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Supplementary Materials for

Hedgehog reciprocally controls trafficking of Smo and Ptc through the Smurf family of E3 ubiquitin ligases

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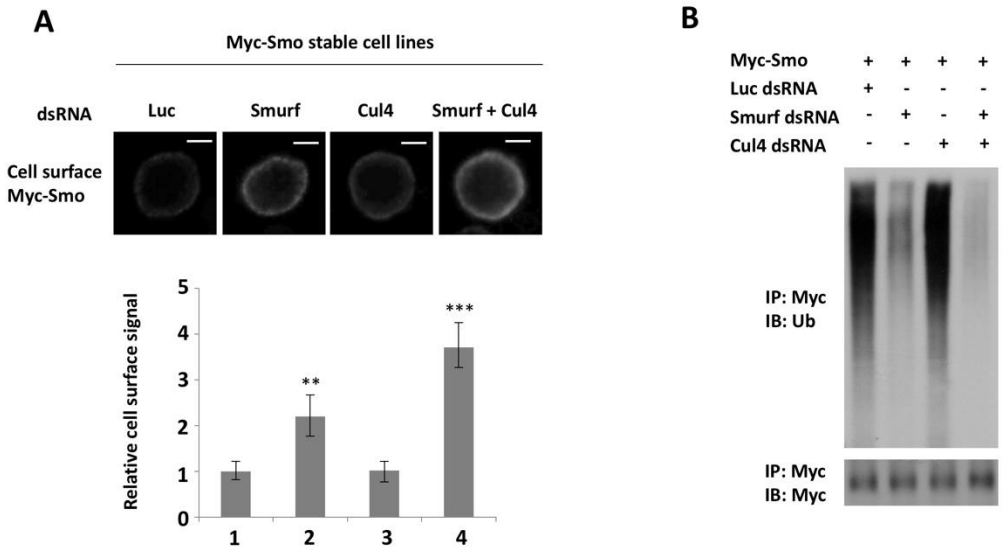

Fig. S1. Cul4 acts in parallel with Smurf to promote Smo ubiquitylation and cell surface clearance.

(**A**) Immunostaining (top) and quantification (bottom) of cell surface Smo in S2 cells expressing Myc-Smo and treated with control (Luc), Smurf, Cul4, or Smurf + Cul4 dsRNA(s). Images are representative of 10 cells per genotype. Data are mean ± SD from 3 independent experiments. n=10 cells for each experimental condition. **, $P<0.01$, ***, $P<0.001$ (student's t-test). (**B**) Western blot showing ubiquitination of Myc-Smo immunoprecipitated from S2 cells that had been treated with the indicated dsRNAs. Blot is representatives of three independent experiments. Scale bars, 5 μm.

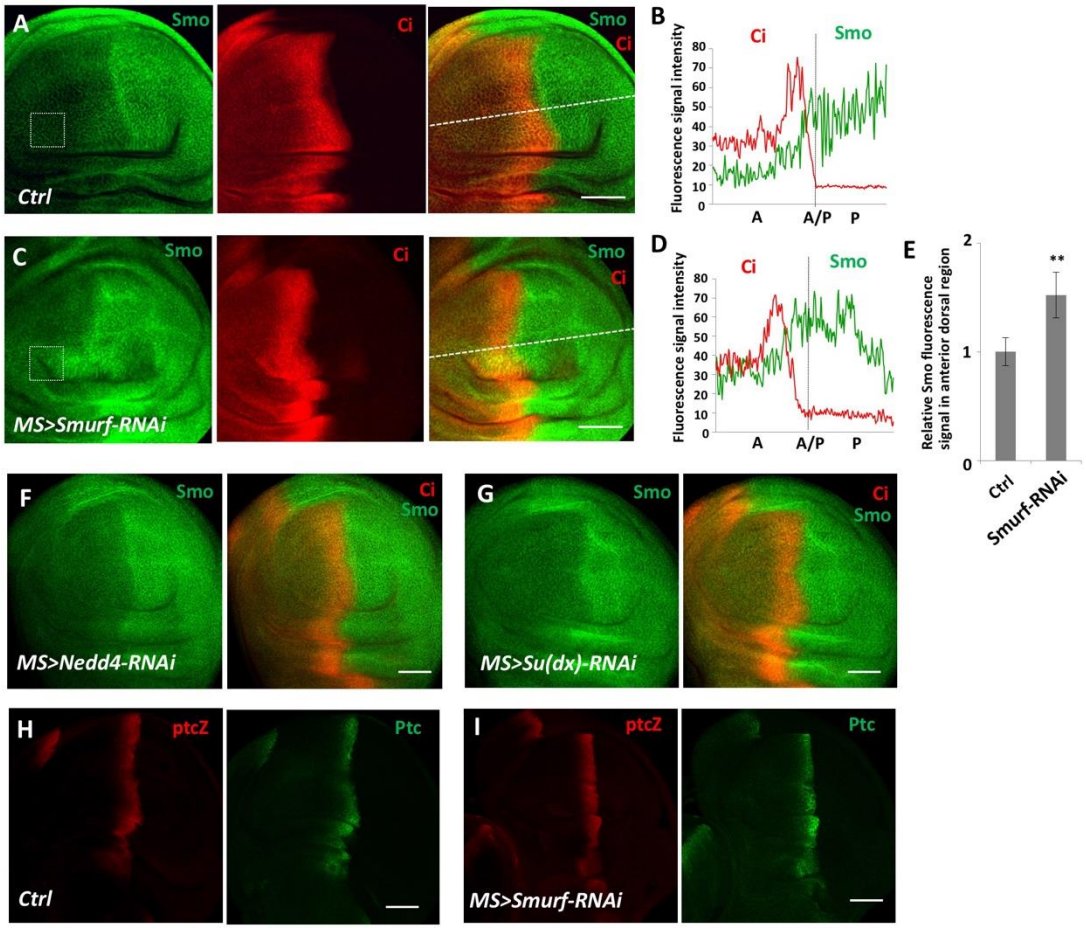

Fig. S2. Effect of Smurf family knockdown on Smo cell surface accumulation and Hh signaling in wing discs.

(**A-G**) Immunostaining for Ci (red) and Smo (green) in a control (ctrl) wing disc (A) or wing discs expressing Smurf (C), Nedd4 (F), or Su(dx) (G) *UAS* RNAi transgenes under the control of the *MS1096* Gal4 driver. Quantification of Ci and Smo fluorescent signal across the anteroposterior axis (dashed line) in control (B) and Smurf RNAi (D) wing discs. Quantification of Smo fluorescent signal in the anterodorsal regions (indicated by the squares) of control or Smo RNAi wing discs (E). Data are mean ± SD from 3 independent experiments. n=6 discs for each genotype. **, P<0.01(student's t-test). (**H-I**) Control (H) and Smurf RNAi (I) wing discs immunostained to show the expression of *ptc-lacZ* (third chromosome insertion line) and Ptc protein. Images are representative of five wing discs per genotype. Scale bars, 50 μm.

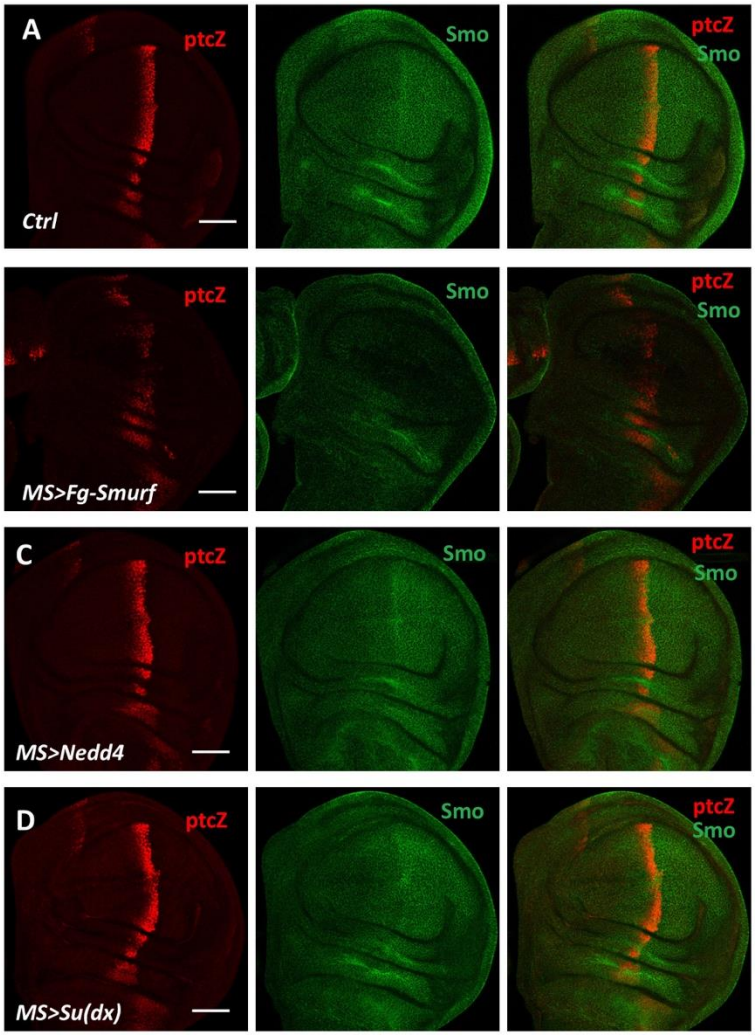

Fig. S3. Effect of overexpression of Smurf family members on Smo cell surface accumulation and Hh signaling in wing discs.

Immunostaining for *ptc-lacZ* (second chromosome insertion) and Smo in a control (ctrl) wing disc (**A**) and wing discs expressing the Fg-Smurf (**B**), Nedd4 (**C**), and Su(dx) (**D**) *UAS* transgenes under the control of the *MS1096* Gal4 driver. Images are representative of five wing discs per genotype. Scale bars, 50 μm.

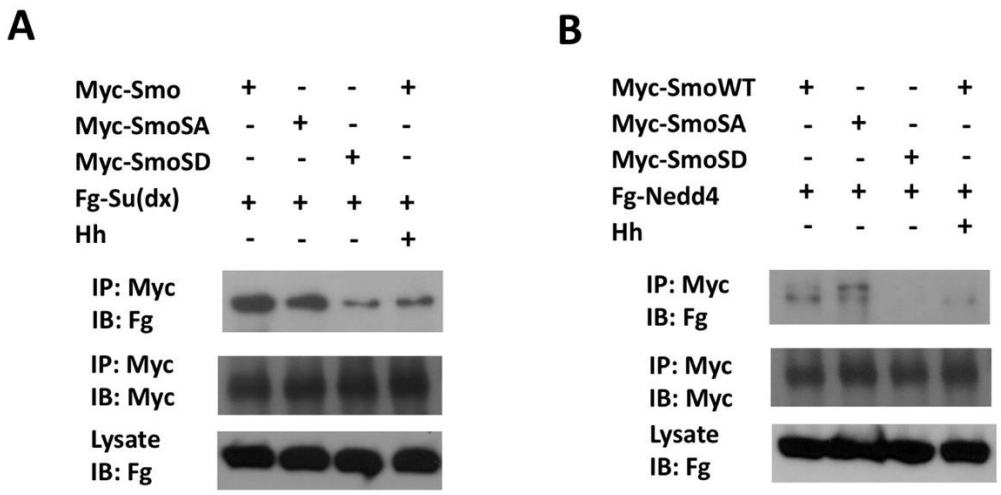

Fig. S4. Hh-stimulated and PKA-mediated phosphorylation of Smo inhibit the recruitment of Su(dx) and Nedd4.

(**A-B**) Flag-tagged Su(dx) (A) or Nedd4 (B) was cotransfected into S2 cells along with Myc-Smo, Myc-Smo^{SA}, or Myc-Smo^{SD} and treated with control or Hh-conditioned medium. After treatment with the proteasome inhibitor MG132, cells were harvested and cell lysates were immunoprecipitated (IP) with an anti-Myc antibody, followed by immunoblotting (IB) with anti-Flag (Fg) and anti-Myc antibodies. Blots are representatives of three independent experiments.

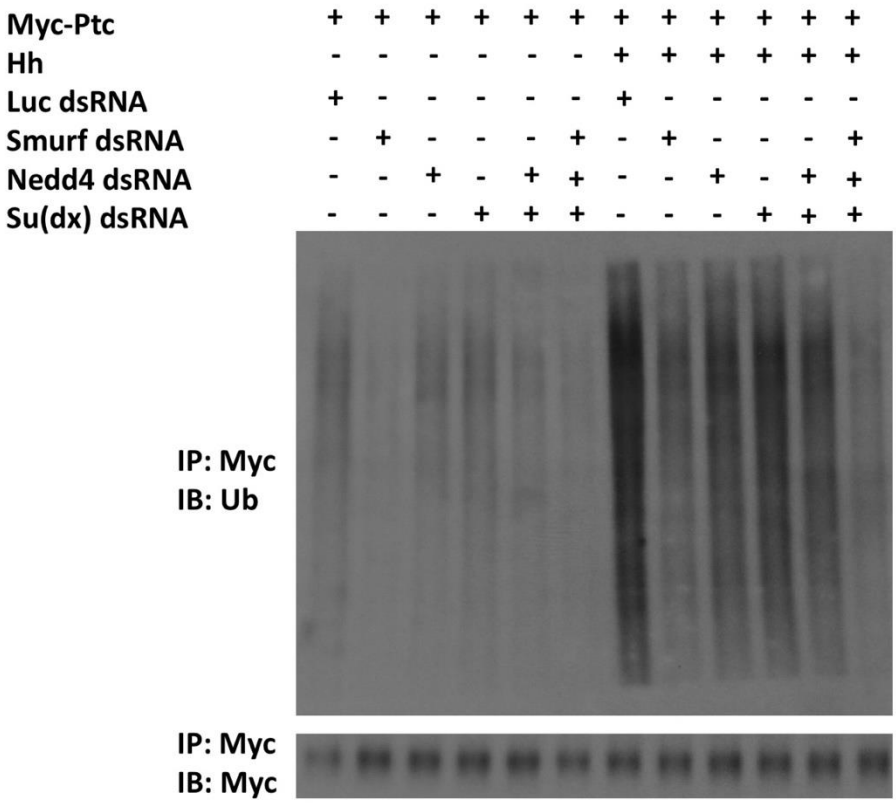

Fig. S5. Hh regulates Ptc ubiquitylation through the Smurf family of E3s.

Ptc ubiquitination assay in extracts from S2 cells treated with control or Hh-conditioned medium in the presence or absence of the indicated dsRNAs. Cell extracts were immunoprecipitated (IP) with an anti-Myc antibody, followed by immunoblotting (IB) with antibodies recognizing ubiquitin or Myc. Blots are representatives of three independent experiments.

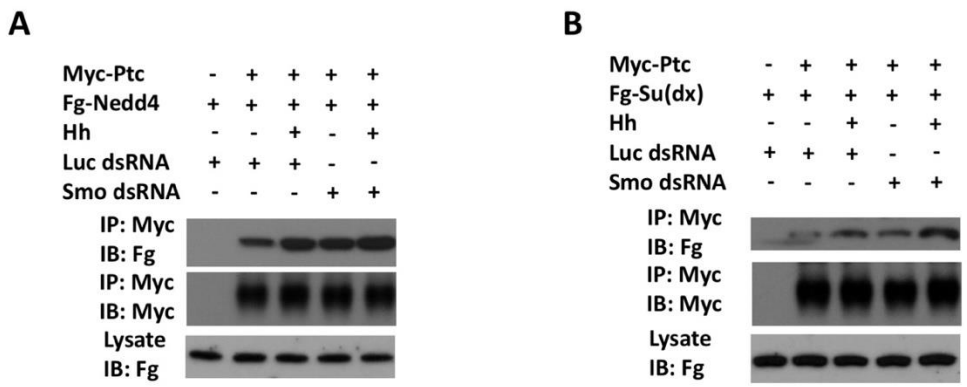

Fig. S6. Regulation of Ptc-E3 interaction by Hh and Smo.

(**A-B**) Immunoblot showing Fg-Nedd4 (A) and Fg-Su(dx) (B) coimmunoprecipitated with Myc-Ptc from extracts of S2 cells treated with the control (Luc) or Smo dsRNA in the absence or presence of Hh-conditioned medium. Blots are representatives of three independent experiments.

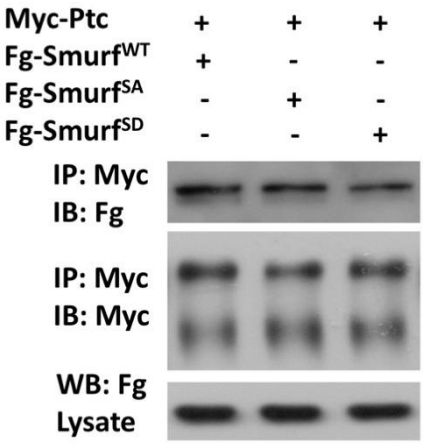

Fig. S7. Ptc interacts with Smurf regardless of Smurf phosphorylation by Gprk2.

S2 cells were transfected with Myc-Ptc and Flag-tagged wild-type (Fg-Smurf^{WT}), phosphorylation-deficient (Fg-Smurf^{SA}), or phosphomimetic (Fg-Smurf^{SD}) forms of Smurf. Cell extracts were immunoprecipitated (IP) with an anti-Myc antibody, followed by immunoblotting (IB) with Flag (Fg) or Myc antibodies, or directly blotted with Fg antibody. Blots are representatives of three independent experiments.