Hedgehog-Interacting Protein is a multimodal antagonist of Hedgehog signalling

Hedgehog (HH) morphogen signalling, crucial for cell growth and tissue patterning in animals, is initiated by the binding of dually lipidated HH ligands to cell surface receptors. Hedgehog-Interacting Protein (HHIP), the only reported secreted inhibitor of Sonic Hedgehog (SHH) signalling, binds directly to SHH with high nanomolar affinity, sequestering SHH. Here, we report the structure of the HHIP N-terminal domain (HHIP-N) in complex with a glycosaminoglycan (GAG). HHIP-N displays a unique bipartite fold with a GAG-binding domain alongside a Cysteine Rich Domain (CRD). We show that HHIP-N is required to convey full HHIP inhibitory function, likely by interacting with the cholesterol moiety covalently linked to HH ligands, thereby preventing this SHH-attached cholesterol from binding to the HH receptor Patched (PTCH1). We also present the structure of the HHIP C-terminal domain in complex with the GAG heparin. Heparin can bind to both HHIP-N and HHIP-C, thereby inducing clustering at the cell surface and generating a high-avidity platform for SHH sequestration and inhibition. Our data suggest a multimodal mechanism, in which HHIP can bind two specific sites on the SHH morphogen, alongside multiple GAG interactions, to inhibit SHH signalling.
The Hedgehog (HH) morphogen pathway fulfills crucial functions in growth and morphogenesis, whilst dysregulation leads to developmental disorders and cancer. The secreted N-terminal domain of SHH (ShhN) is generated from a 45 kDa precursor, undergoing intein-based cleavage to couple an esterified cholesterol molecule to the C-terminus. A subsequent step involves the N-terminal attachment of a palmitoyl moiety to produce the fully active lipid-modified signalling ligand (palmitoylated and cholesterylated ShhN; pShhN). Signalling is activated by binding of pShhNc to the extracellular domains of transmembrane protein PTCH1. Recent structural studies show that PTCH1 and pShhNc form a 2:1 complex, with one molecule of PTCH1 engaging pShhNc at a conserved high-affinity interface involving the conserved SHH zinc- and calcium-binding sites ("protein-protein interface"), and the other at the terminal SHH-palmitoyl and -cholesteryl moieties ("lipid interface"). The PTCH1:pShhNc interaction releases inhibition of the G-protein coupled receptor Smoothened (SMO), which ultimately results in activation of target genes via the GLI transcription factors. When no HH ligand is present, PTCH1 constitutively inhibits SMO signaling, potentially by preventing access to cholesterol or a similar sterol molecule. While the HH ligand is present, PTCH1 constitutively inhibits SMO, preventing the activation of target genes via the GLI transcription factors. When no HH ligand is present, PTCH1 constitutively inhibits SMO, preventing the activation of target genes via the GLI transcription factors. When no HH ligand is present, PTCH1 constitutively inhibits SMO, preventing the activation of target genes via the GLI transcription factors.

Extracellular distribution of pShhNc is key to the activation of correct signalling responses. This is controlled by a combination of co-receptor signalling, the glycosaminoglycan (GAG) chains of heparan sulphate proteoglycans (HSPGs), and the assembly of pShhNc into multimers. The HH pathway is modular, with several other essential cell surface receptors alongside PTCH1. For example, the immunoglobulin superfamily members CDO and BOC bind directly to SHH via the conserved interface involving the pShhNc metal-binding sites. The metal-binding sites are also crucial for SHH interactions with the vertebrate-specific HH antagonist Hedgehog-Interacting Protein (HHIP), one of the most common devastating lung diseases in humans. HHIP is the only secreted inhibitor of HH signalling essential for the development of the lung, cartilage, and brain. HHIP downregulation is associated with HH-dependent tumourigenesis and variants at the HHIP locus are linked to Chronic Obstructive Pulmonary Disease (COPD), one of the most common devastating lung diseases in humans. HHIP is composed of an N-terminal domain (HHIP-N) that shows weak sequence homology to the cysteine-rich domain (CRD) superfamily, typically involved in small molecule-binding. The C-terminal domain of HHIP is composed of a β-propeller and two EGF repeats (HHIP-C). We and others previously determined structures of HHIP-C in complex with human HH ligands ShhN and DhhN, respectively. HHIP utilises a loop inserted into blade 3 of its β-propeller to bind to the HH metal-binding site by directly coordinating the Zn ion. This suggests that HHIP inhibits HH function by sequestering the HH morphogen, and acts as a decoy receptor. Our recent studies identified a role for HHIP—high affinity interaction with the GAG chains of heparan sulphate proteoglycans (HSPGs), as well as uncovering a cluster of residues in HHIP-N involved in this process. However, the role of the HHIP-N CRD and its potential small molecule binding properties remained elusive.

In this work, we use a combination of structural, biophysical and cellular studies to characterise the HHIP N-terminal region, revealing an unexpected GAG binding domain and a CRD with a small molecule-binding fold. We show that HHIP-N is necessary to convey the full signal inhibition by HHIP in response to pShhNc, and that the purified CRD binds to a mimic of the cholesterylolated HH C-terminus. Importantly, we also identify and structurally characterise two distinct GAG binding sites within HHIP-N and HHIP-C, respectively, and show that HHIP-C assembles into large HHIP-GAG oligomers. Our results reveal that HHIP uses a modular mechanism for SHH inhibition. HHIP targets both the SHH metal-binding and lipid-modification sites recognised by PTCH1, while potentially staying localized on the cell surface via HHIP-GAG interactions.

**Results**

**Structure of the HHIP N-terminal domain reveals a CRD fold.** We expressed the N-terminal domain of HHIP (HHIP-N) using mammalian expression in HEK293T cells. Purified HHIP-N was crystallised in the presence of the GAG mimic sucrose octasulphate (SOS). The structure of the HHIP-N:SOS complex was determined using the single anomalous dispersion (SAD) method from native sulphur atoms. A multi-crystal approach was taken in which 24 data sets were collected from 8 isomorphous crystals, utilising both mini-kappa and inverse beam strategies to maximise the observed anomalous signal. This allowed us to determine the structure of HHIP-N in complex with SOS at a resolution of 2.7 Å, with one molecule in the crystallographic asymmetric unit. HHIP-N possesses an elongated globular fold with a unique N-terminal GAG-binding domain (GBD) and a C-terminal CRD (Fig. 1b). HHIP-N is stabilised by a total of 6 disulphide bonds. The GBD of HHIP-N binds SOS, and contains a single helix and flanking loop regions.

CRDs can bind to various small molecules, including folate (folate receptors; FRα/β) and riboflavin (riboflavin-binding protein; RFBP), as well as lipids and sterols, such as cholesterol (SMO, NPC1). HHIP-N is composed of 5 helices (α1-α5) stabilised by three intra-CRD disulphide bonds (IV: C103-C152, V: C141-179 and VI: C145-168). All disulphide bonds were identified as individual sites in the anomalous difference map, and treatment of these as ‘super-sulphurs’ was vital for substructure determination and structure solution (Fig. 1c).

CRDs can bind to various small molecules, including folate (folate receptors; FRα/β) and riboflavin (riboflavin-binding protein; RFBP), as well as lipids and sterols, such as cholesterol (SMO, NPC1). HHIP-N comprises a shallow and elongated binding site consisting of loops that fold over a deep cavity inaccessible to solvent, whereas a ‘groove’ comprises a shallow and elongated solvent-accessible binding cleft, which does not undergo major conformational changes upon ligand binding. We have now included our HHIP-N structure in this analysis and classified it as a member of the pocket-type sub-family, with related structural homologues in the CRDs of NPC1, RFBP and FRα/β.

Previously, we performed an evolutionary structural analysis to classify known CRDs into two distinct sub-families, containing ‘pockets’ or ‘grooves’ as ligand-binding elements. In this context, a ‘pocket’ refers to a CRD in which the ligand-binding site consists of loops that fold over a deep cavity inaccessible to solvent, whereas a ‘groove’ comprises a shallow and elongated solvent-accessible binding cleft, which does not undergo major conformational changes upon ligand binding. We have now included our HHIP-N structure in this analysis and classified it as a member of the pocket-type sub-family, with related structural homologues in the CRDs of NPC1, RFBP and FRα/β. Additionally, this analysis identifies sperm-egg fusion protein JUNO as being a pocket-type CRD which has diverged from typical folate receptors.

The HHIP-N CRD comprises a structural ‘scaffold’ composed of helices α1, α3 and α5 that are stabilised by disulphide bridges, a feature common to all CRD family members. Our HHIP-N structure, we observed two stretches of disordered residues, which link the N-terminal domain and CRD helix α1 (DL1), and the loop regions between helices α2 and α3 (DL2), respectively (Fig. 2a). In FRβ, the equivalent regions form the majority of a folate-binding pocket (Fig. 2b), suggesting that DL1 and DL2 very likely occupy a similar region. The modes of ligand binding by pocket-type CRDs are illustrated in Supplementary Fig. 3b, c and Supplementary Fig. 3f, while ligand binding by groove-type CRDs is detailed in Supplementary Fig. 3d-e. Helices analogous to HHIP-N α1-5 form an equivalent ‘scaffold’ in the pocket-type CRDs NPC1, RFBP and JUNO, within which

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ligand-binding loops are positioned for small molecule binding. A ligand-binding loop comparable to HHIP-N DL1 is also present in the groove-type CRDs of Fz8 and SMO (Supplementary Fig. 3d, e). This structural and evolutionary analysis suggests that loops DL1 and DL2 could potentially form a binding pocket for a physiologically-relevant small molecule ligand within the HHIP-N CRD region.

Next, we tested the function of HHIP-N in a SHH signalling assay, based on measuring the mRNA levels of the HH target gene Gli1 in NIH/3T3 cells. We co-cultured PANC-1 human pancreatic ductal adenocarcinoma cells expressing pShhNc48 to activate the HH pathway. The purified full-length HHIP extracellular domain only lacking the C-terminal helix (residues 39-670, HHIP-ΔHx, Fig. 1a) was able to inhibit HH signalling more efficiently than HHIP-C (Fig. 2c), which lacks HHIP-N but contains the high-affinity SHH binding site26,27. We next asked whether the interaction of HHIP-N with the SHH lipid modifications could account for this increase of inhibition, given the structural similarities with small molecule-binding CRDs. For this, we activated the HH pathway with an N-terminal palmitoylated-SHH peptide (Palm-ShhN15)49, which does not interact with HHIP-C. In this context, HHIP-ΔHx was unable to inhibit signalling (Fig. 2d), excluding a role of the SHH N-terminal palmitoyl moiety in HHIP-N binding.

We recently showed that the SHH C-terminal cholesterol moiety is important for SHH-PTCH1 interactions and activation of HH signalling12. Using a similar isothermal titration calorimetry (ITC) assay, we tested whether a PEGylated cholesterol molecule (mimicking the C-terminus of pShhNc) binds to HHIP. PEG-cholesterol bound specifically to HHIP-N (KD = 23 µM) (Fig. 2e), whereas free PEG did not bind to HHIP-N (Fig. 2f). Due to the high heat of dilution when PEG-cholesterol is titrated into buffer, a series of controls were conducted (Supplementary Fig. 4). Thermodynamic signature plots for HHIP-N compare favourably with those obtained for the recently published interaction of PEG-cholesterol with the canonical Hh receptor PTCH112, revealing a high enthalpic contribution to ΔG suggesting a hydrophobic interaction50.

Fig. 1 The structure of HHIP-N. a HHIP domain organisation and construct design. b Cartoon representation of HHIP-N in complex with SOS. In the top panel, the CRD region is depicted in salmon and the N-terminal GAG-binding domain in dark blue. Two disordered loops (DL1 and DL2) are displayed as dashed salmon lines. In the lower panel, the HHIP-N:SOS complex is shown in rainbow colouring (blue: N-terminus, red: C-terminus). The CRD helices are labelled and disulphide bonds are numbered using Roman numerals. c Maps calculated from final HHIP-N model. The 2Fo-Fc map is shown in blue, contoured at 1σ. An anomalous difference map calculated from S-SAD data used for phasing is displayed as a yellow mesh contoured at 4σ. Corresponding disulphide bonds are numbered using Roman numerals. d Structural phylogenetic analysis of CRDs (see Supplementary Table 2).
This supports that the interaction between PEG-cholesterol and HHIPN is specific. Taken together, the enhancement of HH signalling inhibition by HHIP in the presence of HHIP-N, combined with structural homology to small molecule-binding pocket-type CRDs and observed binding to PEG-cholesterol suggests an interaction between HHIP-N and HH-linked cholesterol. Hence, we propose a model whereby HHIP binds to SHH in a multimodal manner, utilising the SHH metal-binding sites for HHIP-C binding and potentially targeting the cholesterol attachment site for HHIP-N binding. Such an arrangement could be accommodated in a 1:1 SHH-HHIP complex, as HHIP-N and HHIP-C are separated by a 25-residue long, likely flexible linker that could position the SHH-linked cholesterol to interact with HHIP-N and the SHH-metal.
binding site to interact with HHIP-C within the same molecule (Supplementary Fig. 5).

The HHIP N-terminal domain contains a GAG-binding domain. To shed light onto the previously reported GAG-binding properties of HHIP-N\(^{28}\), we determined structures of apo and SOD-bound HHIP-N to resolution of 2.6 Å and 2.7 Å, respectively (Supplementary Table 1). In the apo HHIP-N structure, the N-terminal GAG-binding domain is disordered, while the CRD region is inherently structured (Fig. 3a). This suggests that interaction of HHIP-N with GAG molecules triggers a transition to a folded state, forming an α-helix between residues 50 and 58. Each HHIP-N molecule interacts with 3 SOS molecules (Fig. 3b, Supplementary Fig. 6). SOS is bound to a positively charged surface localised to the HHIP-N N-terminal domain (Fig. 3c), forming electrostatic interactions with a cluster of 6 charged residues in the N-terminal α-helical residues 50–58 to contact a SOS molecule. Mutagenesis of SOS-interacting basic HHIP residues (marked with asterisks in Fig. 3d) were previously observed to weaken the interaction between HHIP and heparin, validating our observed HHIP-N:SOS interface\(^{28}\). The N-terminal domain of HHIP-N is similar to other structurally characterised CRDs, having evolved a GAG-binding function in a discrete domain alongside a conserved small molecule-binding fold. Additionally, we report structural insights into the major secondary structural arrangements of HHIP-N, a GAG-binding protein, upon sugar coordination.

Structure of the HHIP C-terminal domain in complex with heparin. Our previous analysis of HHIP:GAG interactions identified multiple GAG-binding motifs in HHIP, since a construct that contains a deletion of HHIP-N was still able to bind to heparin\(^{27}\). To decipher the GAG-binding properties of HHIP, we conducted binding assays using three domain deletion constructs (Fig. 1a). First, we tested GAG binding using heparin affinity chromatography (Fig. 3e). Both HHIP-C and HHIP-N show binding to a heparin column, albeit with weaker affinity when compared to HHIP-ΔHx. Furthermore, HHIP-N, HHIP-C and HHIP-ΔHx are all cell surface-associated at physiological pH and ionic strength (Supplementary Fig. 7). Taken together, our analysis suggests that both HHIP-N and HHIP-C contribute to the observed affinity for GAG chains.

To structurally characterise HHIP-C:GAG interactions, we determined the 2.7 Å resolution crystal structure of HHIP-C in complex with a 30-mer heparin molecule (Supplementary Table 1, Supplementary Fig. 8a). HHIP-C comprises an N-terminal 6-bladed β-propeller and 2 C-terminal EGF repeats, previously identified in the complex of HHIP-C with HhN ligands\(^{26,27}\). The heparin chain contacts two separate clusters of HHIP surface residues (GAG ‘site 1’ and GAG ‘site 2’) at either end of the molecule (Fig. 4a, Supplementary Fig. 8). In this arrangement, a HHIP-C anti-parallel dimer coordinates one single heparin chain (Fig. 4b) by using a central positively-charged region formed by residues from both HHIP protomers (Fig. 4c). The HHIP-C chains in the dimer are essentially identical (r.m.s.d. of 0.21 Å for 322 equivalent Ca atoms), and show little structural difference to previously published HHIP-C structures (e.g. r.m.s.d. of 0.31 Å for 331 equivalent Ca atoms, PDB ID: 2WFT\(^{36}\)). The heparin backbone displays a right-handed helical structure with roughly 4 sugars per turn, in agreement with previous structural studies\(^{51,52}\). A total of 8 monosaccharides are resolved in the structure, running from a reducing (O1; sugar I) to a non-reducing end (O4; sugar VIII) and forming hydrogen bonds with several polar side chains (Fig. 4d). We also determined the crystal structure of a HHIP-C:SOS complex, which exhibits the equivalent anti-parallel, dimeric HHIP arrangement bound to two SOS molecules coordinated at the same site compared to heparin (Supplementary Table 1, Supplementary Figs. 8b, 9 and 10). In summary, our HHIP-C crystal structures in complex with GAG molecules identify two discrete GAG-binding sites on the surface of HHIP-C, which are distinct and non-overlapping with the HhN-binding interface (Fig. 4e), thus suggesting that both GAG- and HhN-binding can occur simultaneously.

GAG interactions control oligomerisation of HHIP. Our previous study on HHIP:GAG interactions identified low micromolar affinities for the interactions between HHIP-C and the GAGs heparin, hepan sulphate (HS) and chondroitin sulphate (CS)\(^{28}\). To experimentally validate our HHIP-C:GAG complex structures, we generated a HHIP mutant in which 6 positively-charged residues involved in GAG recognition were mutated to glutamate (K277E/R328E/R350E/K569E/R610E/R613E, HHIP-C Glu mutant; Fig. 4d) and analysed binding to GAGs. Using surface plasmon resonance (SPR) with GAGs immobilised on the chip, we observed that the HHIP-C Glu mutant almost completely abolishes binding to heparin, HS and CS, when compared to wild-type (HHIP-C WT) (Fig. 5a). Furthermore, we analysed our observed HHIP-C:GAG interactions by a combination of mutagenesis and heparin affinity chromatography. In addition to the HHIP-C Glu mutant, we generated glycosylation mutants (Supplementary Fig. 10a). N-linked glycans were inserted into the GAG-interacting surfaces of either the HHIP β-propeller (‘ΔAG site 1’) or EGF repeats (‘ΔAG site 2’), as well as combination of both (‘ΔAG sites 1 + 2’). As expected, all mutants showed reduced heparin binding affinity relative to HHIP-C WT (Supplementary Fig. 11b). Next, we tested the effect of HHIP-C WT or HHIP-C Glu mutant in SHH signalling assays using NIH/3T3 cells (Fig. 5b). We observed that the HHIP-C Glu mutant showed significantly less efficient SHH inhibition. To further probe whether this effect results from loss of GAG-binding, we knocked out EXT3, a key enzyme in the biosynthesis of cell-surface heparan sulphate (HS) (Supplementary Fig. 12a–d)\(^{32}\). We confirmed that single Ext3\(^{-/-}\) NIH/3T3 clones lack surface HS chains by showing markedly reduced binding to a scFv antibody.
**Fig. 3** HHIP-N:GAG interactions. **a, b** Comparison of the apo HHIP-N structure (**a**) and HHIP-N:SOS complex. Colour coding is as in Fig. 1b. For HHIP-N:apo, the disordered GAG-binding domain is shown as a dashed line. In the HHIP-N:SOS complex three SOS molecules interact with the GAG-binding domain. **c** Electrostatic surface potential of the HHIP-N:SOS complex, displayed from $-10 kT/e$ to $+10 kT/e$. **d** Close-up view of HHIP-N:SOS binding site showing the region marked in C. Hydrogen bonds are shown as dashed lines (**c, d** rotated 90° around the x-axis relative to B). Mutations previously observed to decrease GAG-binding affinity and cause dysfunction of HHIP are marked with asterisks\(^2\). **e** Heparin affinity chromatography of different HHIP constructs eluted from a linear gradient of NaCl from 20 to 1000 mM (green line). Traces corresponding to different constructs are coloured according to the accompanying legend (inset).
**Fig. 4 The structure of the HHIP-C:heparin complex.**

a Cartoon representation of the HHIP-C monomer structure with the β-propeller domain in slate, the EGF repeats in magenta, the GAG-binding sites in yellow and the HhN-binding site in orange. 

b Arrangement of the two HHIP-C molecules (cartoon representation) around a centrally-coordinated heparin molecule (stick representation).

c Close-up view of the boxed region in B, displaying side chain:heparin interactions. Hydrogen bonds are displayed as black dashed lines and sugar residues are numbered using roman numerals (odd, N,O6-disulphoglucoasamine; even, O2-Sulphoiduronic acid).

d Electrostatic surface potential shown from red (−8 kT/e) to blue (+8 kT/e) with heparin chain shown as sticks.

e Comparison of heparin (yellow sticks) and HhN (orange cartoon) interaction sites on HHIP-C electrostatic surface.
HHIP-C Glu mutant

Fig. 5 Analysis of HHIP-C:GAG interactions and oligomerisation. a Comparative SPR curves for HHIP-C WT (light blue) and HHIP-C Glu mutant (red) binding to heparin, heparan sulphate (HS) and chondroitin sulphate (CS); b Quantification of HH pathway inhibition with the addition of HHIP-C WT or HHIP-C Glu mutant. Relative levels of Gli1 mRNA were quantified from 10 independent experiments, with statistical significance calculated using a two-tailed, paired t-test with \( p = 0.045 \); Data are presented as mean values \( \pm \) SEM. c Model for cell surface HHIP-C oligomeric assembly. GAG chains (represented by heparin, yellow) stabilise a core anti-parallel HHIP-C dimer (surface; black and white) and can then recruit further HHIP-C chains (light blue, violet) to form long oligomeric chains. This facilitates binding and antagonism of multiple HhN ligands (orange). d, e AUC experiments to assess the oligomerisation of HHIP in solution, comprising HHIP-C (d) and HHIP-C:heparin 30-mer complex (e). f, g As in (c, d), comprising HHIP-C Glu mutant (e) and HHIP-C Glu mutant mixed with 30-mer heparin (f). Source data are available as Source Data file.

(HS20) that is known to recognize multiple HS chains\(^{54,55}\) (Supplementary Fig. 12e). These Extl3\(^{−/−}\) clones were responsive to SHH, showing that the machinery required to receive and respond to ligands was unaffected by the loss of HS chains (Supplementary Fig. 12e). Compared to their individual level of SHH activation, wildtype HHIP was a less effective inhibitor in both knock-out cell lines than in their parental cell line. Even more importantly, both HHIP wildtype and Glu mutant inhibited the HH response in the HS-deficient cells to similar degrees, in contrast to the parental cell line where Glu mutant HHIP was a less effective inhibitor (Supplementary Fig. 12e). This demonstrates that the interaction between HHIP-C and GAGs is critical for maximal HH pathway inhibition.

Our HHIP:Cheparin complex structure also suggests that GAGs can mediate the formation of long oligomeric chains of HHIP (Fig. 5c). We observe two HHIP oligomerisation interfaces - an anti-parallel ‘head-to-tail’ heparin-bound HHIP-C dimer (Supplementary Fig. 13a) with a total buried surface area of 603 Å\(^2\) (Supplementary Fig. 13a), and a ‘head-to-head’ dimer with a buried surface area of 981 Å\(^2\) (Supplementary Fig. 10b) in the crystal. The ‘head-to-tail’ dimer interface is smaller than physiological dimer interfaces and is likely stabilised by the heparin molecule (PISA ΔG\(^{\text{P-value} = 0.41, \text{shape complementarity score} = 0.75}\)\(^{45,46}\), while the larger ‘head-to-head’ interface is more likely to be physiological (PISA: ΔG\(^{\text{P-value} = 0.43, \text{shape complementarity score} = 0.52}\)). Previous studies using multi-angle light scattering (MALS) showed that the apo HHIP-C is monomeric at a concentration of approximately 10 μM\(^{26}\). To test whether oligomers observed in the crystal structure exist in solution at higher concentrations (potentially mimicking the local concentrations at the cell membrane), we performed sedimentation velocity analytical ultracentrifugation experiments (AUC). This revealed a predominantly monomeric apo HHIP-C population (4 S, 53 kDa), with minor populations of dimers (5 S, ~100 kDa) and trimers (6 S, ~150 kDa) also observed (Fig. 5d). As expected, addition of the heparin 30-mer triggers a drastic shift to increased sedimentation rates, and causing the sedimentation of an array of higher-order oligomeric species from 50 kDa up to 6 MDa (4 S–50 S; Fig. 5e).

Higher-order oligomer formation in response to the addition of heparin is completely ablated in the HHIP-C Glu mutant (Fig. 5f-g), consistent with GAG-binding induced oligomerisation of HHIP. Taken together, our biophysical and cellular data suggest that HHIP-GAG complexation leads to HHIP clustering, and these assemblies might fine-tune inhibition of the HH signal.
HHIP-N can also bind GAGs with high affinity and despite crystallising as a monomer, forms weak dimers in solution when analysed using MALS (Supplementary Fig. 1Aa). Interestingly, apo HHIP-N is predominantly monomeric in solution up to 200 μM when studied by AUC (Supplementary Fig. 1Ab), and can also form oligomers in the presence of heparin (up to a size consistent with tetramers, as shown from c(s, I/θθ) plots to analyse molecular weights in solution; Supplementary Fig. 1C). This is consistent with our crystal packing analysis (Supplementary Fig. 4), and suggests that GAGs stabilise a contact between HHIP-N GAG-binding domains in solution to assist oligomerisation (Supplementary Fig. 1D). In conclusion, HHIP is regulated through both protein:protein- and proteinate:GAG-mediated oligomerisation at several different sites, that can be linked to dynamic modulation of HH signalling.

**Discussion**

The function of the N-terminal domain of HHIP has remained a long-standing mystery. Previous work in our laboratory and by others showed that HHIP-C binds to the metal-binding site of SHH with high (low nanomolar) affinity, and inhibits HH signalling by functioning as a decoy receptor. Our structural, biophysical and cellular work reveals that HHIP-N contains a CRD, which acts as an additional module within HHIP to inhibit HH signalling. Moreover, HHIP-N has evolved a structured region engaged in cell surface heparan sulphate proteoglycan binding. Recent pioneering work on the PTCH1:pShhNc interaction has greatly enhanced our understanding of HH signal reception and transduction. PTCH1 and SHH interact in a 2:1 stoichiometry, with one PTC1 molecule (PTCH1-A) binding to the high-affinity metal-binding site of SHH and the other PTC1 molecule (PTCH1-B) grasping the two lipid modifications to form the full signalling complex (Fig. 6a). None of these interactions alone is sufficient to fully activate PTCH1 function in cellular assays.

This mechanism shows parallels to WNT morphogen signalling, in which WNT interacts with its receptor Frizzled (Fz) with both its covalently-linked palmitoleate (via the Fz-CRD, evolutionarily related to the HHIP-N CRD), and also via a protein-protein interface. WNT inhibition is, in part, achieved by interactions with secreted Fz-related proteins (sFRPs) (via both palmitoleate- and protein-protein contacts), which act as secreted decoy receptors. Our structural and functional analysis suggests that HHIP has potentially evolved a similar role, targeting the SHH cholesterol moiety (via HHIP-N) and the metal-binding site (via HHIP-C). Thus, a two-pronged engagement with both protein- and lipid-based interfaces seems to be a common theme in recognition of morphogens.

Insertion of the SHH palmitoyl and cholesteryl moieties into the ectodomain of PTCH1-B blocks a proposed conduit for cholesterol/sterol transpory. Shielding of the pShhNc cholesterol modification by HHIP-N would be an effective mechanism to release SHH-mediated inhibition and transport function of PTCH1. This is complemented by HHIP-C and PTCH1-A competing for the SHH metal-binding site, which also overlaps with the binding sites for co-receptors CDO, BOC and GAS1. These two modes of signal antagonism constitute a fail-safe mechanism by which HHIP regulates HH signalling. HHIP-C binds to the SHH metal-containing site some 100-fold tighter compared to CDO and PTCH1, and thus could outcompete both CDO and PTCH1 from this site. This could result in the formation of a ‘negative receptor complex’, facilitating for example SHH endocytosis (Fig. 6b).

Various studies have identified HHIP as the only known secreted diffusible inhibitor of HH signalling. We previously showed that HHIP secretion is dependent on interaction with GAGs, and that HHIP can bind to various types of GAGs. Here, we have delineated the molecular basis for this interaction. Both HHIP-N and HHIP-C contain discrete GAG-binding sites, which combine to modulate cell surface affinity. Together, they constitute additional minor regulatory sites of HH signalling. Cell surface-attached GAGs such as HS and CS organise secreted proteins into gradients, varying local concentrations to enable graded signal activation. Here, we show that GAGs are able to cluster HHIP-C into large assemblies, linked to the potency of HHIP-mediated HH inhibition (which is further potentiated by HHIP-N). In addition, the HHIP C-terminal helix (residues 671–700) that contributes to cell surface binding via formation of a predicted leucine zipper-type assembly may enhance clustering even further, combined with the dimerisation abilities observed for both HHIP-N and HHIP-C. HHIP clustering by GAGs at the cell surface generates a high-avidity platform for SHH binding. This platform might limit diffusion and loss of HHIP into extracellular space and position HHIP to inhibit SHH signalling at the cell surface (Fig. 6c). This process can be further regulated by SHH-mediated internalisation of HHIP, which potentially can happen at both SHH producing or responding cells. From our structural analysis, we also note that there is a long flexible linker between HHIP-N and HHIP-C, which we previously described as being proteolytically sensitive. Cleavage of this linker may regulate the overall cell surface affinity of HHIP in vivo. Taken together, our results indicate that HHIP regulation.

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**Fig. 6 Models of extracellular HH signalling antagonism by HHIP.** a A 2:1 interaction of PTCH1:pShhNc represents the active signalling complex. The pShhNc lipid appendages insert to seal channels in PTCH1-B. b HHIP-C targets the conserved high-affinity metal ion-binding site on the pShhNc surface (protein-protein interaction), competing with binding from PTCH1-A and other co-receptors, whilst HHIP-N is able to contribute to this by targeting the C-terminal cholesterol moiety, possibly re-establishing PTCH1 cholesterol transport ability. It is also of note that HH signalling is activated via SMO CRD-cholesterol interaction, and competition of this by the HHIP-N CRD represents a possible additional mode of inhibition. Additionally, HHIP-mediated receptor endocytosis may act to remove SHH from the cell surface. c Cell surface GAG-mediated clustering represents a second modality for HH regulation by HHIP. The avidity of HHIP is increased by cluster formation at the proteoglycan layer, similar to how SHH lipoprotein multimers act to increase local morphogen concentration. The GAG-binding site in HHIP-N is also important for this process.
of HH signalling is dynamic, and inhibitory processes are modulated through distinct functionalities within the HHIP N- and C-terminal domains.

Aberrant expression of SHH has been linked to the initiation and progression of numerous cancers, and HH inhibitors targeting SMO are in the clinic against basal cell carcinomas. The SHH-specific mouse antibody 5E1 binds tightly to the SHH-metal-binding site, overlapping with HHIP and has been shown to inhibit SHH activity in vivo and to reduce tumour growth in a pancreatic cancer mouse model. HHIP could be used in a similar way, working as an engineered biologic to inhibit HH signalling. Here, we provide the framework for the development of HHIP-based HH inhibitors that can specifically preserve or block the different SHH-receptor interaction sites, and this interaction mode can be further fine-tuned by the GAG-binding properties and resultant HHIP clustering. Various biomedically-important secreted signalling proteins are reported to undergo GAG-dependent clustering, and drugging such mechanisms could present an unexploited avenue for therapeutic discovery.

Methods

Protein expression and purification. Constructs of human HHIP (Uniprot ID: Q69QV1) comprising the N-terminal domain (HHIP-N, 39-209), C-terminal domain (HHIP-C, 213-670) and a full-length variant only lacking the C-terminal to undergo GAG-dependent clustering and drugging such interactions could be further tuned by the GAG-binding properties and resultant HHIP clustering. Various biomedically-important secreted signalling proteins are reported to undergo GAG-dependent clustering, and drugging such mechanisms could present an unexploited avenue for therapeutic discovery.

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Surface plasmon resonance. SPR experiments were performed using a Biacore T200 machine (GE Healthcare) in 10 mM HEPES pH 7.5, 120 mM NaCl, 0.05% (v/v) polyvory, 20 at 25 °C. Proteins were buffer exchanged into running buffer and concentrations were calculated from the absorbance at 280 nm using predicted molar extinction coefficients. Heparin (Iduron; average molecular weight >9000 Da) and chondroitin sulphate (CS) sodium salt from shark cartilage (Sigma) were biotinylated using EZ-Link Biotin-LC-Hydrazide (Thermo Fisher Scientific) in a solution containing 17% (v/v) DMSO for 26 hours at 20 °C. GAGs were then extensively dialysed, first against water and then SPR running buffer (120 mM NaCl, 10 mM HEPES pH 7.5, 0.05 % (v/v) Tween 20), similar to a procedure described previously. Biotinylated sugars were immobilised on CM5 sensor chips to which 3600 RU of streptavidin were covalently coupled. After each binding experiment, the chip was regenerated with running buffer supplemented with 1.5 M NaCl at 30 μL min⁻¹ for 120 s. In all experiments, the trace returned to baseline following regeneration. HHEP constructs were injected at a flow rate of 5 μL min⁻¹. All data were analysed using SCRUBBER2 (Biologic) and GraphPad Prism Version 6.04 (GraphPad Software, La Jolla California USA). Best-fit binding curves were calculated using non-linear curve fitting of a one-site–total binding model \( Y = [\text{max}] X/(K_D + X) + Y_S \) where \( X \) is analyte concentration and the level of non-specific binding is assumed to be proportional to the analyte concentration; hence \( N \) equals the slope of non-specific binding). The background value was set to zero as the data had been previously referenced. \( [\text{max}] \) and \( K_D \) values quoted are determined for the specific binding portion only.

 Isothermal titration calorimetry. Experiments were performed using a MicroCal PEAQ-ITC (Malvern) at 25 °C in 10 mM HEPES, pH 7.5, 150 mM NaCl and 3% isopropanol, with a differential power of 10 μcal s⁻¹. Experiments consisted of an initial test injection of 0.4 μL, followed 150 s later by 18 injections of 2 μL, spaced 150 s apart. Owing to the low solubility of cholesterol, a PEG-cholesterol was used for affinity measurements. HHHIP was dialysed against 0.15 M NaCl, 10 mM HEPES and 3% isopropanol, final pH 7.5. Lyophilized PEG-cholesterol and PEG200 were resuspended in dialysis solution to a concentration of 1 mM. Protein concentrations were determined from the absorbance at 280 nm using calculated molar extinction coefficients. Cell concentrations of 1 μM HHIP protein and syringe concentrations of 1 mM PEG-cholesterol or PEG200 were used for all experiments. Thermograms were integrated and corrected for heats of dilution using PEAQ-ITC analysis software (Malvern). Isotherms were fitted with the \( A = B / AB \) model, where cell and syringe concentrations and baselines of each experiment were fitted locally. All figures were prepared using PEAQ-ITC analysis software (Malvern).

Immunofluorescence microscopy. HEK 293 T cells were seeded at a concentration of 10,000 cells mL⁻¹ in poly-D-lysine coated 35 mm dishes (MatTek) and incubated at 37 °C, 5% CO₂ for 18 h. At 18 h, the cells were washed with PBS, d-buffered with a differential power of 10 μcal s⁻¹ and quenched in 0.3 M glycine for 3 min. Fixed cells were stored overnight at 4 °C for PBS. For the staining process, fixed cells were blocked for 10 min on 1% BSA/ PBS, before HA tag probing using a Mouse HA Epitope Tag Antibody (Thermo Fisher) at 1 μg mL⁻¹ in 1% BSA/PBS at 25 °C for 1 h. Cells were washed in PBS 3 times for 10 min, before incubation with an Alexa Fluor® 633-conjugated goat anti-Mouse IgG antibody (A633; 633 nm; A647; 647 nm) (Thermo Fisher) at a concentration of 2 μg mL⁻¹ in 1% BSA/PBS/PBS at 1 h at 25 °C. Excess antibody was removed via washing 3 times in PBS for 10 min, with one final wash containing 0.5 μg mL⁻¹ Hoechst 33342 nuclear stain (A633; 355 nm; A488; 485 nm) Immuno- fluorescence was detected using a Leica TCS SPB WLL Confocal SMD Microscope. Images were processed in Fiji.

Multiangle light scattering (MALS). A total of 100 μL protein samples were injected onto an 8200 10/30 column (GE Healthcare) equilibrated in a running buffer of 10 mM HEPES pH 7.5, 150 mM NaCl over a concentration range of 48–192 μM. A Wyatt Dawn HELEOS-II MALS detector and Wyatt Optilab rEX refractive index detector were coupled to this system, and both were run in parallel with a gradient of elution buffer (10 mM HEPES pH 7.5, 100 mM NaCl) at 1418 rpm, 14 in 418 minutes. A Wyatt Dawn CE-21203) for 30 minutes at 4 °C. Cells were then washed three times with SB before being resuspended in PBS for flow cytometry analysis. Flow cytometry was performed on a BD Acuri C6 Flow Cytometer using a 552 nm laser for excitation and a 610/20 nm bandpass filter to collect emitted light. Forward and side scatter plots were used to select a population of live, mostly single cells, which were then analyzed. A population of at least 7500 selected cells was analyzed for each sample.

HH signalling assay. Gli1 mRNA measurements were used as a readout for HH pathway activation and were performed as in. Briefly, NIH/3T3 cells (ATCC, CRL-1658) were grown to confluency and then serum-starved for 24 h in reduction of serum to 0.5% NBCS. The HH pathway was either induced by adding 50 mM purified ShhN-C212,180 or, of NIH/3T3 cells were co-cultured with the full-length HH-producing human cell line Panc-1 (ATCC, CRL-1469)89, with addition of HHHIP-C constructs or HHHIP-AHx at 100 mM in each case. For HH pathway stimulation by the palmitoylated N-terminal Shh peptide (Palm-ShhN15), peptides were added at 10 μM and HHHIP-AHx at 1 μM (n = 2). Reverse transcription quantitative PCR (RT-qPCR) was carried out with the Power SYBR® Green Cells-to-CT™ kit (Life Technologies) according to manufacturer’s instructions. Every experiment was performed with two biological replicates and three technical replicates and results were calculated according to the ΔΔCt method and maximal pathway activation was normalised to 100. The PCR primers for Gli1 (forward primer, 5’-caagcaacttatgaccgg3’; reverse primer, 5’-aagcagccttattgatta3’; and Gapdh (forward primer, 5’-aggtgagctgatt3’; reverse primer, 5’-ggtagctcggaa3’) are specific for murine DNAs. Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. A list of data and structure factors for HHIP-N:SO, HHIP-N apo, HHIP-Cheparin and HHIP-CS that have been deposited is the Protein Data Bank (PDB) (www.rcsb.org) under accession numbers 7PGK, 7PGL, 7PGM and 7PGQ, respectively. Any other data supporting the findings of this manuscript are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions
C.S., S.C.G., and R.A.S. designed the project. S.C.G. and B.B. expressed and purified the proteins for crystallization and carried out biophysical experiments. T.M. contributed to protein production. S.C.G. and R.J.C.G. performed AUC analysis. S.C.G. crystallized the proteins. S.C.G. and C.S. collected and processed the X-ray data. S.C.G., K.E.O and C.S. solved and refined the crystal structures. R.A.S. conceived the cellular experiments and performed SHH signalling assays. E.J.I., R.D., and R.R. generated the extl-/- cell line, M.Q. and D.F.C. synthesized the PEG-cholesterol. S.C.G. and C.S. prepared the manuscript with input from all authors.

Competing interests
The authors declare no competing interests.

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