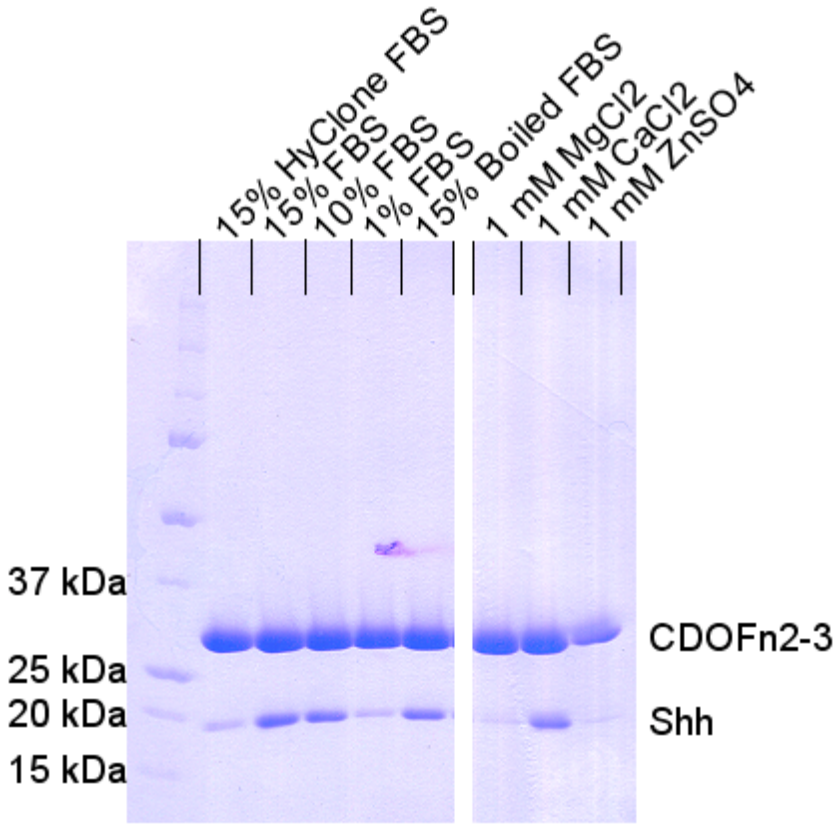
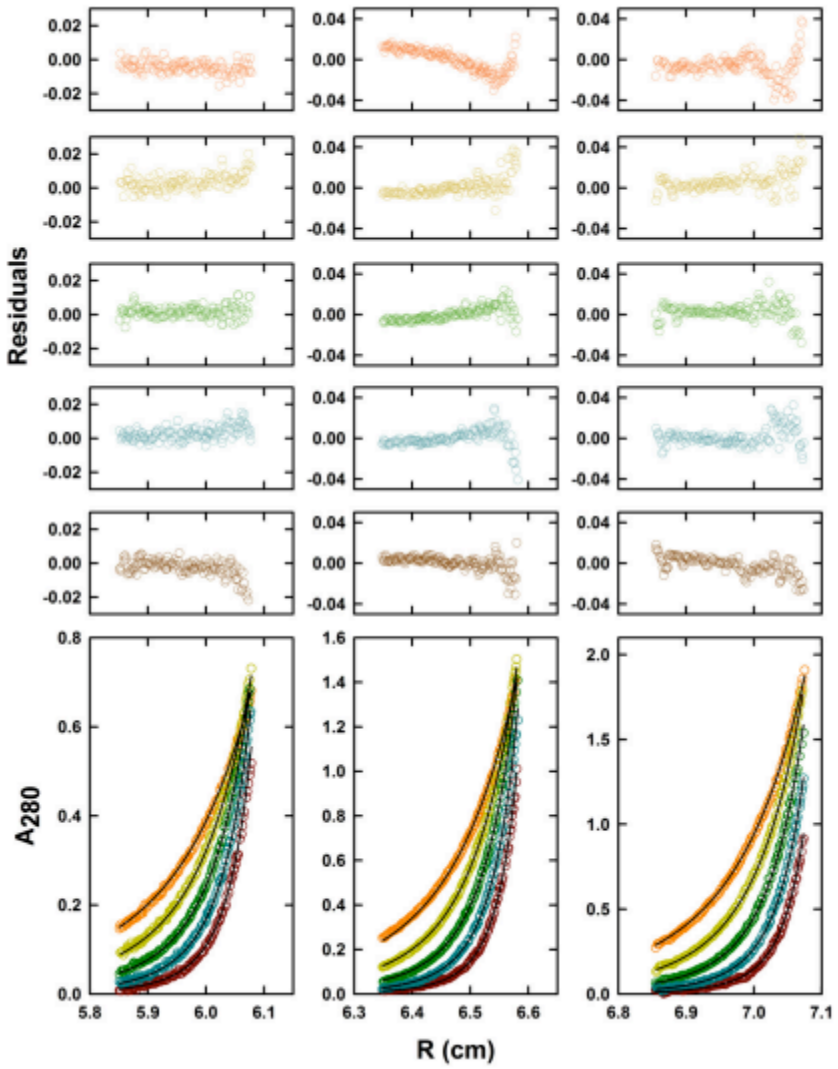
Supplementary Figures and Legends



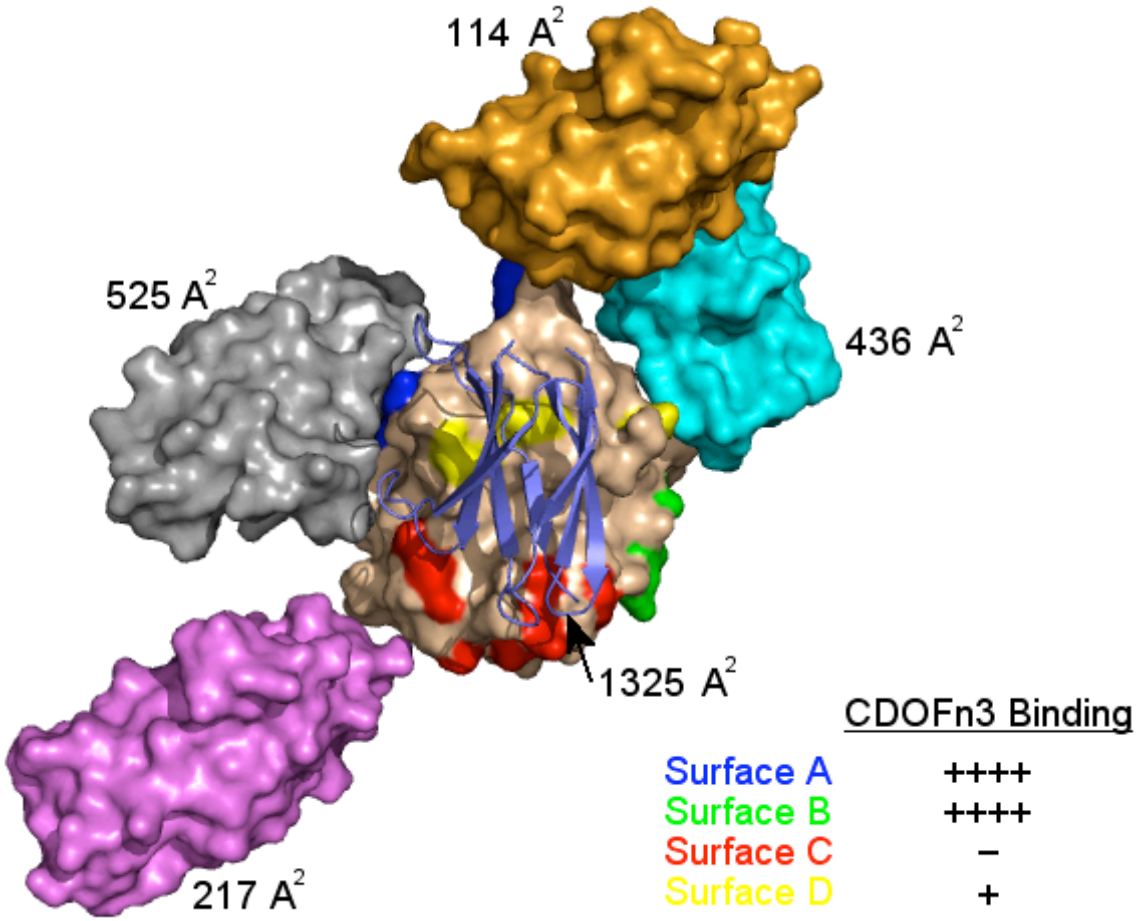
Supplementary Figure 1 | Calcium ions in fetal bovine serum (FBS) promote Shh binding to CDOFn2-3. The interaction between ShhN and CDOFn2-3 was assayed by pull-down experiments and analyzed by SDS-PAGE. Pull-down experiments were performed by incubating ShhN with CDOFn2-3-coupled resin in the presence of LMW heparin plus the additives listed above each lane. Molecular weight standards are in the leftmost lane. Results from a single gel are shown with some middle lanes omitted for clarity. HyClone FBS refers to HyClone "Fetal Clone I Bovine Serum Product", which has been treated to reduce calcium ion concentration to facilitate growth of cells in suspension. The concentration of free calcium ion in Normal HyClone FBS is ~3.4 mM and <0.5 mM in Fetal Clone I.



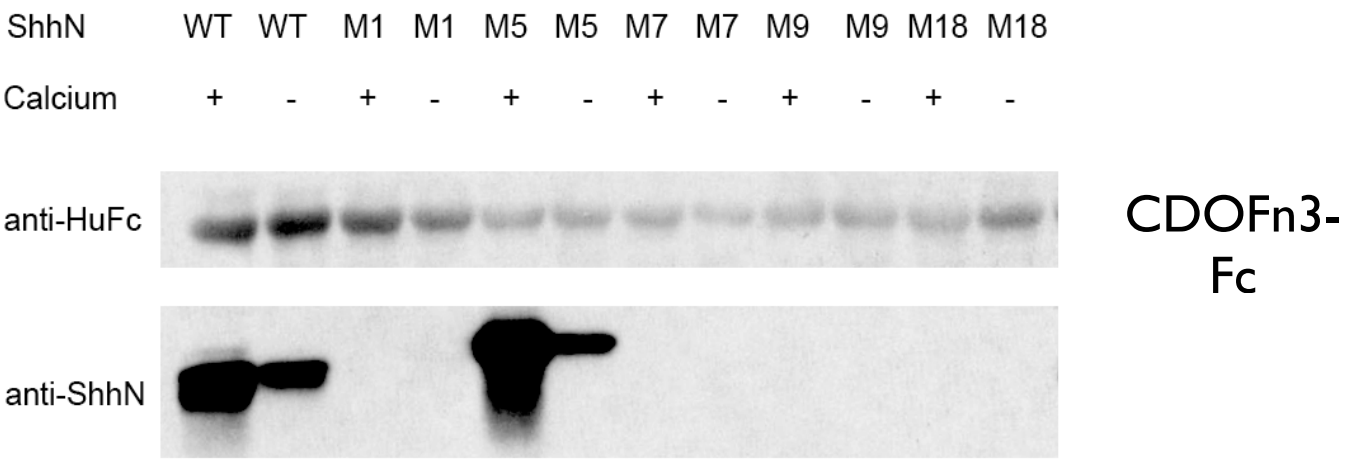
Supplementary Figure 2 | ShhN and CDOFn3 interact to form a 1:1 complex in the presence of added calcium. Sedimentation equilibrium profiles shown in terms of A_{280} versus the radius r for 1:1 stoichiometric mixtures of CDOFn3 and ShhN each at 6.9 μM (left), 13.8 μM (center) and 20.7 μM (right). All samples contained 1 mM calcium chloride and data were collected at 20 (orange), 24 (yellow), 28 (green), 32 (cyan) and 36 (brown) krpm and 4.0°C. The data were analyzed globally in terms of a reversible A + B ↔ AB interaction using mass conservation. The best fit is depicted by black lines through the experimental points. The

corresponding distributions of the residuals are shown above the plot. Analysis in terms of reversible A + A + B ↔ AAB equilibria with either CDOFn3 or ShhN as A does not return better data fits. Sedimentation equilibrium data collected in a similar fashion for 2:1 and 1:2 stoichiometric mixtures of CDOFn3 and ShhN were also best fit in terms of reversible A + B ↔ AB equilibria returning an average 4 ± 2 μM (data not shown).

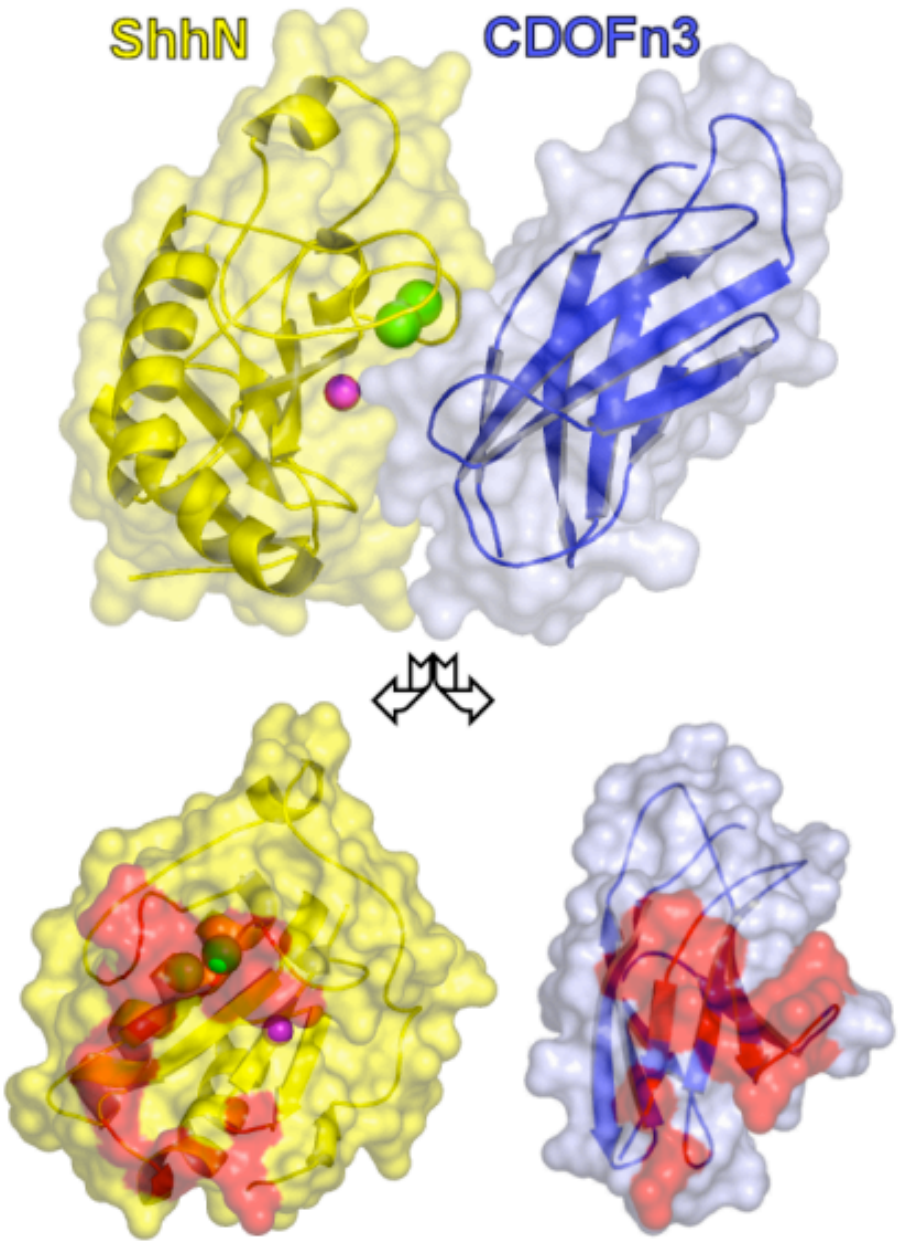
a


114 A2
525 A2
436 A2
1325 A2
217 A2
CDOFn3 Binding
Surface A ++++
Surface B ++++
Surface C –
Surface D +

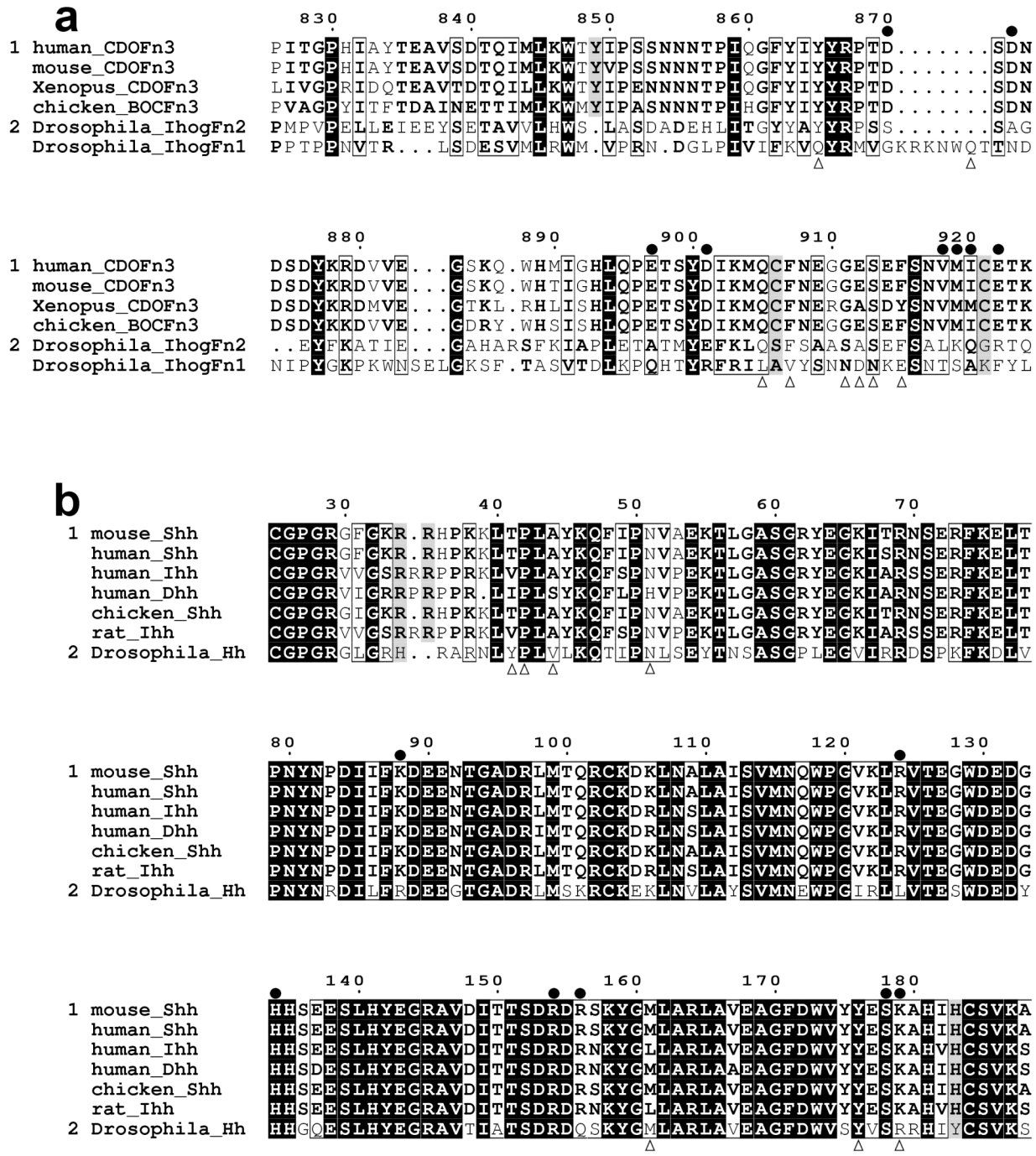
b


ShhN WT WT M1 M1 M5 M5 M7 M7 M9 M9 M18 M18
Calcium + - + - + - + - + - + -
anti-HuFc
anti-ShhN
CDOFn3-Fc

Supplementary Figure 3 | Shh surface mutations identify a functional CDO interface. (a) Shh is shown as a wheat-colored molecular surface with 4 groups of point mutations colored blue (Surface A), green (Surface B), red (Surface C), and yellow (Surface D). The functionally relevant CDOFn3 molecule as judged by mutagenesis is shown as a blue ribbon diagram. The other four CDOFn3 molecules that contact Shh in the crystal lattice are displayed as different colored molecular surfaces. The qualitative effects of each set of surface mutations on CDOFn3 binding are shown in the lower-right corner. (b) Pull-down experiments in which the ability of a CDOFn3-Fc fusion protein to pull down various mutant forms of ShhN are shown (M1: Shh K88A, R124A, R154A, S178A; M5: A44V, T41Y, K179R, Y45L; M7: E90Q, E91Q, E127Q, D132N; M9: Shh D89V; M18: E127K). The top row of lanes shows an anti-human Fc Western blot and relative expression levels. The bottom row of lanes shows an anti-ShhN Western blot and the ability of various Shh mutants to interact with CDOFn3. All Shh mutants but M5 contain residues at the Shh-CDOFn3 interface shown in Figure 1.

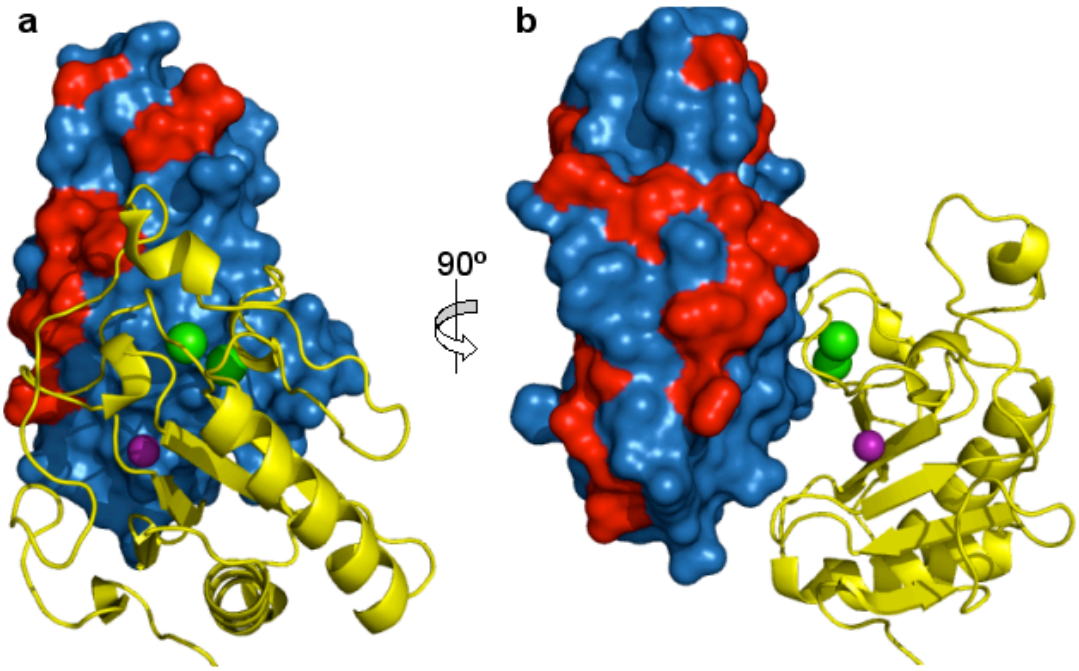


Supplementary Figure 4 | Contact surfaces on ShhN and CDOFn3. (Top) Surface representation of ShhN (yellow) complexed with CDOFn3 (blue). Calcium and zinc ions are shown as green and purple spheres, respectively. (Bottom) The ShhN and CDOFn3 surfaces displayed with the molecules separated as if opening a book relative to the top image. Surfaces composed of contact residues are colored red.



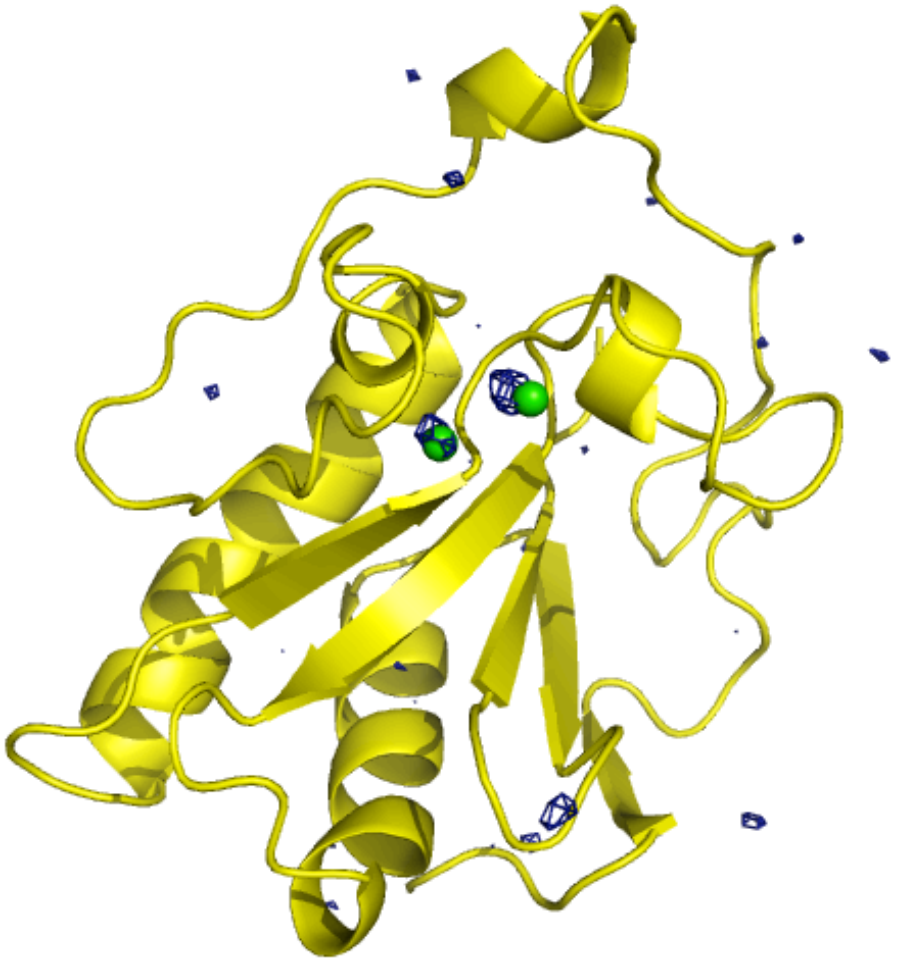
Supplementary Figure 5 | ShhN and CDO/BOC sequence alignments with contact residues indicated. (a) Alignment of amino acid sequences of the third FNIII domains of CDO and BOC from the indicated species. Residues with atoms within 4 Å of ShhN atoms in the CDOFn3/ShhN crystal structure are indicated by filled black circles. Residues in IhogFn1 within

4 Å of Hh atoms are indicated by open triangles. (b) Alignment of Hh sequences with ShhN residues with atoms within 4 Å of CDOFn3 atoms indicated by filled black circles. *Drosophila* HhN residues with atoms within 4 Å of IhogFn1 atoms are indicated by open triangles.

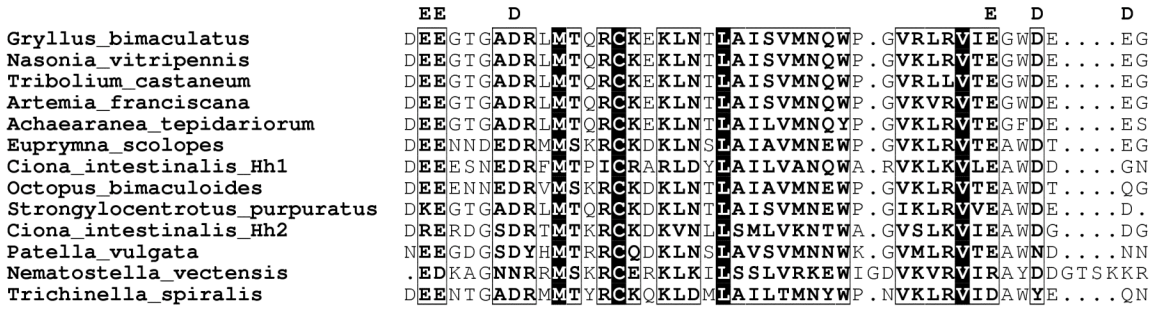


Supplementary Figure 6 | Shh binding-site is conserved between CDO and BOC.

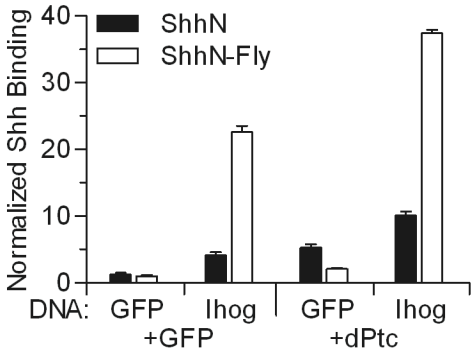
a, Complex of Shh bound to CDOFn3. Shh is shown as yellow ribbons, while calcium and zinc ions are depicted as green and purple spheres, respectively. The molecular surface of CDOFn3 is shown, with residues that are identical between human CDO and BOC colored blue and non-identical residues colored red. **b**, A view of this complex rotated 90º about a vertical axis relative to the view in **a**.



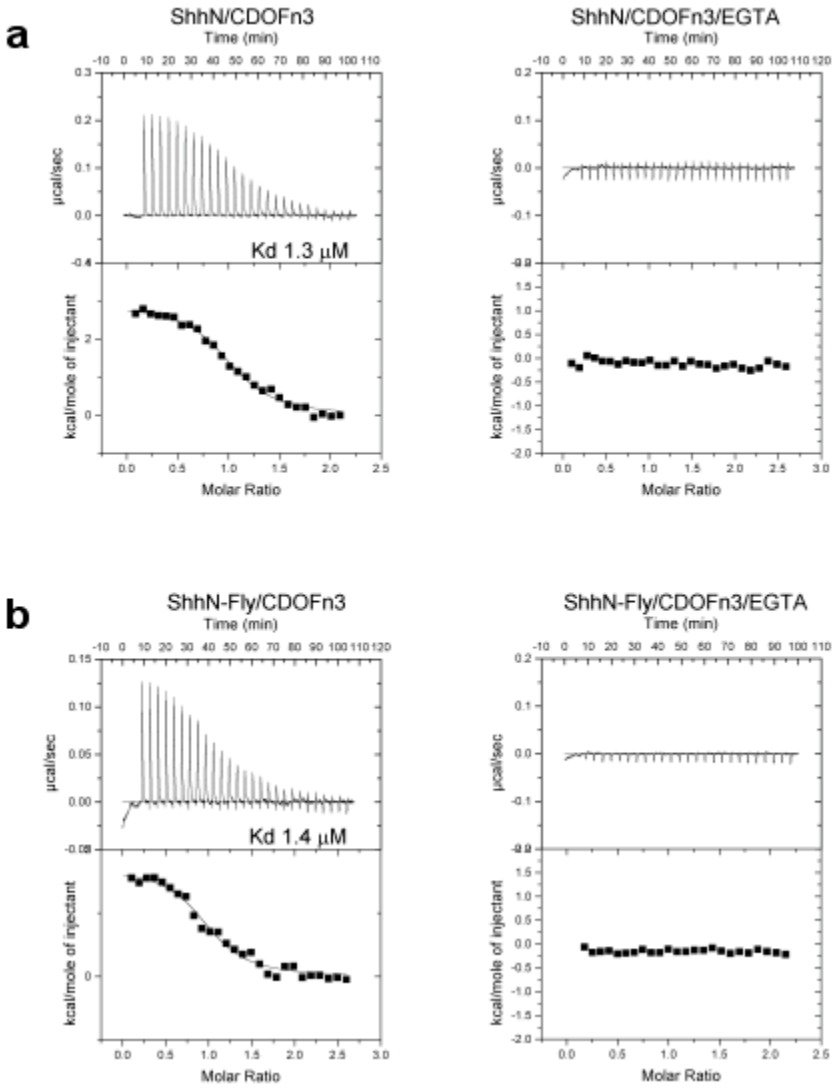
Supplementary Figure 7 | Anomalous difference Fourier map confirms identification of calcium ions. An anomalous difference Fourier map calculated with data collected at the Cu K_α edge (λ=1.54 Å) is shown in dark blue contoured at 3.2 σ. Shh is shown as yellow ribbons and the two calcium ions are depicted as green spheres. At this wavelength, calcium has a modest anomalous signal (f' = 1.28), while magnesium (f' = 0.18) and zinc (f' = 0.67) do not.



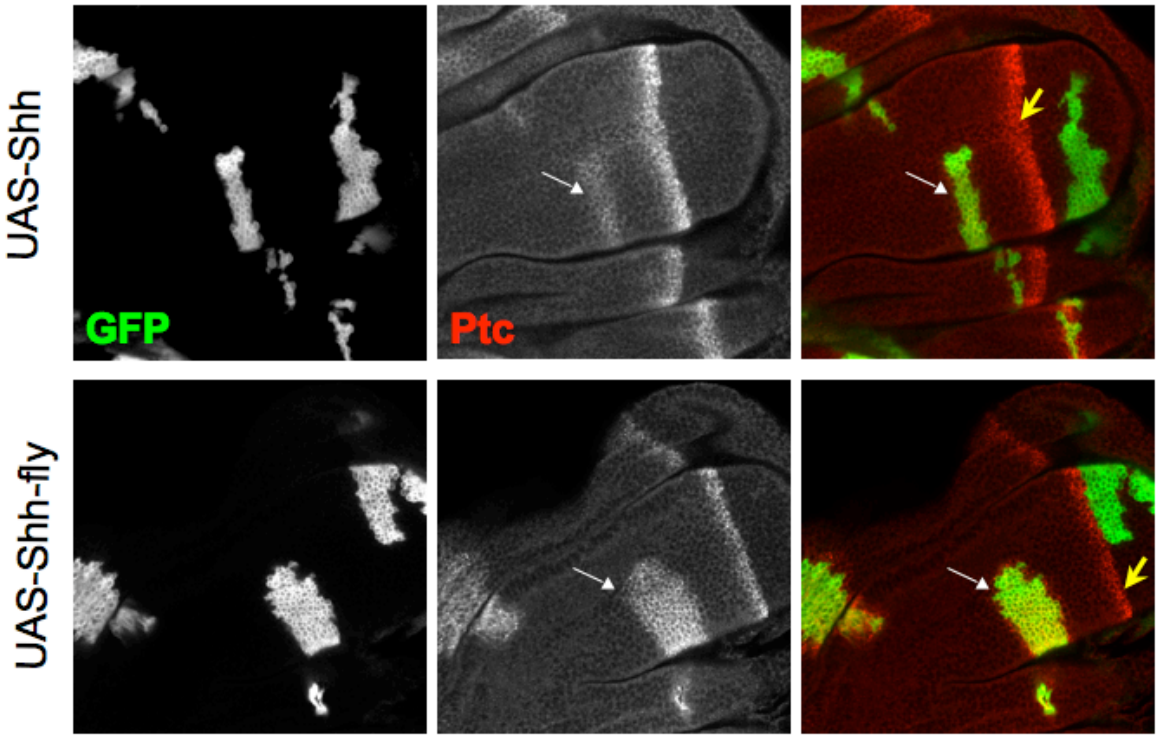
Supplementary Figure 8 | The Hh calcium-binding site is highly conserved. Of 77 identifiable Hh sequences, 64 conserve all six calcium-binding residues observed in Shh (shown in bold letters above the alignment); the other 13 sequences are shown here. The first eight sequences have a single substitution for the last aspartic acid residue (six to E, one each to G and Q), and the *Ciona intestinalis*-Hh2 and *Strongylocentrotus purpuratus* sequences have a single substitution for the first glutamic acid residue. The three remaining Hh sequences each contain more than one mutation to the calcium-binding residues.



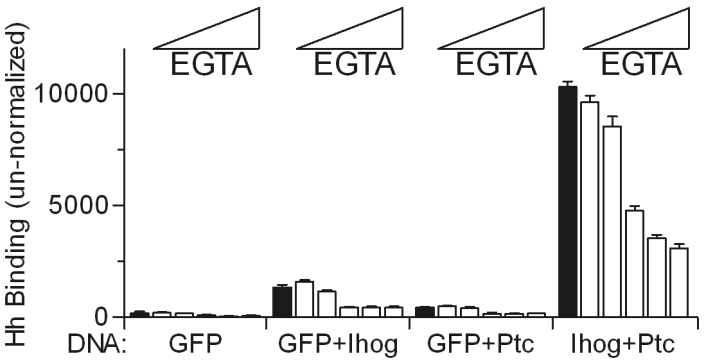
Supplementary Figure 9 | ShhN and ShhN-Fly do not bind well to dPtc. Binding of ShhN- or ShhN-Fly-luciferase to fly cells transfected with DNA encoding the indicated proteins.



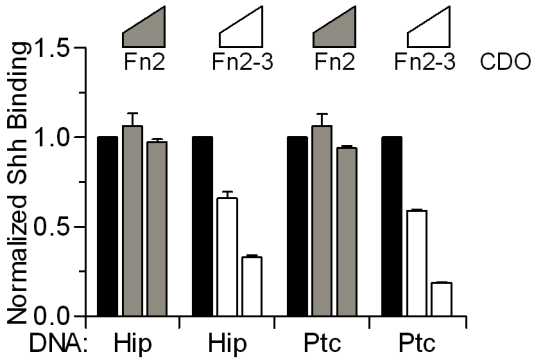
Supplementary Figure 10 | ShhN and ShhN-Fly require calcium to bind CDOFn3. ITC data for **a,** ShhN and **b**, ShhN-Fly binding to CDOFn3 in the presence (left) and absence (right) of calcium. The binding of both ShhN and ShhN-Fly to CDOFn3 is endothermic.



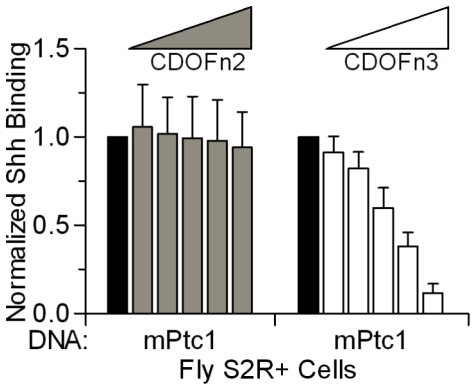
Supplementary Figure 11 | Expression of Shh-Fly induces Ptc expression to a greater extent than wild-type Shh. Expression of the Hh pathway target gene Ptc in the anterior compartment of fly wing imaginal discs carrying clones expressing wild type Shh (top panel) or Shh-Fly (bottom panel). Clones expressing Shh/Shh-Fly are marked by GFP expression. Ptc expression induced by Shh (white arrow, top panel) is much weaker than expression of endogenous Ptc at the compartment boundary (yellow arrow). Ptc expression induced by Shh-Fly (white arrow, bottom panel) is comparable to the level of endogenous Ptc (yellow arrow), and can be detected several cell diameters outside the clone. Anterior is left, ventral is up.



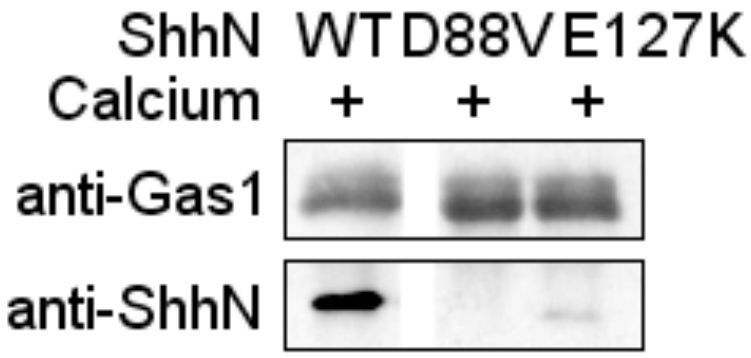
Supplementary Figure 12 | EGTA inhibits Hh binding to Ptc. *Drosophila* S2R+ cells expressing the indicated proteins were assayed for Hh binding in the presence of 1.8 mM Ca^{2+} (black bars) and increasing EGTA concentrations (1.8-2.7 mM, white bars). S2R+ cells express Ptc, and RNAi targeting of Ptc reduces binding of HhN to Ihog-overexpressing cells to near background levels. Since EGTA has no effect on binding of HhN to Ihog *in vitro*, the effects of EGTA on HhN binding to transfected S2R+ cells is likely attributable to effects of EGTA on HhN/Ptc interactions.



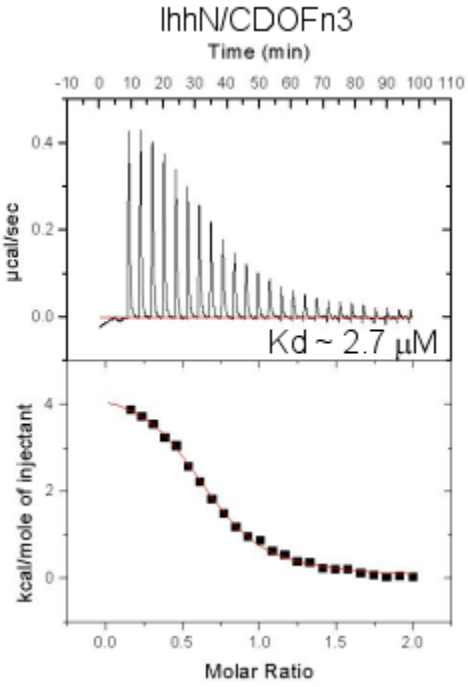
Supplementary Figure 13 | CDOFn23 competes for Shh binding to cell surface Hip and Ptc. Cell-based binding assays with wild-type Shh were performed in the presence of calcium (black bars) plus 5 and 30 μM soluble CDOFn2 (gray bars) or CDOFn23 (white bars).



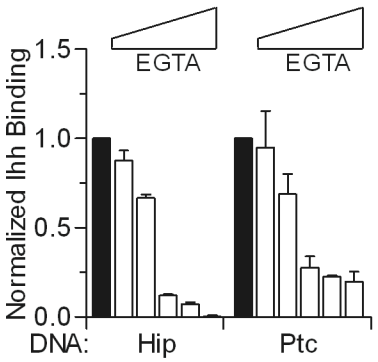
Supplementary Figure 14 | CDOFn23 competes for Shh binding to mPtc on the surface of Fly S2R+ cells. Cell-based binding assays with wild-type Shh were performed in the presence of calcium (black bars) 0.5-30 μM soluble CDOFn2 (gray bars) or CDOFn3 (white bars).



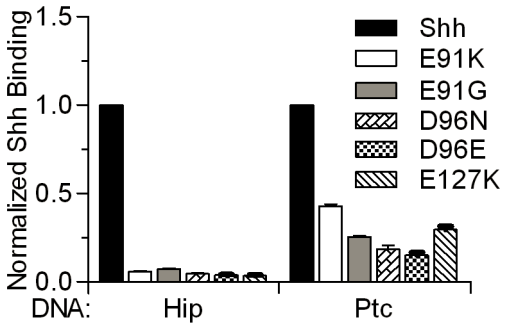
Supplementary Figure 15 | D88V ShhN binds to Gas1 less well than wild-type ShhN. Western blot of Gas1 Fc-fusion proteins binding to ShhN, ShhD88V, and ShhN harboring a mutation homologous to hIhhE131K (E127K). Intervening lanes were omitted for clarity.



Supplementary Figure 16 | IhhN binds CDOFn3 in the presence of calcium. ITC data for IhhN binding to CDOFn3 in the presence of 1 mM calcium chloride.



Supplementary Figure 17 | EGTA inhibits Ihh binding to cell surface Hip and Ptc. Cells expressing Hip or Ptc were assayed for Ihh binding in the presence of 1.8 mM Ca^{2+} (black bars) and increasing EGTA concentrations (1.8-2.7 mM, white bars).



Supplementary Figure 18 | Substitutions homologous to Ihh BDA-causing substitutions reduce Shh binding to Hip and Ptc. Normalized binding of wild-type Shh (black bars), and Shh variants harboring substitutions homologous to the Ihh BDA-causing substitutions to cells expressing Hip or Ptc.

Supplementary Tables

Table 1 X-ray refinement statistics	
Resolution (Å)	30.6-1.7
R_{work}/R_{free}	0.186/0.229
Number of atoms	
Protein	3911
Ligand/ion	6
Water	410
B-factors	
Protein	33.1
Ligand/ion	22.4
Water	34.2
R.m.s deviations	
Bond lengths (Å)	0.014
Bond angles (°)	1.42

Table 2. ShhN and CDOFn3 are monodisperse monomers in solution

Sample	Experimental $s_{20,w}$ (S)	Mass (kDa) (SV)	Calculated $s_{20,w}$ (S)	Mass (kDa) (SE)	Mass (kDa) (Sequence)
CDOFn3	1.59 ± 0.01	12.3 ± 0.5	1.54	11.5 ± 0.3	11.405
ShhN	2.08 ± 0.02	18.0 ± 0.5	1.99	18.1 ± 0.6	18.699
ShhN D89V	2.20 ± 0.03	18.0 ± 1.3	--	18.5 ± 0.4	18.683

Sedimentation velocity (SV) and equilibrium results (SE) are based on a single ideal species analysis. Calculated sedimentation coefficients are determined in HYDROPRO. The sedimentation coefficient calculated for ShhN is based on the co-ordinates of a construct truncated at the N-terminus (15 residues) with a calculated mass of 16.897 kDa.