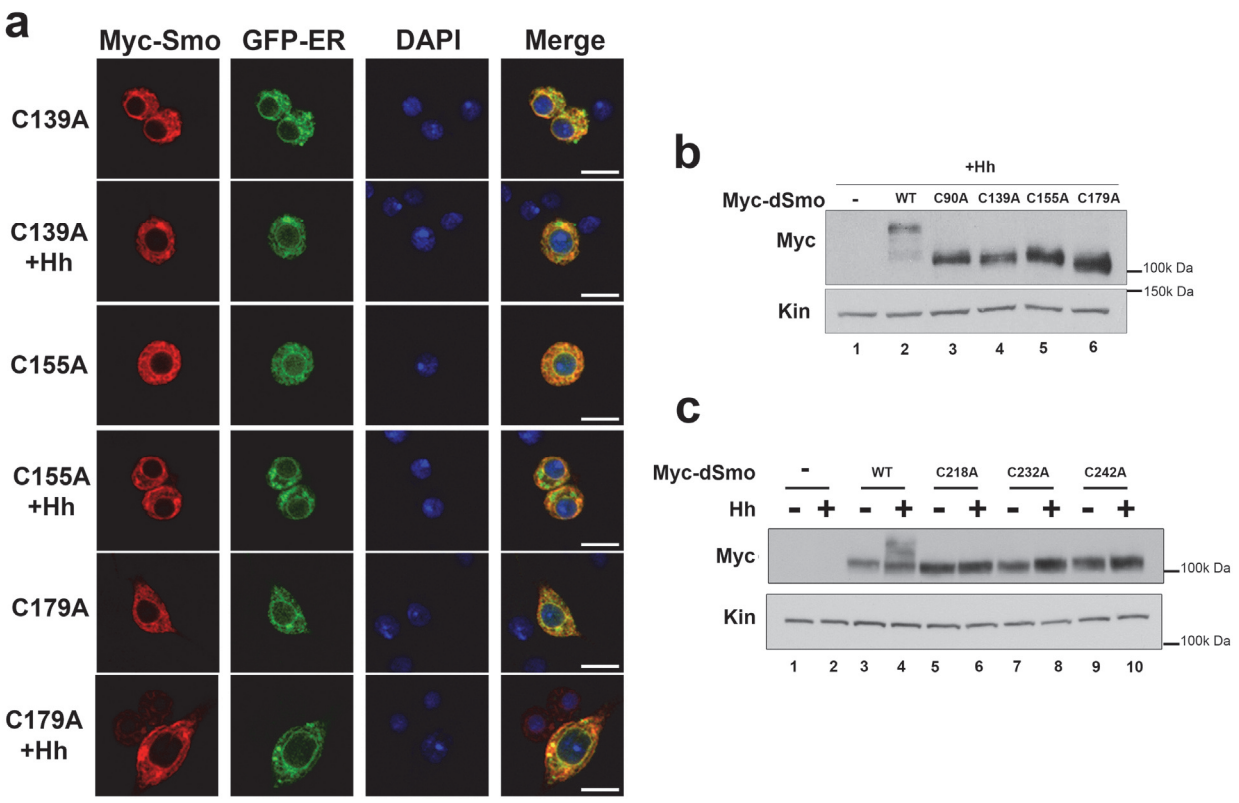
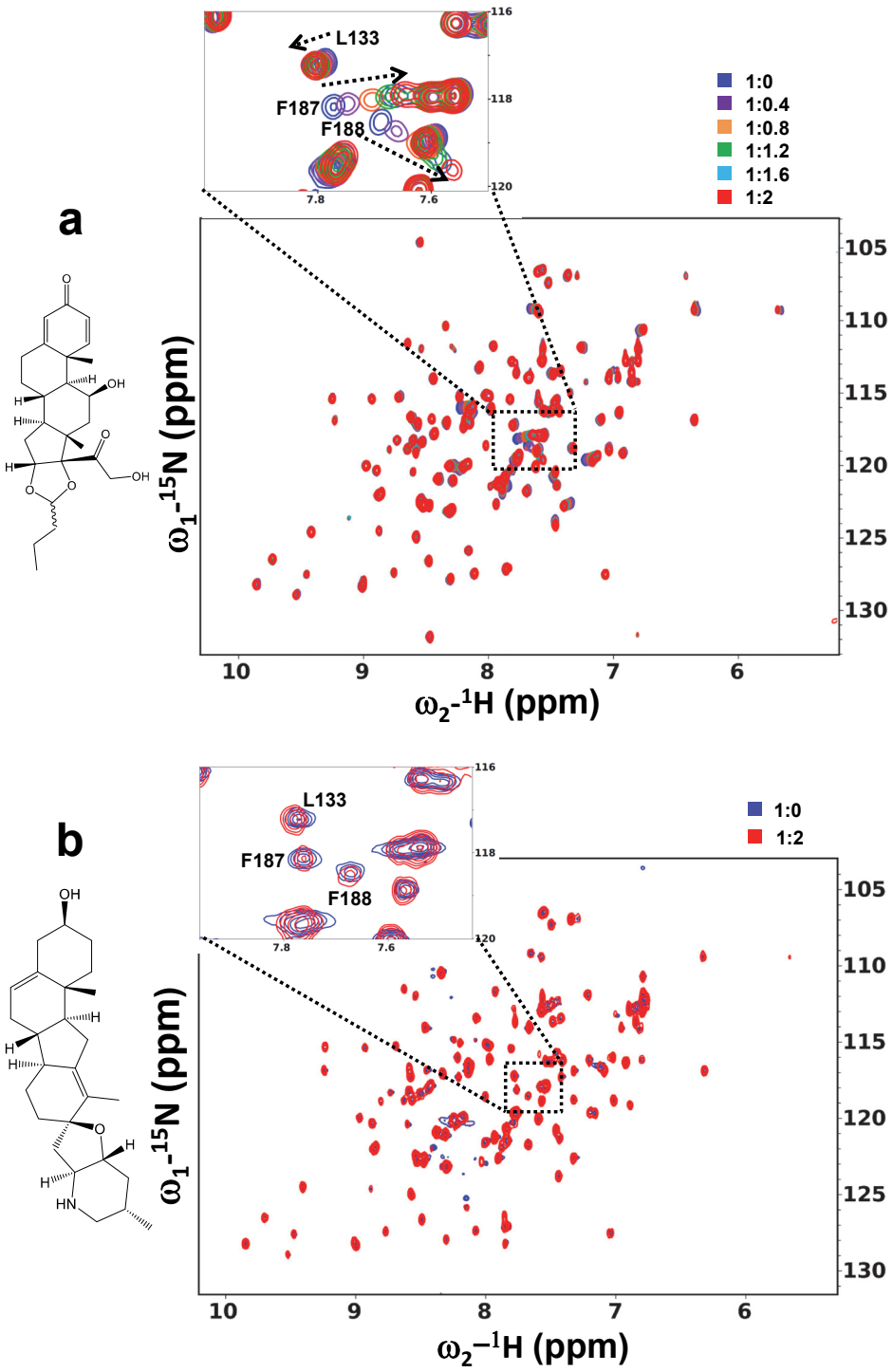
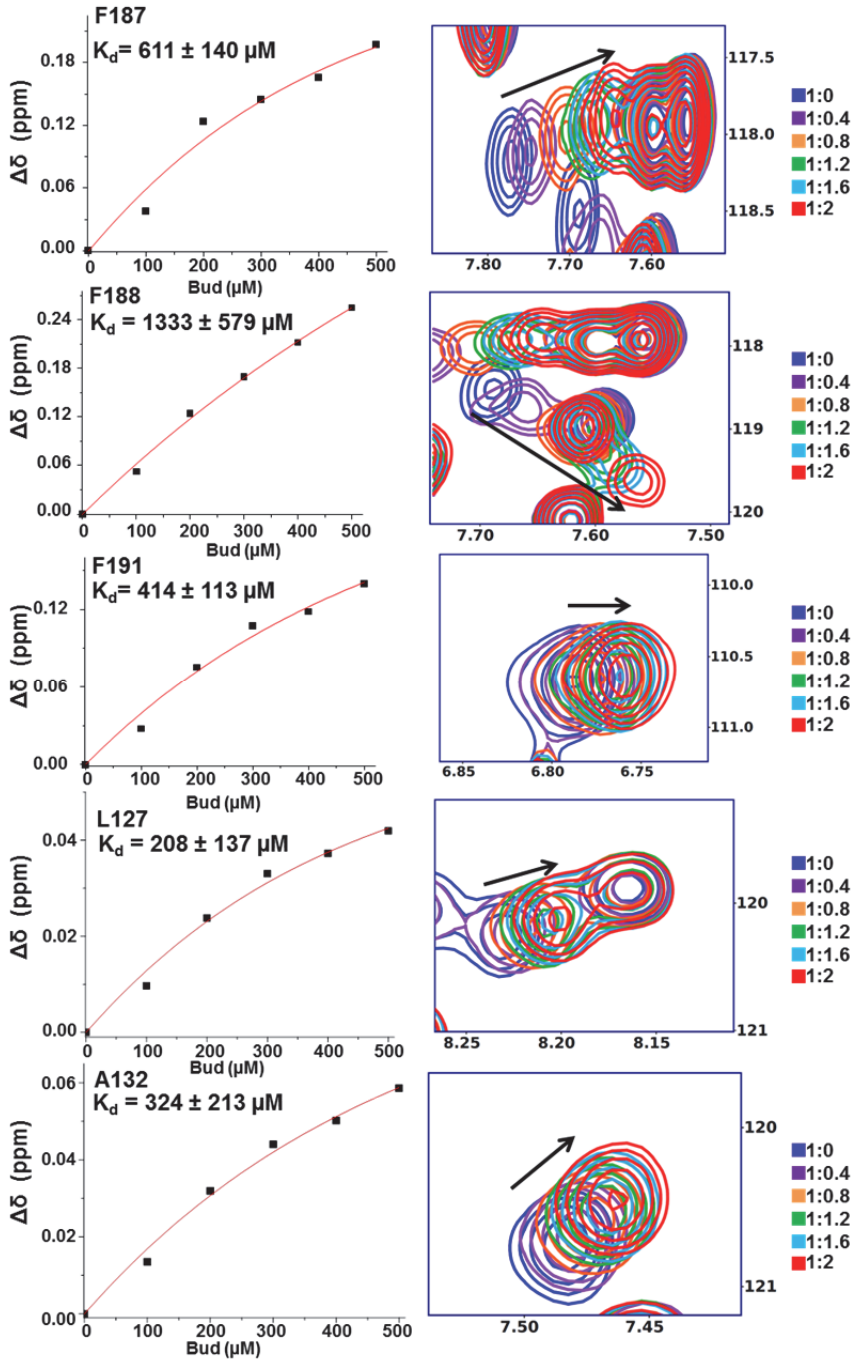


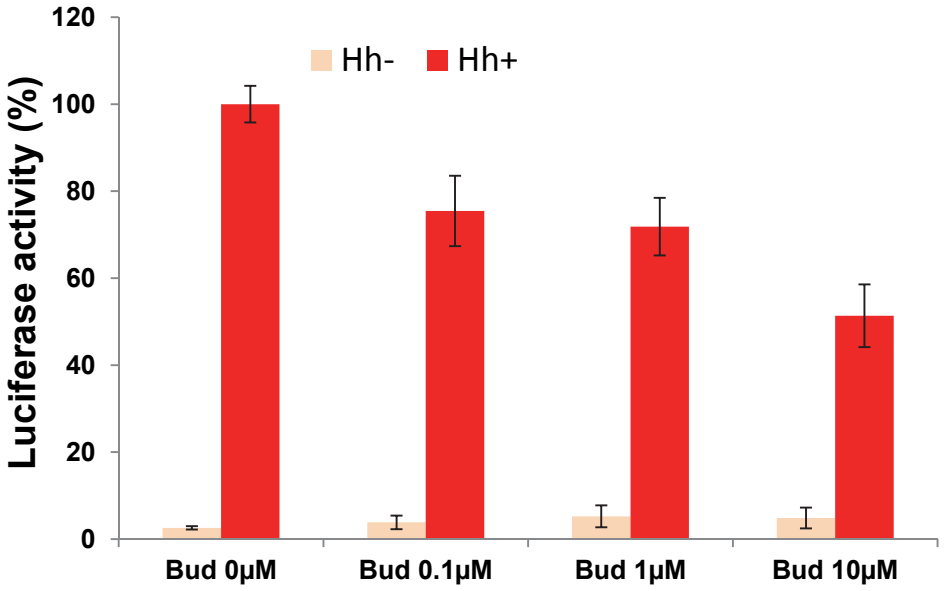
Supplementary Figure S1: Cysteines essential for Smo signalling. **(a)** CRD mutants with compromised signalling activity have altered sub cellular localization. Cl8 cells expressing wild type or the indicated Myc-Smo mutant protein, in the presence of Hh (+) or empty vector control, were examined by indirect immunofluorescence. Wild type Smo translocates to the plasma membrane in response to Hh, whereas Smo CRD cysteine to alanine mutants that were required for maximal Hh reporter gene induction (C139A, C155A and C179A) were largely retained in the ER. Smo was detected using anti-Myc (red), Calreticulin-GFP-KDEL marks the ER (green) and DAPI (blue) marks the nucleus. Scale bar: 10uM **(b)** CRD mutants with compromised signalling activity were expressed similar to wild type protein. Lysates were prepared from Cl8 cells transfected with the indicated *pAc-myc-smo* construct plus *pAc-hh* (+). The Smo CRD cysteine to alanine mutants that were required for maximal Hh reporter gene induction (C90A, C139A, C155A and C179A) expressed robustly when compared to wild-type Smo. Lysates were normalized to total protein and Smo protein was examined by SDS-PAGE and western blot against the myc tag (Myc). **(c)** The Smo ECLD C218, 238 and C242 to alanine mutants expressed similar to wild type protein (lanes 5-10 compared to3-4). Lysates were normalized to total protein and Smo protein was examined by SDS-PAGE and western blot against the myc tag (Myc).



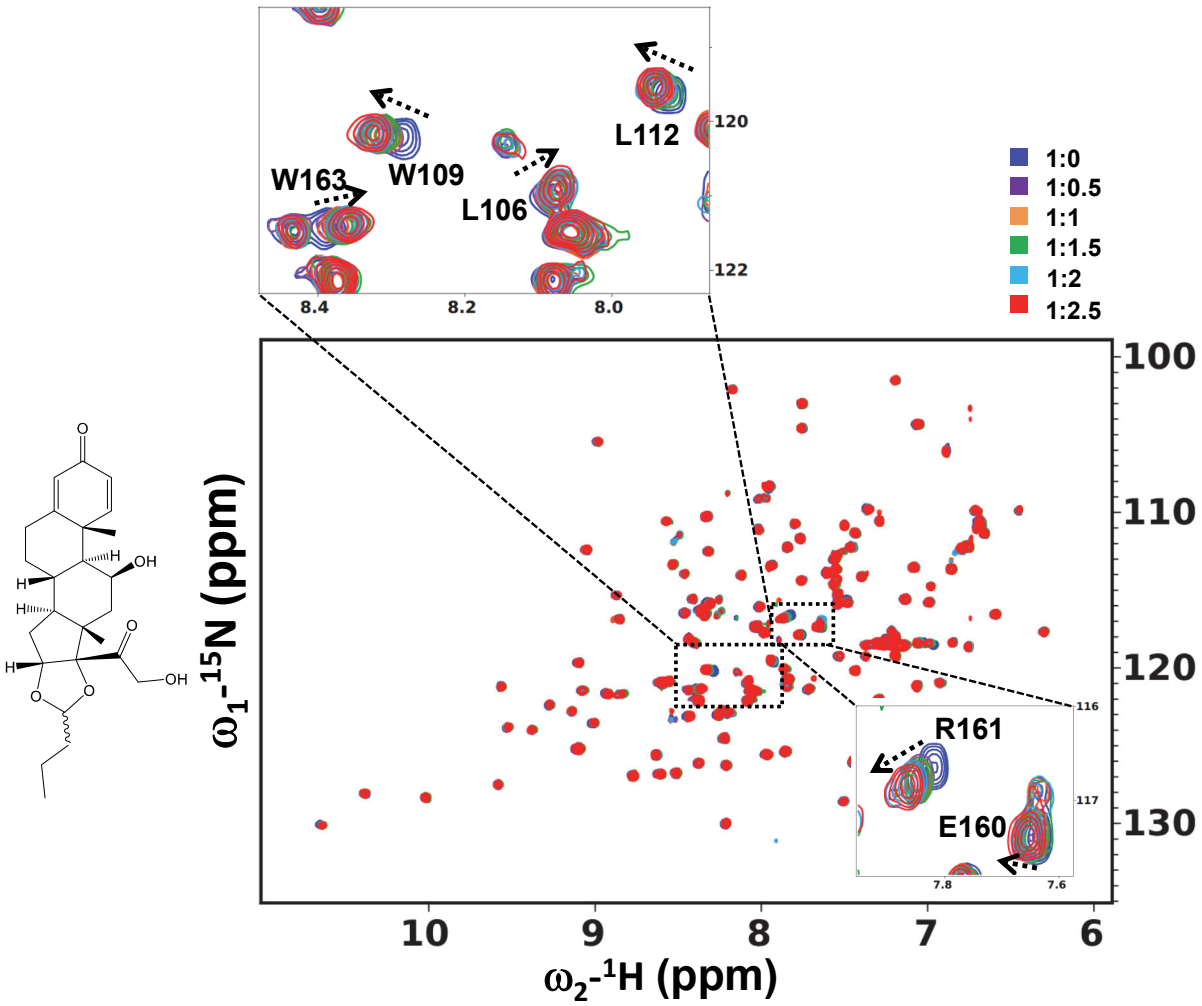
Supplementary Figure S2: NMR analysis of the binding of Bud and cyclopamine to *Drosophila* Smo CRD. (a) ^{1}H-^{15}N HSQC spectra of the Smo CRD in the absence and presence of an increasing concentration of Bud. The protein concentration (250µM) is constant and the increasing ratio of ligand is shown in different colours. The upper inset represents examples of the CSPs in Smo CRD on titration with Bud. **(b)** ^{1}H-^{15}N HSQC spectra of the Smo CRD in the absence and presence of cyclopamine. The upper inset shows that there were no significant changes in the chemical shifts of Smo CRD on titration with cyclopamine at a molar ratio of 1:2 (Smo CRD: cyclopamine).



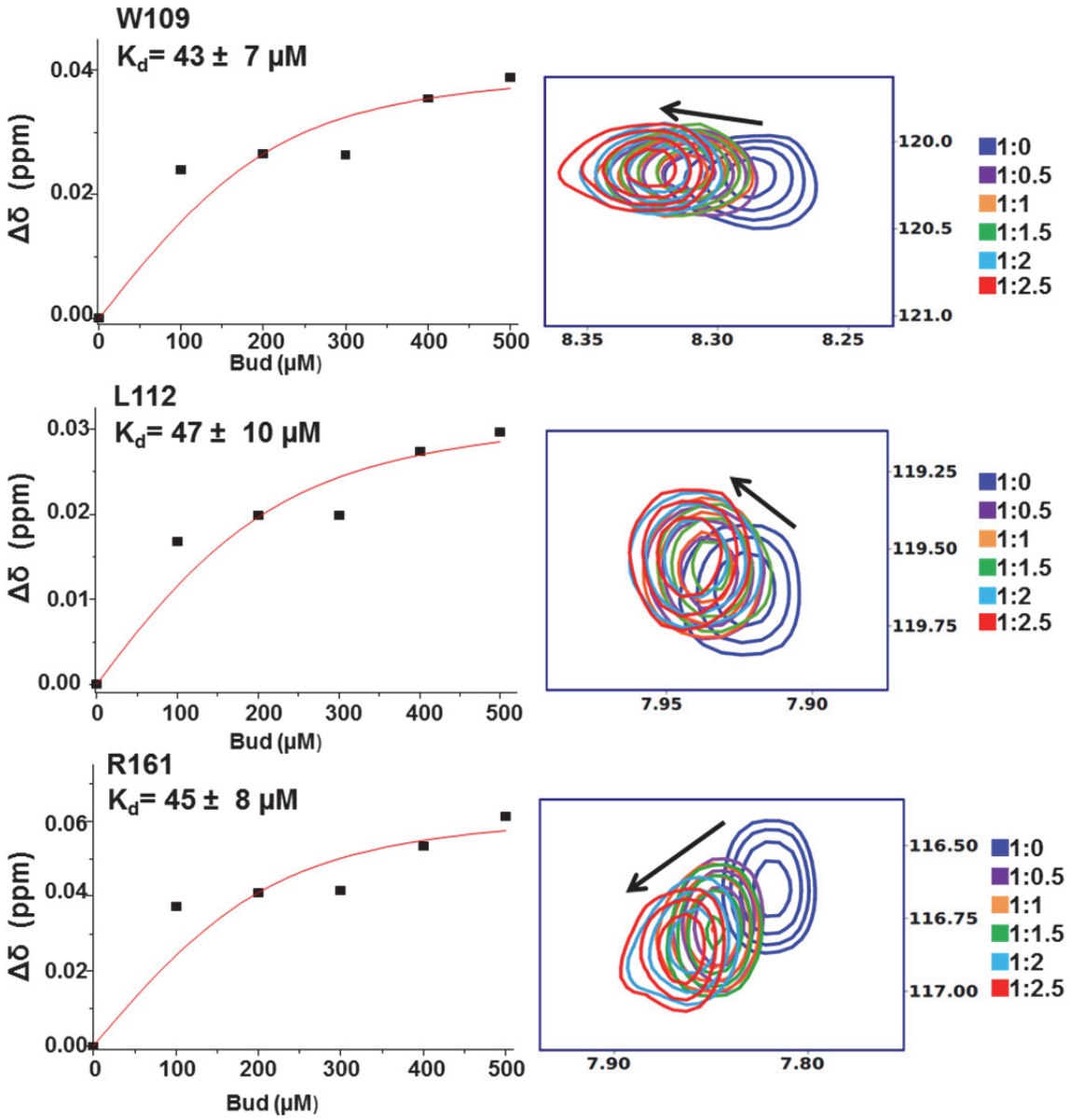
Supplementary Figure S3: The residues of *Drosophila* Smo CRD bind to Bud. Enlarged ^{1}H-^{15}N HSQC spectra of F187, F188, F191, L127, and A132 during the titration of *Drosophila* Smo CRD and the ligand Bud. The spectra are colour coded according to the protein: Bud ratio. The chemical shift change is plotted against the concentration of Bud and the corresponding K_d values are shown.



Supplementary Figure S4: Bud attenuates Hh-induced reporter gene activity. Cl8 cells were transfected with empty vector control (-Hh) or *pAc-Hh* (+Hh), as indicated. Cells were treated with vehicle (DMSO) or increasing concentrations of Bud in serum-free media 24 hours after transfection. Cells were lysed 24 hours post Bud treatment, and *ptcΔ136-luciferase* activity was determined. Bud triggered a ~20-40% reduction in *ptcΔ136-luciferase* activity. Luciferase activity was normalized to *pAc-renilla* control. Experiments were performed at least three times in duplicate and all data pooled. Error bars indicate standard error of the mean (s.e.m).

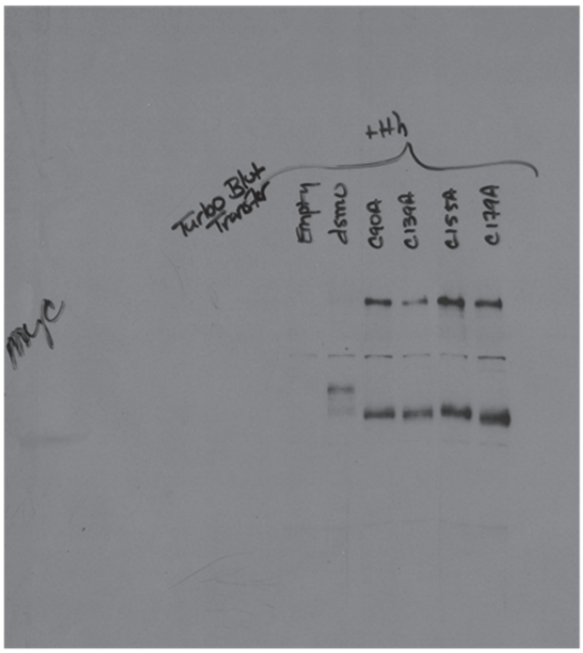


Supplementary Figure S5: NMR analysis of the binding of Bud to human Smo CRD. ^{1}H-^{15}N HSQC spectra of the human Smo CRD in the absence and presence of an increasing concentration of Bud. The protein concentration (200µM) is constant and the increasing ratio of ligand is shown in different colours. The upper and lower inset represents examples of the CSPs in human Smo CRD on titration with Bud.



Supplementary Figure S6: Human Smo CRD binds to Bud. Enlarged ^{1}H-^{15}N HSQC spectra of W109, L112, and R161 during the titration of human Smo CRD and the ligand Bud. The spectra are colour coded according to the protein: Bud ratio. These are the same residues which interact with 20-OHC in vertebrate Smo. The chemical shift change is plotted against the concentration of Bud and the corresponding K_d values are shown. The chemical shift change is greatest at the first point and gets saturated at higher ligand concentrations suggesting a higher binding affinity.

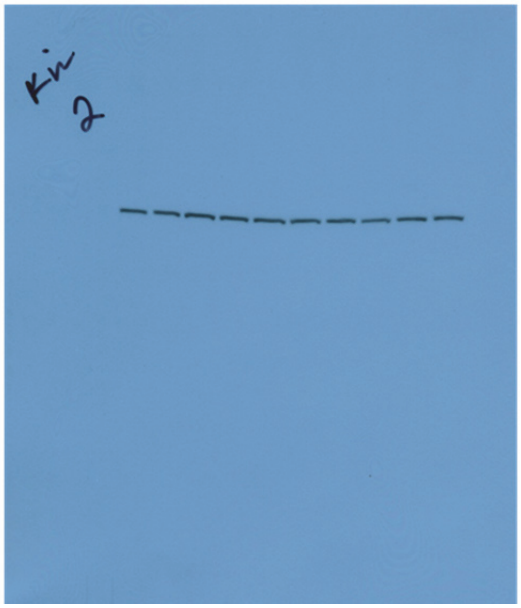
S2b.Smo Blot



S2b.Kinesin Blot

S2c.Smo Blot

S2c.Kinesin Blot



Supplementary Figure S7: Full scan of western blots reported in Supplementary Fig.S1b and S1c.

Supplementary Table S1: The HADDOCK input details for used for docking Bud on Drosophila smo CRD are summarized.

	Residue Number
Active Residues	D129, A132, F187, F188, and F191
Passive Residues	Y131, K134, K138, N184, and T186.
Fully flexible regions	F187-L192
Binding energy	-2694±37 kcal/mol
Buried Surface area	575 ± 13 $Å^2$