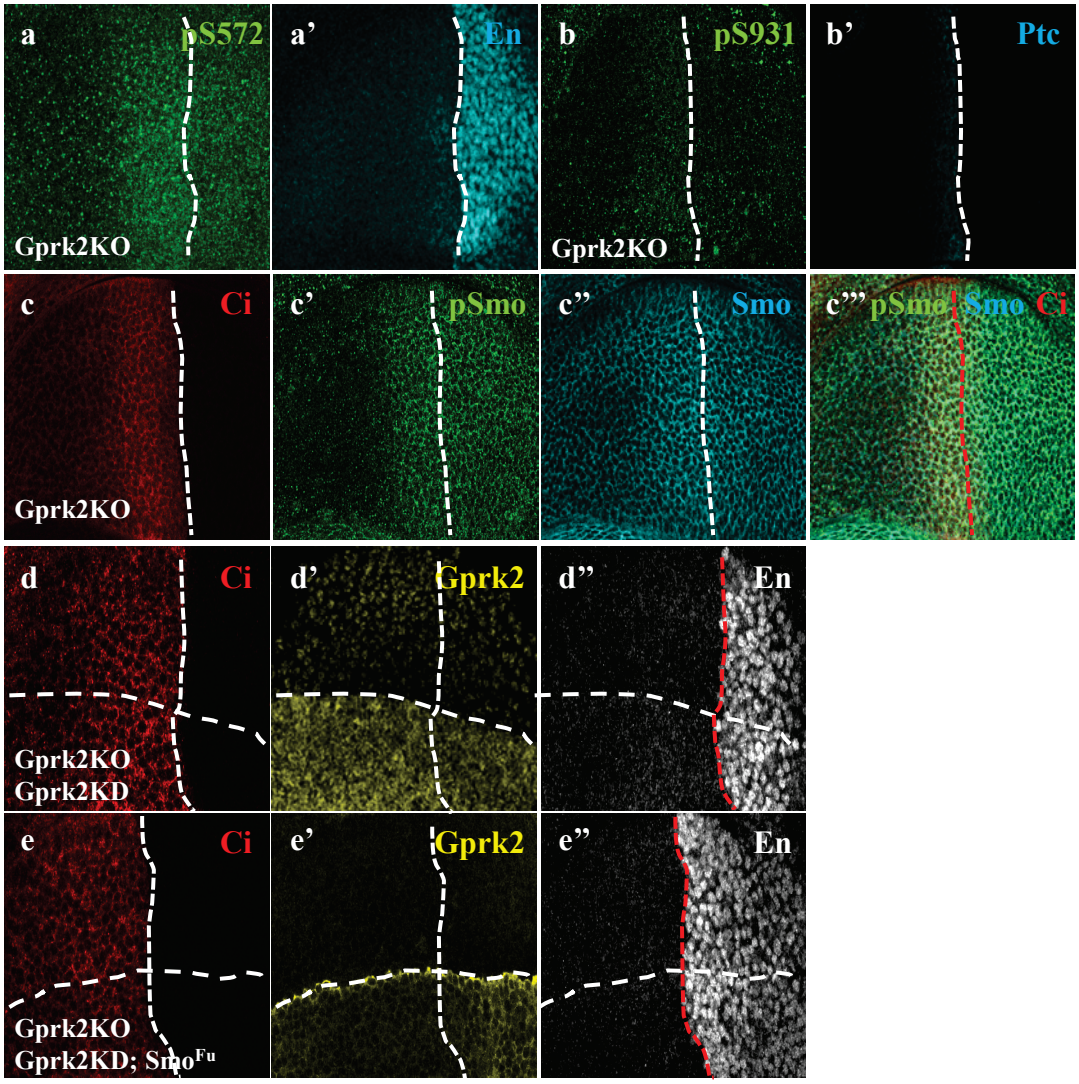


Figure S3. The very last amino acids of Smo promote Fu kinase activity *in vitro*

(**a**) S2R+ cells were transfected with indicated constructs, and lysates were analyzed by Western blotting for the presence of pS572, pS931, Fu and Cos2. (**b**) S2R+ cells were transfected with indicated Smo constructs and Fu-WT. Endogenous Cos2 is depleted by RNAi (lanes 4 to 6). After transfection, Fu immunoprecipitations were analyzed for the presence of Smo variants. Note that SmoΔFu weakly binds Fu even in absence of endogenous Cos2 (lane 6 compared to lane 3). (**c**) Wing discs expressing SmoΔFu driven with *apt*-Gal4 were stained for Cos2 (red, c, c'') and pSer572 (green, c, c'). (**d**) S2R+ cells expressing Cos2-Flag, Fu-HA, Smo-WT or SmoΔFu were treated with or without Hh in which endogenous Smo has been removed using a dsRNAi against the 3'UTR of Smo as it is described in the figure. Lysates were analyzed by Western blot for the phosphorylation on of pS572, pS931 and for the presence of Fu and Cos2. Note that the phosphorylation on S931 is lost when SmoΔFu is expressed (lane 8 compared to lane 4). (**e**) From the figure 2h-I, relative amount of pS931 staining of SmoΔFu and Smo^{Fu}Myc + SmoΔFu in both compartments (n=8 discs).



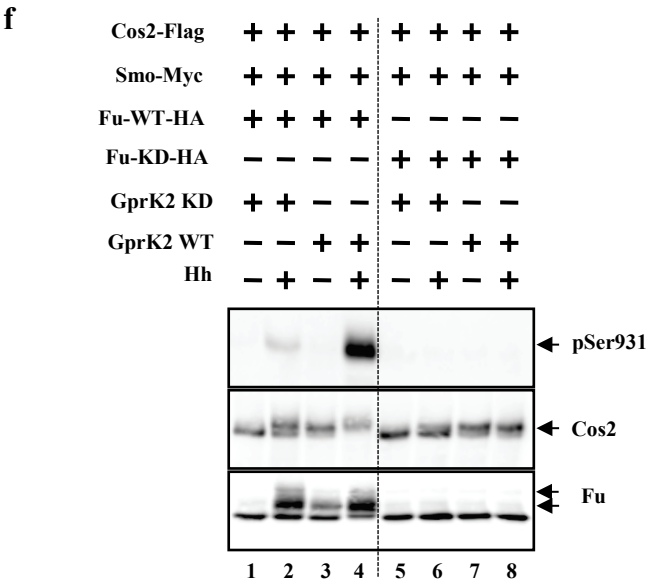


Figure S4. Gprk2 modulates the activity of the kinase Fu by acting on Smo

(**a-c**) Wing discs from *gprk2* KO mutant were immunostained for pS572 (green, a), En (blue, a'), pS931 (green, b), Ptc (blue, c'), Ci155 (red, c, c'''), phospho-Smo (green, c', c''') and Smo (blue, c'', c'''). (**d-e**) Wing discs from the *gprk2* KO mutant expressing Gprk2-KD alone (d) or with Smo^{Fu}-Myc (e) by using *ap*-gal4 driver were stained for Ci155 (red, d, e), Gprk2 (yellow, d', e') and En (grey, d'', e''). **(f)** S2R+ cells were transfected with Smo, Cos2, Fu-WT, Fu-KD, GprK2-WT and GprK2-KD in absence or presence of Hh. Lysates were analyzed by Western blotting for the presence of pS931, Fu and Cos2. Note that the absence of shift electromobility of Fu-KD protein in any conditions of transfection.

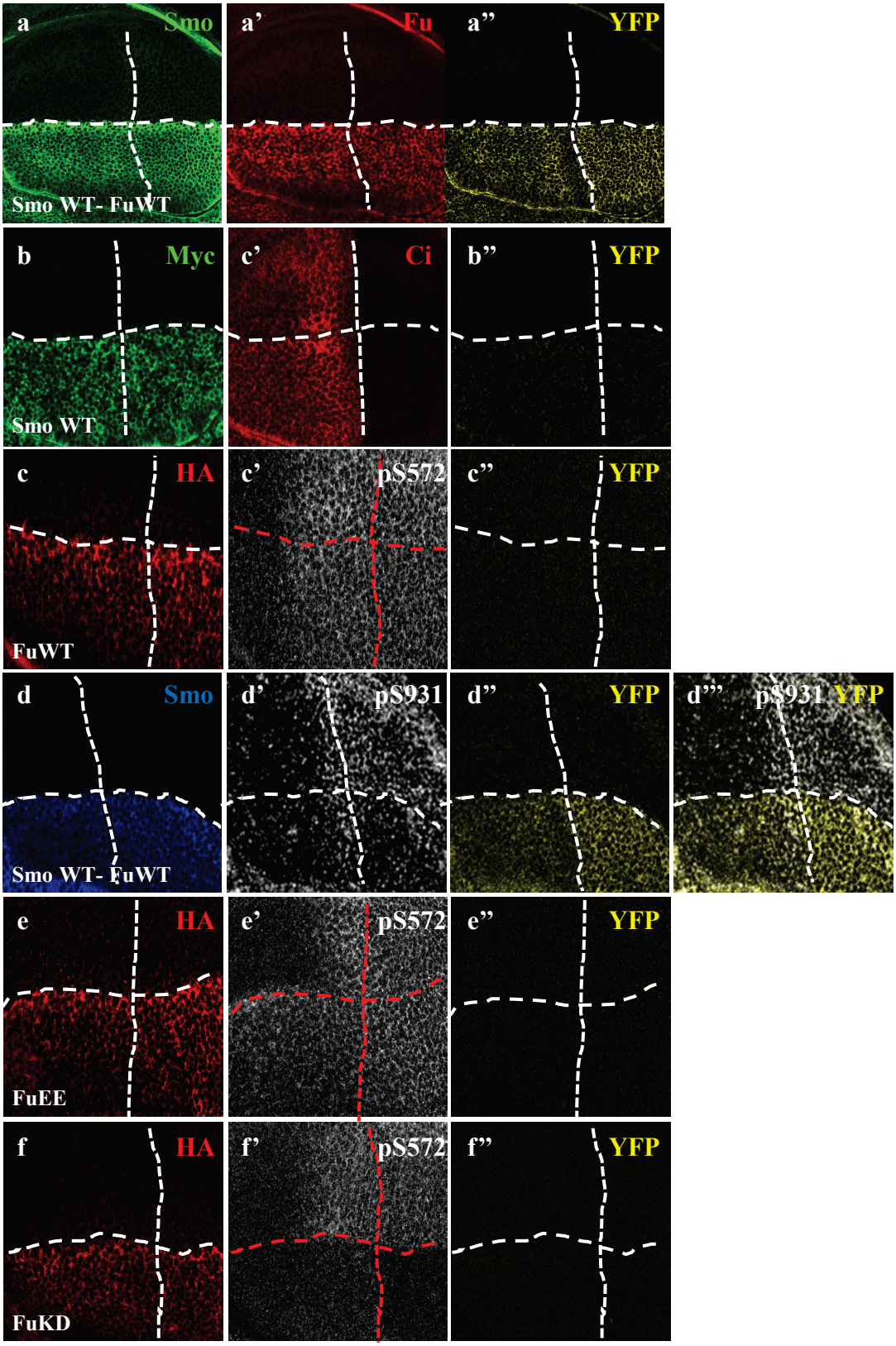


Figure S5. Smo and Fu regulate the phosphorylation of Cos2 *in vivo*

(**a**) Wing imaginal discs co-expressing Smo-WT-N-YFP-Myc and HA-C-YFP-Fu-WT in the dorsal compartment, were stained for Smo (green, a), Fu (red, a'). YFP signal was shown in yellow for a''. (**b-c**) Wing imaginal discs expressing individually Smo-WT-N-YFP-Myc (**b**) or HA-C-YFP-Fu-WT (**c**) in the dorsal compartment using *ap*-Gal4 driver were stained for Myc (green, b), Ci (red, b'), HA (red, c) and pS572 (grey, c'). YFP signal was detected in yellow for b" to c". (**d**) Wing imaginal discs co-expressing Smo-WT-N-YFP-Myc and HA-C-YFP-Fu-WT in the dorsal compartment, were stained for Smo (blue, d), pS931 (grey d,' d"'). YFP signal was shown in yellow for d"-d"'. (**e-f**) Wing imaginal discs expressing individually HA-C-YFP-Fu-EE (**e**) or HA-C-YFP-Fu-KD (**f**) in the dorsal compartment using *ap*-Gal4 driver were stained for HA (red, e, f) and pS572 (grey, e', f'). YFP signal was detected in yellow for e" to f".

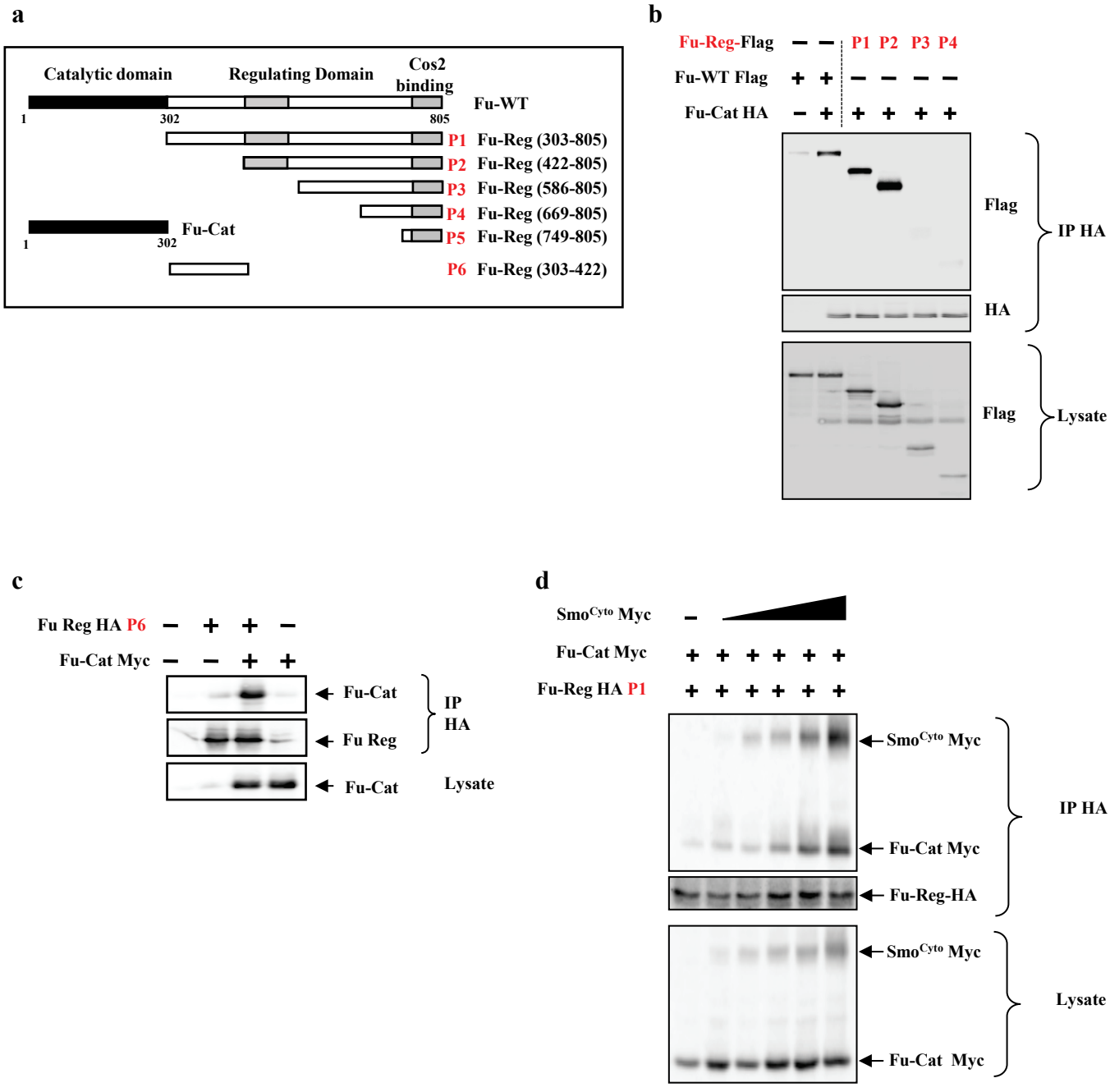


Figure S6. Association of the Fu regulatory domain with Fu catalytic domain and Smo

(**a**) Schematic representation of Fu-WT and Fu variants with various deletions of its regulatory domain. (**b**) S2R+ Cells were transfected with indicated Fu-Reg-Flag constructs described in (a) in presence of Fu-Cat-HA. After Fu-Cat immunoprecipitations using a HA antibody, the extracts were analyzed for the presence of Fu-Reg variants using a Flag antibody. (**c**) S2R+ cells were transfected with Fu-Reg-P6 in presence of Fu-Cat. Fu-Reg immunoprecipitates were analyzed for the presence of Fu-Cat. (**d**) After transfection with the indicated constructs, a fixed amount of Fu-Reg P1-HA and Fu-Cat in presence of an increasing amount of Smo^{Cyto}, Fu-Reg HA immunoprecipitates were analyzed for the presence of Fu-Cat and Smo^{Cyto} using Myc antibody.

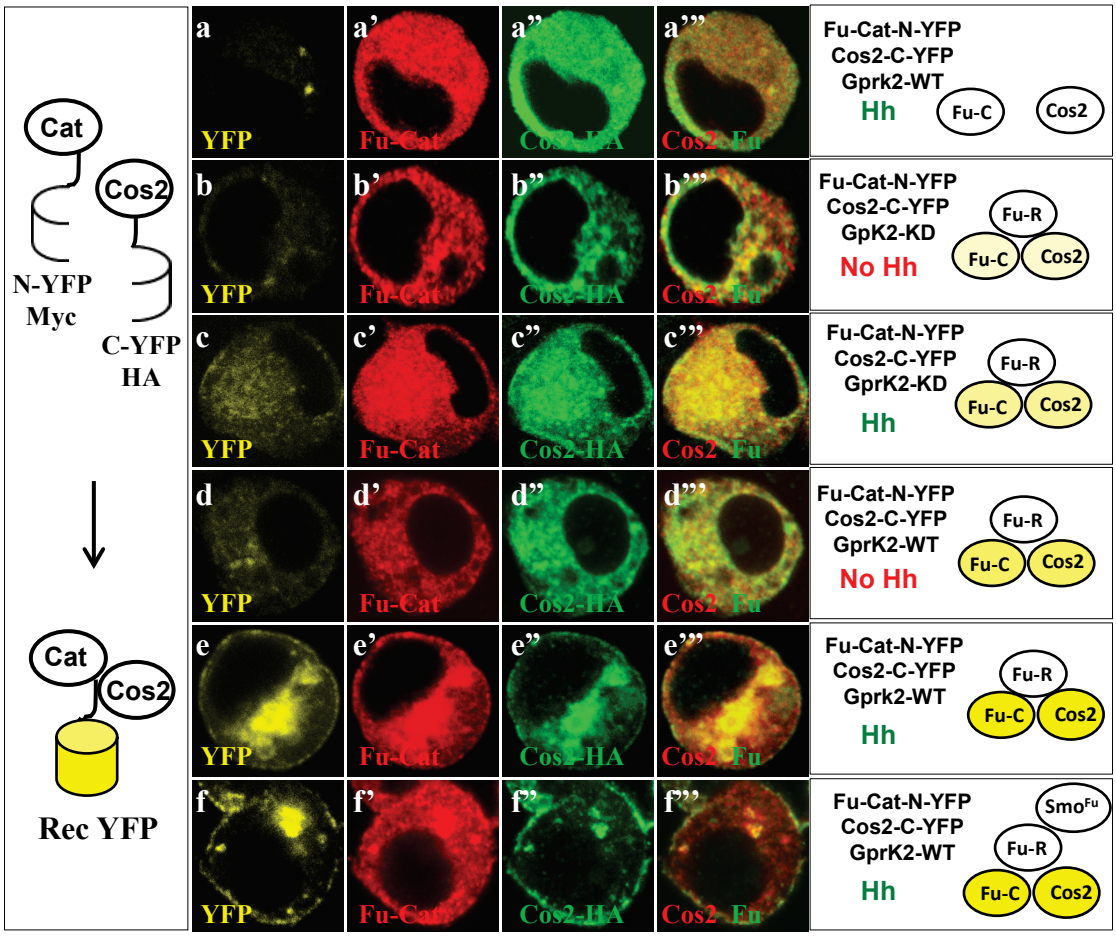


Figure S7. The proximity of Fu-Cat with Cos2 is enhanced in presence of Hh

(**a-f**) The panels of cells illustrate the BiFC experiments from the figure 8. S2R+ cells with or without Hh treatment were transfected with Fu-Cat-N-YFP-Myc and Cos2-C-YFP-HA in absence (a) or presence of Fu-Reg (b-f) or with Smo^{Fu} (f). Schematic representations of YFP reconstituted activity are represented on the left, while the experimental conditions and structure of the protein complex are indicated on the right of each panel. Note that different GprK2 variants (GprK2-WT or GprK2-KD) are showed in each "right" panel. Transfected cells were stained for Myc (red in a'-f', a'''-f''') and HA (green, a''-f'', a'''-f'''). From **a** to **f**, YFP signals were detected in yellow.

Table S1. Reagent type (species) or resource

Reagent or resource	Source	Identifier
Antibodies		
mouse monoclonal anti-Smo 1:200	DSHB	Cat# 20C6
rabbit polyclonal anti-Cos2 1:500	Ruel et al., 2003	
monoclonal 2A1 rat anti-Ci155 1:10	Motzny and Holmgren, 1995	2A1
rabbit polyclonal anti-Fu 1:200	Ruel et al., 2003	
rabbit polyclonal anti-Smo 1:200	Ruel et al., 2003	
rabbit polyclonal anti-Cos2 1:500	Ruel al., 2003	
mouse monoclonal anti-Ptc 1:200	DSHB	5E10
mouse monoclonal anti-En 1:400	DSHB	4D9
rabbit polyclonal anti-En 1:1000	Santa Cruz	sc-28640
rat monoclonal anti-HA 1:1000	Roche	11 867 423 001
mouse monoclonal anti-HA 1:25	Homemade	12CA5
rabbit polyclonal anti-Myc 1:100	Santa-Cruz	A-14
anti-pSer931-Cos2 1:250	Ranieri et al., 2012	
anti-pSer931-Cos2 1:250	Ranieri et al., 2012	
guinea pig monoclonal anti-Gprk2 1:1000	Cheng et al., 2010	
Alexa Fluor 405 anti-antibody	Life technologies	
Alexa Fluor 488 anti-antibody	Life technologies	
Alexa Fluor 568 anti-antibody	Life technologies	
Alexa Fluor 647 anti-antibody	Life technologies	
Bacterial and Virus Strains		
Top10 E. Coli	Invitrogen	C404003
Biological Samples		
Drosophila wing imaginal discs	This study	
Chemicals, Peptides, and Recombinant Proteins		
Bovine Calf Serum	Gibco	10270-106
Schneider's Drosophila Medium	Gibco	21720-024
Lipofectamine LTX	Invitrogen	15338-100
Okadaic Acid (OA)	Sigma-Aldrich	07760
Halt Protease and Phosphatase Inhibitor	Thermo Scientific	1861284

Reagent or resource	Source	Identifier
Commercial Assays		
Pierce ECL Western Blotting Substrate	ThermoFisher Scientific	32106
DC Protein Assay	Bio-Rad	5000112
Qiagen plasmid maxi kit	Qiagen	12163
Experimental Models: Cell Lines		
Drosophila Schneider R+ cells	DGRC	FBrf0024118
Experimental Models: Organisms/Strains		
Drosophila melanogaster wild-type		
D. melanogaster UAS-Smo-N-YFP	Ranieri et al., 2014	
D. melanogaster *UAS-C-YFP-Fu-WT*	This paper	
D. melanogaster UAS-C-YFP-Fu-EE	This paper Malpel	
D. melanogaster UAS-SmoΔFu	et al., 2007	
D. melanogaster UAS-Smo^{Fu}	This paper	
D. melanogaster UAS-Smo^{Cyto}	This paper	
D. melanogaster UAS-Smo$^{SAID-Fu}$	This paper	
D. melanogaster gprk2 KO	Cheng et al., 2010	
D. melanogaster UAS GFP Smo WT	Zhang et al., 2004	
D. melanogaster UAS GFP Smo AAA	Zhang et al., 2004	
D. melanogaster UAS-Smo dsRNA	VDRC	108351
D. melanogaster UAS-Gprk2 dsRNA	VDRC	109241
D. melanogaster apterous-Gal4	FlyBase	FBal0284995
D. melanogaster rotund-Gal4	FlyBase	FBal0291355
D. melanogaster UAS-Dicer2	Bloomington Drosophila Stock center	24650
D. melanogaster UAS-Flag-gprk2 WT	Jiang et al., 2016	
D. melanogaster UAS-Flag-gprk2 KD	Jiang et al., 2016	
D. melanosgaster UAS Smo SD123	Chen et al., 2010	
D. melanosgaster UAS Smo GPSA	Chen et al., 2010	
D. melanosgaster UAS DsRNA Smo 3'UTR	Maier et al., 2014	
Oligonucleotides		
Forward primer for *Smo^{Cyto}*, CACCGGTACCATGGGCTGGACACCTTCTTCAATTGAGACTT	This paper	N/A

Reagent or resource	Source	Identifier
Reverse primer for *Smo* ^{Cyto}, AAGCTCGAGTTTTGAAGGCAGCAATAACATTTTGAGTTTGTCCGAC	This paper	N/A
Forward primer for Smo $^{\Delta SAID}$, CACCGGTACCATGGGCTCGGAGGAGGATAATTCCAGAGCATCC	This paper	N/A
Reverse primer for *Smo* $^{\Delta SAID}$, AAGCTCGAGTTTTGAAGGCAGCAATAACATTTTGAGTTTGTCCGAC	This paper	N/A
Forward primer for *Smo* ^{Fu}, CACCGGTACCATGGGCAACGCAGCCAGCAGACAAAGAAC	This paper	N/A
Reverse primer for *Smo* ^{Fu}, AAGCTCGAGTTTTGAAGGCAGCAATAACATTTTGAGTTTGTCCGAC	This paper	N/A
Forward primer for *Fu-Cat*, *CACCGGATTCAGGATGGGCAACCGCTACGCGGTAAGCTCG*	This paper	N/A
Reverse primer for *Fu-Cat*, ACTGAATTCAAAGTCCAAAGCGGCCAGGGCCTCGTCC	This paper	N/A
Forward primer for *Fu-R*, CACCAGATCTACTAGTACCATGGGCGAGTCGCGACAGGAAAACTTGACC	This paper	N/A
Reverse primer for *Fu-R*, CCGAGATCTACTAGTGGTGACGAAAAAAGTGAAGTGACTGAT	This paper	N/A
Forward primer for *Gprk2*, CACCGGTACCATGGAATTAGAGAATATTGTGGCCAA	This paper	N/A
Reverse primer for *Gprk2*, AAGTCTAGAGCTTTCGACCGTCGTGGAGGACACGCTGTGA	This paper	N/A
Forward primer for *Fu and variants*, *CACCGAATTCAGGATGAACCGCTACGCGGTAAGCTCG*	This paper	N/A
Reverse primer for *Fu and variants*, AAGGAATTCGGTGACGAAAAAAGTGAAGTGACTGAT	This paper	N/A

Reagent or resource	Source	Identifier
Forward primer for *PKA*, CACCGGTACCGAATTCAAGATGGGCAACAACGCCACCACG TCGAATAAG	This paper	N/A
Reverse primer for *PKA*, *AAGTCTAGAGAATTCAGCAAACTCCTTGGCACACTTCTC*	This paper	N/A
Forward primer for *Smo*, CACCGGTACCATGCAGTACTTAAACTTTCCGCGCAT	This paper	N/A
Reverse primer for *Smo*, *GAACTCGAGTTTTGAAGGCAGCAATAACATTTTGAGT*	This paper	N/A
Forward primer for *Cos2*, CACCGGTACCATGGAAATACCCATTCAGTTAGCGGTGCGC	This paper	N/A
Reverse primer for *Cos2*, *AAGTCTAGAGTTTCGACGACTTGCGTCCTGGAT*	This paper	N/A
Recombinant DNA		
pUAST Smo^{Cyto} Myc	This paper	N/A
pUAST Smo $^{\Delta SAID}$ Myc	This paper	N/A
pUAST Smo^{Fu} Myc	This paper	N/A
pUAST Smo-WT with Myc or HA tags	This paper	N/A
pUAST GFP Smo-WT	Zhang et al., 2004	
pUAST GFP Smo AAA	Zhang et al., 2004	
pUAST Smo ΔFu	Malpel et al., 2007	
pUAST Smo-Myc-N-YFP	Ranieri et al., 2014	N/A
pUAST C-YFP-HA-Fu WT	This paper	N/A
pUAST C-YFP-HA-Fu KD	This paper	N/A
pUAST C-YFP-HA-Fu EE	This paper	N/A
pUAST Gprk2-Myc-N-YFP	This paper	N/A
pUAST Flag-Gprk2-WT	Jiang et al., 2016	
pUAST Flag-Gprk2-KM	Jiang et al., 2016	
pUAST Gprk2 with Myc or HA tags	This paper	N/A
pAct Ptc	Yao et al., 2006	N/A
pUAST Fu-Cat with HA or Myc tags	This paper	N/A
pUAST Fu-Reg with HA or Myc tags	This paper	N/A

Reagent or resource	Source	Identifier
pUAST Fu-WT or KD HA	This paper	N/A
pUAST N-YFP-Myc-Fu-Cat	This paper	N/A
pUAST PKA	This paper	N/A
pUAST Cos2 Myc	Ruel et al., 2007	
pUAST Cos2-Myc-N-YFP	This paper	N/A