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 μ M (left), 13.8 μ M (center) and 20.7 μ M (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 \leftrightarrow 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 $A + A + B \leftrightarrow AAB$ 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 $A + B \leftrightarrow$ AB equilibria returning an average $4 \pm 2 \mu M$ (data not shown).





a

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.

	а	830	840	850	860	870	•
1 2	human_CDOFn3 mouse_CDOFn3 Xenopus_CDOFn3 chicken_BOCFn3 Drosophila IhogFn2	PITGPHIAYT PITGPHIAYT LIVGPRIDQT PVAGPYITFT PMPVPELLEI	EAVSDTQIM EAVSDTQIM EAVTDTQIL DAINETTIM EEYSETAVV	KWTYIPSSN KWTYVPSSN KWTYIPENN KWTYIPASN KWMYIPASN	NTPIQGFYI NTPIQGFYI NTPIQGFYI NTPIHGFYI DEHLITGYYA	(YRPTD (YRPTD YRPTD YRPTD YRPSS	. SDN . SDN . SDN . SDN . SDN
-	Drosophila_IhogFn1	PPTPPNVTR.	LSD esv M	RWM.VPRN.I	GLP IV I F KV	2 YR M V GKRKNWQ A	TTND
1	human_CDOFn3 mouse_CDOFn3	880 DSDYKRDVVE DSDYKRDVVE	8: GSKQ.WI GSKQ.WI	90, HMIGHLQPETS HTIGHLQPETS	9: • • • • • • • • • • • • • • • • • • •	LO EGGESEFSNVMI EGGESEFSNVMI	СЕТК СЕТК
2	Xenopus_CDOFn3 chicken_BOCFn3 Drosophila_IhogFn2 Drosophila_IhogFn1	DSDYKRDMVE DSDYKKDVVE EYFKATIE NIPYGKPKWN	GTKL.R] GDRY.W] GAHARS] SELGKSF.T/	HLISHLQPETS HSISHLQPETS FKIAPLETATN ASVTDLKPOHT	SYDIKMQCFNI SYDIKMQCFNI 4YEFKLQSFS YRFRILAVY	ERGASDYSNVMM EGGESEFSNVMI AASASEFSALKQ SNNDNKESNTSA	CETK CETK GRTQ KFYL
	L-						
	b		40 			70. /	v n T n
	human_Shh human_Ihh human_Dhh chicken_Shh rat_Ibh	CGPGRGFGKR CGPGRVVGSRI CGPGRVIGRRI CGPGRGIGKR	. RHPKKLTP RRPPRKLVP PRPPR.LIP . RHPKKLTP	LAYKQFIPNV LAYKQFSPNV LSYKQFLPHVE LAYKQFIPNV LAYKQFIPNV	EKTLGASGRY EKTLGASGRY EKTLGASGRY EKTLGASGRY	EGKIISRNSERF (EGKIARSSERF (EGKIARNSERF (EGKITRNSERF (EGKITRNSERF	KELT KELT KELT KELT
	2 Drosophila_Hh	CGPGRGLGRH	. RARNLYPI	<u>V</u> IKOTIPNIS	SEYTNSASGPI	JEGVIRRDSPKF	KDLV
		80	9 <u> </u>		10 1	13	<u> </u>
	1 mouse_Shh human_Shh human_Ihh human_Dhh chickor, Shb	PNYNPDIIFK PNYNPDIIFK PNYNPDIIFK PNYNPDIIFK DNYNPDIIFK	DEENTGADR DEENTGADR DEENTGADR DEENTGADR	LMTORCKDKLN LMTORCKDKLN LMTORCKDRLN IMTORCKDRLN	VALAISVMNOV VALAISVMNOV VSLAISVMNOV VSLAISVMNOV	VPGVKLRVTEGW VPGVKLRVTEGW VPGVKLRVTEGW VPGVKLRVTEGW VPGVKLRVTEGW	DEDG DEDG DEDG DEDG
	rat_Ihh 2 Drosophila_Hh	PNYNPDIIFK PNYNPDIIFK PNYNRDILFR	DEENIGADR DEENTGADR DEEGTGADR	LMSKRCKEKLN	ISLAISVMNO ISLAISVMNO IVLAYSVMNE	VPGVKLRVTEGW VPGIRLLVTESW	DEDG DEDG DEDY
		140	150	160	170	180	
	1 mouse_Shh human_Shh human_Ihh human_Dhh	HHSEESLHYEO HHSEESLHYEO HHSEESLHYEO HHSDESLHYEO	GRAVDITTSI GRAVDITTSI GRAVDITTSI GRAVDITTSI GRAVDITTSI	DRDRSKYGML DRDRSKYGML DRDRNKYGLL DRDRNKYGML	ARLAVEAGFDW ARLAVEAGFDW ARLAVEAGFDW ARLAAEAGFDW ARLAAEAGFDW	VYYESKAHIHC VYYESKAHIHC VYYESKAHVHC VYYESKAHVHC	SVKA SVKA SVKS SVKS
	rat_Ihh 2 Drosophila_Hh	HHSEESLHYE HHGQESLHYE	GRAVDITISI GRAVDITISI GRAVTIATSI	DRDRNKYGLL DRDQSKYGML A	ARLAVEAGFD ARLAVEAGFD ARLAVEAGFD	VYYESKAHVHC VYS <u>YVSRRH</u> IYC	SVKA SVKS SVKS

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 90° 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.

	EE	D								Е	D	D
Gryllus_bimaculatus	DEEGI	GADRI	MTQF	CKE	KLNI	I A I	SVMN	2₩ P.	GVRLR	VIE	WDE	EG
Nasonia_vitripennis	DEEGI	[G ADR]	MTQF	CKE	KLNI	ΪΑΙ	SVMN	Q₩P.	GVKLR	VTE 🤆	WDE	EG
Tribolium_castaneum	DEEGI	[G ADR]	MTQF	CKE	KLNI	ΙΑΙ	SVMN	2₩P.	GVRLL	VTE 🤆	WDE	EG
Artemia_franciscana	DEEGI	[G ADR]	<u>, M t q f</u>	CKE	KLNI	ΙΑΙ	SVMN	2₩P.	GVKVR	VTE 🤆	WDE	EG
Achaearanea_tepidariorum	DEEGI	[G ADR]	MTQF	CKE	KLNI	ΪΑΙ	LVMN	QYP.	GVKLR	VTE	FDE	ES
Euprymna_scolopes	DEENN	ID EDR M	1 MS KF	CKD	KLNS	ΓΑΙ	AVMNE	EWP.	GVKLR	VTE 🛛	WDT	EG
Ciona_intestinalis_Hh1	DEEES	5 N EDR F	MTPI	CRA	RLDY	ΊΑΙ	LVAN	2WA.	RVKLK	VLE Z	WDD	GN
Octopus_bimaculoides	DEEEN	1NEDR	MSKF	CKD	KLNI	ΙΑΙ	AVMNE	EWP.	GVKLR	VTEZ	WDT	QG
Strongylocentrotus_purpuratus	DKEGI	rg adr i	MTQF	CKD	KLNI	ΊΑΙ	SVMNE	EWP.	GIKLR	VVE A	WDE	D.
Ciona_intestinalis_Hh2	DRERI	G SDR I	MTKF	CKD	KVNI	ISM	ILVKN:	CWA.	GVSLK	VIE 🛛	WDG	DG
Patella_vulgata	NEEGI	GSDYH	IMTRF	CQ D	KLNS	L AV	SVMN	NWK.	GVMLR	VTE Z	WND	NN
Nematostella_vectensis	. EDKA	A G NNR F	MSKF	CE R	KLKI	ISS	LVRKE	E₩IG	DVKVR	VIRZ	YDD	GTSKKR
Trichinella_spiralis	DEENI	[G ADR]	1 MT YF	CK Q	KLDM	1 A I	LTMN	ζ₩Ρ.	NVKLR	VID P	WYE	$\dots QN$

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 Ca²⁺ (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 Ca²⁺ (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	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				

Sample	Experimental	Mass (kDa)	Calculated	Mass (kDa)	Mass (kDa)
	s _{20,w} (S)	(SV)	s _{20,w} (S)	(SE)	(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

Table 2. ShhN and CDOFn3 are monodisperse monomers in solution

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.