Hedgehog (Hh) signaling is crucial for establishing complex cellular patterns in embryonic tissues and maintaining homeostasis in adult organs. In Drosophila, Interference hedgehog (Ihog) or its close parologue Brother of Ihog (Boi) forms a receptor complex with Patched to mediate intracellular Hh signaling. Ihog proteins (Ihog and Boi) also contribute to cell segregation in wing imaginal discs through an unknown mechanism independent of their role in transducing the Hh signal. Here, we report a molecular mechanism by which the Ihog proteins mediate cell–cell interactions. We found that Ihog proteins are enriched at the site of cell–cell contacts and engage in trans-homophilic interactions in a calcium-independent manner. The region that we identified as mediating the trans-Ihog–Ihog interaction overlaps with the Ihog–Hh interface on the first fibronectin repeat of the extracellular domain of Ihog. We further demonstrate that Hh interferes with Ihog-mediated homophilic interactions by competing for Ihog binding. These results, thus, not only reveal a mechanism for Ihog-mediated cell–cell interactions but also suggest a direct Hh-mediated regulation of both intracellular signaling and cell adhesion through Ihog.

Hedgehog (Hh) signaling is essential for establishing the complex cellular patterns in various embryonic tissues and plays key roles in maintaining adult organ homeostasis. Hh pathway dysfunction during development can cause birth defects in humans, such as holoprosencephaly (1), and postembryonic malfunction of this pathway is linked to various proliferative disorders, such as the growth of malignant tumors (2).

The mature Hh ligand is derived from the Hh protein precursor by autoprocessing and lipid modification (3). Pathway activity is triggered by binding of the dually lipidated Hh ligand to Patched (Ptc), a transporter-like protein that, in the absence of Hh, suppresses the activity of Smoothened (Smo). The release of Smo inhibition upon Hh binding to Ptc activates an intracellular signal cascade that stimulates transcriptional activation of pathway target genes (4).

The Drosophila Hh receptor is composed of Interference hedgehog (Ihog) proteins, or the related Brother of Ihog (Boi) proteins, and Ptc. Ihog or Boi is required for Hh reception and stimulation of biological responses as well as for sequestration of Hh to limit long-range signaling (5–11). Drosophila Ihog proteins are type I single-span transmembrane proteins with four or five extracellular immunoglobulin (Ig) domains and two extracellular fibronectin type III (FNIII) domains; consequently, these proteins resemble cell adhesion molecules (12). Previously, our laboratory identified a function of the Ihog proteins that is independent of their role in transducing the Hh signal (13). Specifically, ectopic Ihog expression leads to aggregation of otherwise nonadherent cells, and loss of Ihog activity in the context of the Drosophila wing disc disrupts cell segregation, even in the presence of downstream genetic rescue of the intracellular response to Hh (13). Considering the structural similarity between Ihog proteins and cell adhesion molecules, we proposed that Ihog proteins function similarly to cell adhesion molecules to directly mediate cell–cell interactions. However, mechanistic understanding of this function, how the dual roles of Ihog proteins (transduction of the Hh signal and cell–cell interaction) are coordinated, and their functional interplay is lacking.

To address these questions, we explored the cell adhesion and homophilic interaction properties of Ihog and the effect of Hh on these properties. We used Drosophila S2 cells, which lack a Hh signal response and are intrinsically nonadhesive, to investigate the properties of ectopically expressed Ihog in cell–cell interactions. We found that Ihog proteins are enriched at the site of cell–cell contacts and engaged in calcium-independent homophilic trans-interactions. By mapping the Ihog–Ihog trans-homophilic binding site, we determined that it overlaps with the Ihog–Hh interface on the first fibronectin repeat in the extracellular domain of Ihog. We further demonstrated that Hh...
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Results

Ihog proteins concentrate at the site of cell–cell contact and engage in trans-homophilic interactions

The Hh co-receptors Ihog and Boi resemble typical cell adhesion molecules with a single transmembrane domain, four Ig domains, and two FNIII domains (12). Our laboratory reported that the Ihog proteins possess cell adhesion functions independent of their role in transducing the Hh signal (13). To investigate the molecular mechanism, we expressed Ihog in Drosophila S2 cells. Hh ligands and the intracellular Hh mediator Cubitus interruptus (Ci) are absent from S2 cells, and these cells are intrinsically nonadhesive (14–16), thus enabling the assessment of Ihog-mediated cell–cell interactions without the complication of co-occurring Hh signaling.

Consistent with our previous observations (13), Drosophila S2 cells transiently transfected with Ihog tagged with hemagglutinin (HA) at the C-terminal intracellular side formed multicellular aggregates, whereas the untransfected cells remained mostly dispersed (Fig. 1A). When we stained the cells with an antibody recognizing the extracellular domain (anti-IhogECD) and an antibody recognizing the intracellular HA tag (anti-HA), we found that anti-IhogECD detected Ihog along the outer surface of the multicell aggregates and that anti-HA revealed Ihog at both the outer surface of the clusters and along the contacting cell surfaces (Fig. 1B and C). We predicted that the extensive contacts formed among the Ihog-expressing cells prevented antibodies from diffusing into the multicell aggregates, thus excluding the anti-IhogECD antibodies from cell–cell contacts (Fig. 1B). The anti-HA staining pattern suggested that Ihog proteins were enriched at cell–cell contacts (Fig. 1C), and quantification of fluorescence intensity in the stained cells confirmed this enrichment (Fig. 1D). We also showed that the enrichment of Ihog proteins at cell–cell contacts was several-fold higher than the enrichment of coexpressed membrane-localized mCD8-GFP proteins (Fig. S1), suggesting that the increased signal of Ihog at the cell–cell contacts was not simply due to membranes of adjacent cells being in close proximity.

Drosophila S2 cells, which are derived from phagocytic hematopoietic cells, lack DE-cadherin at the cell surface and do not form Ca\(^{2+}\)-dependent cell aggregates (17). To exclude the possibility that Ihog caused S2 cell aggregation by indirectly activating or inducing the production of other endogenous adhesion molecules, we stained S2 cells for DE-cadherin, DN-cadherin, and fasciclin II (Drosophila neural cell adhesion molecule (NCAM)). As expected, the S2 cells had only background staining for these proteins, and their abundances were unaffected by transfection with Ihog-YFP constructs (Fig. 2, A–C). Furthermore, Ihog-dependent cell aggregation occurred when Ca\(^{2+}\) in the medium was eliminated with the addition of EGTA (Fig. 2, D and E). Thus, the data indicated that the Ihog proteins, in addition to functioning as a Hh co-receptor (5), engage in Ca\(^{2+}\)-independent, homophilic trans-interactions to mediate cell–cell interactions.

The first FNIII domain is essential for Ihog-mediated formation of cell–cell contacts

The Ig (Ig1–4) domains and the FNIII (Fn1 and Fn2) domains in the extracellular portion of Ihog proteins are potentially capable of mediating cell adhesion (12). To map the region in the extracellular portion of Ihog required for the formation of cell–cell contacts, we generated a series of truncations of the extracellular domain of Ihog-YFP, expressed them in S2 cells, and monitored their distribution in live cells using YFP fluorescence. We also monitored the distribution of the small, single-transmembrane-domain protein CD8 tagged with GFP (mCD8-GFP (18)) as a control protein that localizes to the membrane. As expected, mCD8-GFP was found in intracellular structures (likely intermediates along the biosynthetic and trafficking pathway) and along the cell surface (Fig. 3A). The distribution of mCD8-GFP was similar in individual cells, two closely positioned cells, and clusters of multiple cells (Fig. 3A). Similar to the full-length Ihog protein, Ihog truncated at the cytoplasmic C-terminal domain (CTD) was enriched along the contacting sides of cells that both expressed the truncated Ihog (Fig. 3, B and C), indicating that the intracellular domain is not necessary for the enrichment and homophilic interaction. We found that all the extracellular domain–truncated Ihog variants with an intact Fn1 domain were enriched at the sites of cell–cell contact in closely positioned two-cell and multiple-cell aggregates (Fig. 3, C–F). In contrast, Ihog lacking the Fn1 domain was
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Figure 2. Ihog-mediated cell aggregation is independent of other cell adhesion molecules. A–C, S2 cells transfected with plasmids expressing Ihog-YFP (green; YFP fluorescence) were fixed, and nuclei were stained with DAPI (blue) and other indicated cell adhesion molecules (red). Yellow stars indicate Ihog-expressing cells; blue stars indicate nontransfected cells. Scale bar, 5 μm. D, S2 cells were transfected with plasmids expressing both Ihog and GFP. Cells were dissociated by trypsin treatment and then mixed for 4 h in the presence of the indicated concentration of EGTA. Scale bar, 100 μm. E, the aggregation effect from experiments like those in A was quantified as the ratio of transfected cells within a cluster to total transfected cells. Each bar shows the mean ± S.D. from n = 30 different images. Unpaired two-tailed t test was used for statistical analysis. n.s., not significant, p > 0.05. Error bars represent S.D.

present along the plasma membrane but did not concentrate at the site of cell–cell contact (Fig. 3, G–I). Similar results were also seen for cells cotransfected with the Ihog parologue Boi.

We tested the ability of the truncated Ihog variants to induce aggregation of transfected S2 cells. We monitored aggregation induced by coexpression of mCherry with Ihog or Ihog lacking the CTD (IhogΔCTD), lacking the Fn2 domain (IhogΔFn2), lacking all four Ig domains (IhogΔIG), and lacking the Fn1 domain (IhogΔFn1) (Fig. 4A). Control cells expressed mCherry without any coexpressed Ihog. We dissociated the cells and then quantified the percentage of cell clusters 4 h after mixing (Fig. 4B). Cells expressing wildtype (WT) Ihog protein or any of the Ihog truncated proteins containing the Fn1 domain (IhogΔCTD, IhogΔFn2, and IhogΔIG) exhibited significant aggregation of the transfected S2 cells (Fig. 4, A and B). In contrast, cells expressing IhogΔFn1 did not form any recognizable cell aggregates and were indistinguishable from those expressing mCherry alone (Fig. 4, A and B). We confirmed by immunoblotting that the transfected cells used for the aggregation assays had similar amounts of Ihog or Ihog mutant (Fig. 4C).

To test for the Ihog–Ihog interaction biochemically, we assessed whether the extracellular domain of Ihog binds to itself using a pulldown assay. We used only the extracellular domain to avoid any aggregation due to the hydrophobic transmembrane domain or unphysiological interactions between the extracellular domain and the intracellular domain. We expressed variants of the extracellular domain of Ihog tagged with FLAG (FLAG-tagged IhogECD) along with YFP-tagged WT IhogECD in S2 cells and collected the media. We captured FLAG-tagged IhogECD variants with FLAG antibody–coated beads and detected the amount of YFP-tagged IhogECD bound by immunoblotting. Consistent with the cellular data, FLAG-tagged IhogECD variants with the Fn1 domain, but not those with Fn1 deletions, pulled down YFP-tagged IhogECD (Fig. 4D). Thus, S2 cell–based results and the biochemical data indicated that Ihog proteins bind to each other through the Fn1 domain, which serves as the basis for Ihog-mediated homophilic cell–cell interactions.

The Ihog trans-homophilic binding site overlaps with the Ihog–Hh interface

Our data showed that the Ihog Fn1 domain is essential for Ihog–Ihog homophilic interactions, and previous biochemical and structural studies showed that the Ihog Fn1 contributes to the binding site for the N-terminal Hh signaling domain (HhN) in a heparin-dependent manner (7, 8). We explored the structural properties that enable the same FNIII domain to possess these two distinct functions of ligand binding and homophilic interaction. We used the IhogFn1-2 (PDB code 2IBB) and the IhogFn1-2–HhN (PDB code 2IBG) complex structures (8) to identify surface residues on the Ihog Fn1 domain. We divided those residues that are nonoverlapping with the Hh–Ihog interface into six major regions, which we called M1–6, and subjected these regions to mutagenesis (Fig. 5A and Table S1). Based on previous reports (5, 8), we also tested mutant Ihog proteins with surface residue substitutions that disrupt Ihog–Hh (Ihog×Hh) or Ihog–Ptc (Ihog×Ptc) interactions, respectively (Fig. 5A and Table S1).

Because the side chains of the selected residues are solvent-exposed, we expected that the folded structures of these proteins would not be affected. Indeed, the abundances of the mutant proteins were similar to that of WT Ihog expressed in the S2 cells, and the mutants properly localized to the cell surface of S2 cells (Fig. S2). We expressed mCherry alone or mCherry with WT or the mutated Ihog proteins and assessed their ability to induce cell clustering by quantifying cell cluster formation 4 h after cell dissociation (Fig. 5, B and C). The only region that impaired cell aggregation activity when mutated was the region where Ihog interacts with heparin and mediates the Hh interaction (Ihog×Hh). Furthermore, S2 cells expressing Ihog×Hh mutants neither formed clusters with S2 cells expressing WT Ihog proteins (Fig. 6, A–C) nor concentrated at the cell–cell contact sites (Fig. 6D). In contrast, Ihog×Ptc was enriched at cell–cell contacts (Fig. 6E), and Ihog×Ptc-expressing cells formed clusters at the same proportion as WT Ihog–
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expressing cells. Together, these data with the S2 cells indicated that the Ihog homophilic binding site involves the heparin-binding site and overlaps with the Ihog–Hh interface in the Ihog Fn1 domain.

Figure 3. The Fn1 domain targets Ihog protein to the cell–cell contacts. A–I, S2 cells were transfected with plasmids expressing GFP-tagged mCD8 or YFP-tagged Ihog variants. The top row shows images of transfected single cells, the middle row shows closely positioned pairs of cells, and the bottom row shows clusters of multiple cells. Scale bar, 5 μm. Data are representative of n > 3 independent experiments and at least n = 30 of each class of cell or cluster. A diagram of the domains of Ihog-YFP is presented to the left, and schematic representations of each construct are provided for reference above each panel.

Figure 4. The Fn1 domain is essential for Ihog-mediated cell aggregation. A, S2 cells were transfected separately with empty vector (Ev; negative control) or plasmids expressing HA-tagged Ihog variants and mCherry. Forty-eight hours after transfection, cells were dissociated by trypsin treatment and then mixed for 4 h to allow aggregation to occur. Note that the brightfield channel shows both transfected and untransfected S2 cells. Scale bar, 100 μm. B, the aggregation effect from experiments like those in A was quantified as the ratio of transfected cells within a cluster to total transfected cells. Each bar shows the mean ± S.D. from n = 30 different images from three experiments. Unpaired two-tailed t test was used for statistical analysis. ****, p < 0.0001; n.s., not significant (p > 0.05). C, Western blot analysis for Ihog of lysates from S2 cells from experiments like those described in A. Tubulin served as a loading control. D, in vitro analysis of the IhogECD. Media from Drosophila S2 cells transfected with plasmids expressing IhogECD-HA-YFP and a FLAG-tagged IhogECD variant were collected and incubated with FLAG antibody (anti-FLAG)–conjugated agarose beads. The amount of bound IhogECD-HA-YFP was assessed by blotting with an antibody against GFP (anti-GFP). Cells transfected with an empty vector (Ev) served as a negative control. IP, immunoprecipitation. Error bars represent S.D.

Hh binding and trans-homophilic interactions are simultaneously incompatible for a single Ihog molecule

Intriguingly, our data indicated that the two distinct functions of Ihog proteins involve the same extracellular domain on
an overlapping surface of Ihog (Figs. 3–6), suggesting that the signal transduction and trans-homophilic binding activities are simultaneously incompatible for a single Ihog molecule. Thus, we predicted that the presence of Hh ligands would compete for the Ihog homophilic binding or vice versa, depending on the relative abundance and affinities of the Fn1 domain for Hh and another Ihog Fn1 domain. Indeed, we found that transient coexpression of HhN by S2 cells reduced the interaction of differentially tagged IhogECD in a coimmunoprecipitation assay (Fig. 7A). A competitive binding assay using purified HhN revealed a concentration-dependent interference between the interactions of differentially tagged IhogECD proteins (Figs. 7B and S3). These results are consistent with competition between homophilic Ihog–Ihog interactions and heterophilic Ihog–Hh interactions.

The published HhN–IhogFn1-2 crystal structure reveals a 2:2 complex, in which each HhN molecule contacts a single Ihog molecule, and a pair of 1:1 Hh–Ihog complexes forms a dimeric 2:2 complex that is entirely mediated by cis-interactions between the Ihog proteins (8). In this complex, Hh does not interfere with the cis-Ihog–Ihog interaction. Consistent with a cis-interaction that is not destabilized by Hh, the HhN-mediated disruption of Ihog–Ihog binding was incomplete, even when the concentration of HhN was 10 times higher than the reported dissociation constant of IhogFn1–2 for HhN (8) (Fig. 7B, lane 2). The Ihog–Ihog interaction that persisted in the presence of excess HhN was likely due to Ihog–Hh homophilic cis-interactions that were not competed by HhN (8). Furthermore, due to the coexistence of Ihog–Ihog cis-homophilic interactions that are not competed by Hh, in vitro competition assays, such as that used here (Fig. 7), cannot determine the binding affinity of Ihog–Hh versus Ihog–Ihog trans-homophilic interactions. Taken together, we showed that the Hedgehog co-receptor Ihog mediates trans-homophilic binding and signal reception via an overlapping surface, and the presence of excess Hh ligands interferes with Ihog-mediated homophilic interactions by competing for Ihog binding.

**Discussion**

The Ihog family proteins are type I single-span transmembrane proteins with Ig and FNIII domains, resembling typical cell adhesion molecules in the Ig cell adhesion molecule (Ig-CAM) superfamily. We previously found that Ihog proteins not only play an essential role in Hh signal transduction but also contribute to cell segregation in the *Drosophila* wing imaginal
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**A** Schematic diagram of the mutation sites on the Ihog proteins lacking Hh- or Ptc-binding capacity. The Hh-binding site (Ihog\textsuperscript{Hh}) and Ptc-binding site (Ihog\textsuperscript{Ptc}) were muta ted separately for analysis. 

**B** S2 cells were transfected with plasmids expressing WT or mutant Ihog along with GFP or mCherry as indicated. Cells were dissociated by trypsin treatment and then mixed to allow aggregation to occur. Scale bar, 100 µm. 

**C** Bar graph showing the ratio of transfected cells within a cluster to total transfected cells. Each bar shows the mean ± S.D. from n = 30 different images. Unpaired two-tailed t test was used for statistical analysis. **n.s.**, not significant, p > 0.05; ****, p < 0.0001. Scale bar, 5 µm. Error bars represent S.D.

**D** and **E** S2 cells transfected with plasmids expressing HA-tagged mutant Ihog\textsuperscript{Hh} or Ihog\textsuperscript{Ptc} were fixed and stained with an HA antibody. Scale bar, 5 µm.

**Figure 6. Mutations that disrupt Hh binding also abolish Ihog-mediated trans-homophilic interactions.** A, schematic diagram of the mutation sites on the Ihog proteins lacking Hh- or Ptc-binding capacity. The Hh-binding site (Ihog\textsuperscript{Hh}) and Ptc-binding site (Ihog\textsuperscript{Ptc}) were mutated separately for analysis. B, S2 cells were transfected with plasmids expressing WT or mutant Ihog along with GFP or mCherry as indicated. Cells were dissociated by trypsin treatment and then mixed to allow aggregation to occur. Scale bar, 100 µm. C, the aggregation effect from experiments like those in B was quantified as the ratio of transfected cells within a cluster to total transfected cells. Each bar shows the mean ± S.D. from n = 30 different images. Unpaired two-tailed t test was used for statistical analysis. **n.s.**, not significant, p > 0.05; ****, p < 0.0001. D and E, S2 cells transfected with plasmids expressing HA-tagged mutant Ihog\textsuperscript{Hh} or Ihog\textsuperscript{Ptc} were fixed and stained with an HA antibody. Scale bar, 5 µm. Error bars represent S.D.

In this study, we showed that, when transfected into the relatively nonadhesive *Drosophila* S2 cells lacking the ability to transduce the Hh signal, Ihog proteins concentrate at cell–cell contacts and mediate cell–cell interactions in a homophilic, calcium-independent manner.

The region that we identified as mediating the trans-Ihog–Ihog interaction overlaps with the region that mediates the interaction with Hh and includes the region where the negatively charged glycan heparin binds. Heparin is required for the Ihog–HhN heterophilic interaction by bridging positively charged patches of both proteins at the interaction interface (8). In the *in vitro* immunoprecipitation assay and competition assay (Figs. 4 and 7), binding between differentially tagged Ihog ECDs and Hh-mediated disruption of Ihog–Ihog interactions required heparin in the immunoprecipitation buffer (see “Experimental procedures”). In the cell-based assays, heparan sulfate proteoglycans are present because these are naturally produced by the S2 cells (19). Thus, the Ihog–Ihog homophilic *trans*-interactions likely occurred through heparin-dependent bridging of positively charged surfaces on the two opposing Fn1 domains, in a manner similar to heparin-bridged Ihog–Hh interactions (Fig. S4). These results thus provided a mechanistic basis for the role of Ihog proteins in wing disc cell segregation (13).

Hh release occurs at the apical or basal side of the single-layered disc epithelium (20–25), providing Hh at sites where Ihog–Ihog *trans*-interactions are unlikely to happen. In contrast, the Ihog–Ihog *trans*-interactions would contribute to cell–cell interactions along the lateral sides of epithelia, which are farther from the source of secreted Hh and less likely affected by the apically or basally secreted Hh. We thus propose that the dominant function of Ihog proteins depends on their subcellular localization and the availability of the Hh ligands (Fig. 8).

Of note, this dual function is not unique to Ihog proteins. Like Ihog, other members of the Ig-CAM family, such as the Netrin receptor Deleted in Colorectal Cancer (DCC), the Slit receptor Robo, and NCAM, have dual roles. These proteins act as “glue” that holds cells together and as molecular sensors to mediate cellular responses, such as motility, proliferation, and survival (26, 27). Whereas ligand binding and cell adhesion are often structurally separated involving different extracellular domains (28–30), the Ihog protein couples two distinct functions within the same region. Further studies are needed to puzzle out the physiological relevance of coupling the two distinct functions of the Ihog proteins in the same region of the protein.

Cdo and Boc are vertebrate homologs of the *Drosophila* Ihog proteins. Both Cdo and Boc contribute to aspects of Hh signaling (7, 31–33) by binding to mammalian Hh proteins through a nonorthologous FNIII repeat (7, 33, 34). Cdo and Boc may not mediate cell–cell adhesion (35–37), based on the lack of cell aggregation when Cdo was overexpressed in the fibroblast-derived cell lines Rat 6 and C1-T24 (35). However, recombinant, soluble fusion proteins that contain the entire Cdo extracellular region coupled to either alkaline phosphatase or the Fc region of human IgG not only bind to the surface of various cell lines (37) but also interact with the extracellular domain of Boc (36). Furthermore, a secreted form of the extracellular domain of Cdo functions as a dominant-negative form of Cdo and inhibits myogenic differentiation, whereas an analogous form of Boc or the full-length Boc promotes such differentiation (36). It is possible that Boc and Cdo interact in a heterophilic complex and that their interactions with themselves or each other may be affected by ligands or other proteins with which they interact.
Our study identified a ligand-dependent competitive mechanism for coordinating the biophysical (cell–cell contact) and biochemical (subunit of a signaling receptor) functions of a single protein. Bound proteins were detected by Western blotting with the indicated antibodies. Input shows the amount of protein, detected with the indicated antibodies, in the medium samples. B, the effect of recombinant HhN on homophilic IhogECD interactions. Drosophila S2 cells were transfected with plasmids expressing differentially tagged IhogECD or IhogECD-HA-YFP. Media were collected separately 48 h after transfection. Medium containing IhogECD-HA-YFP was first mixed with recombinant MBP-HhN at the indicated concentrations, and then the samples were mixed with medium containing IhogECD-FLAG. The mixed medium containing differentially tagged Ihog and HhN was then incubated with anti-FLAG antibody–conjugated agarose beads. Bound proteins were detected by Western blotting with the indicated antibodies. Input shows the amount of protein, detected with the indicated antibodies, in the medium samples. Quantified data from multiple experiments like those shown in B are available in Fig. S3. IP, immunoprecipitation.

Figure 8. A model for the Ihog-mediated homophilic and heterophilic interactions in cell adhesion and Hh reception. The diagram illustrates Ihog-mediated homophilic interaction across the epithelium, Ihog–Hh heterophilic interaction at the apical or basal surfaces of cells sending Hh and cells responding to Hh, and the Ihog–Ptc–Hh receptor complex at the apical or basal surfaces of Ihh-responding cells. Note that both cis- and trans-Ihog–Ihog homophilic interactions can occur simultaneously for a single Ihog molecule.
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the mechanisms by which Hh regulates cell adhesion through both transcriptional and nontranscriptional (as described here) processes may be relevant in developmental, homeostatic, and pathological contexts.

**Experimental procedures**

**Constructs**

Expression constructs of GFP, mCherry, mCD8-GFP, HhN, Hh, and differentially tagged Igog variants used in *Drosophila* cell culture were cloned into the pAcSV expression vector. The IgogECD-3XFLAG and IgogECD-HA-YFP variants were prepared by replacing the Fc tag in the pIB/Fc vector (38) with 3XFLAG tag and HA-YFP tag, respectively.

**Antibodies**

The following antibodies and dilutions were used: anti-β-tubulin E7 (1:5000) (Developmental Studies Hybridoma Bank), rat anti-DE-cadherin (DACK2, Developmental Studies Hybridoma Bank), rat anti-DN-cadherin (DN-Ex #8, Developmental Studies Hybridoma Bank), mouse anti-fasciclin II (ID4, Developmental Studies Hybridoma Bank), mouse anti-HA (1:1000) (HA11, Covance), mouse anti-FLAG (1:1000) (M2, Sigma), rabbit anti-GFP (1:1000) (A-11122, Invitrogen), and rat anti-Ihog antibody (1:500). HRP-conjugated and fluorophore-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories.

**Cell culture and transfection**

*Drosophila* S2 cells (*Drosophila Genomics Resource Center*) were cultured in *Drosophila* Schneider’s medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine (Thermo Fisher) at 25 °C in a humidified incubator. Transfection was performed with FuGENE 6 transfection reagent (Promega).

**Cell aggregation assay**

S2 cells were transfected separately with plasmids expressing desired proteins. Forty-eight hours after transfection, S2 cells were washed with PBS and dissociated by 0.05% trypsin treatment for 5 min at 25 °C. The dissociated cells were resuspended in fresh medium with 10% fetal bovine serum or supplemented with EGTA or purified HhN at concentrations indicated in the figure legends. The resuspended cells were then incubated in 1.5 ml ultra-low-adhesion microcentrifuge tubes with gentle rotation at room temperature for the time indicated in the figure legends. Cells were then transferred into glass-bottom dishes (D35-20–1.5-N, In Vitro Scientific) for live imaging by microscopy. In the experiments involving mixing differentially labeled red and green cells, cells coexpressing GFP or mCherry with the plasmid expressing the protein of interest were counted under microscope and mixed with an equal number of transfected cells prior to incubation with rotation.

To assess cell aggregation, low-magnification fields of similar cell density were randomly taken from each cell aggregation experiment, and the cell clusters were scored if they contained three or more cells. The aggregation effect was quantified as the ratio of certain transfected cells within clusters to total transfected cells (both clustered and nonclustered). Each bar shows the mean ± S.D. from 20–30 different images. Unpaired two-tailed t test was used for statistical analysis. Statistical analysis was performed using GraphPad Prism software.

**Cell immunostaining and imaging**

Forty-eight hours after transfection, dissociated S2 cells were allowed to settle and adhere for 60 min on a glass coverslip. Cells were then washed twice with PBS, fixed in 4% formaldehyde (Ted Pella) in PBS, blocked and permeabilized by 1.5% normal goat serum and 0.1% Triton X-100 in PBS, incubated with primary antibody in PBS containing 1.5% normal goat serum and 0.1% Triton X-100 for 1 h at room temperature, washed three times with 0.1% Triton X-100 in PBS, incubated with secondary antibody with DAPI, and washed with 0.1% Triton X-100 in PBS. Cell surface staining was carried out in the absence of Triton X-100.

**MBP-HhN purification**

The MBP-HhN expression plasmid was a gift from Dr. Daniel Leahy (The University of Texas at Austin). A DNA fragment encoding the *Drosophila melanogaster* Hh residues 85–248 (HhN) was cloned into the MBP-HTSHP expression vector, which was modified based on the pMal-c2x vector (New England Biolabs) by including a linker region with various tags (His-TEV-Strep-His-PreScission). Similar to the procedure described previously (8), the fusion proteins were expressed in *Escherichia coli* strain B834 (DE3) by induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside overnight at 16 °C. Cells were harvested, lysed, and centrifuged, and the supernatant was passed over nickel-nitrilotriacetic acid resin (Qiagen). Proteins were eluted with imidazole according to the manufacturer’s suggestions. The elution was then placed into 6000–8000 molecular weight–cutoff 40 mM dialysis tubing and dialyzed against 20 mM Tris (pH 8.0) and 200 mM NaCl.

**Immunoprecipitation**

S2 cells were cotransfected with HA- and YFP-tagged IgogECD (residues 1–707, representing the entire extracellular domain of Igog) and FLAG-tagged IgogECD variants in the presence or absence of Hh as indicated in the figure legend. Media were harvested 48 h post-transfection by centrifuging for 5 min at 500 g (4 °C) to remove cells and then for 15 min at 18,000 g (4 °C) followed by filtering (0.2 µm low-molecular-weight heparin (Sigma)). Cells were harvested, lysed, and centrifuged, and the supernatant was passed over nickel-nitrilotriacetic acid resin (Qiagen). The filtered media were further preclanned by incubating with Protein A/G–Sepharose beads (GE Healthcare) for 3 h. The preclanned media were then incubated overnight at 4 °C with EZviewTM Red Anti-FLAG® M2 Affinity Gel (Sigma) to capture FLAG-tagged protein. Beads were washed with PBS containing 0.1% Tween 20 supplemented with 40 µM low-molecular-weight heparin (Sigma). Proteins were recovered directly in SDS-PAGE sample buffer. Proteins samples were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore) for Western blot analysis.

**Western blot analysis**

Forty-eight hours after transfection, S2 cells were lysed in 1% Nonidet P-40 (50 mM Tris-HCl at pH 6.8, 150 mM NaCl, and
protease inhibitors) for 30 min at room temperature. The lysate was clarified by centrifugation, and proteins were recovered directly in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE under reducing conditions and then transferred onto PVDF membranes (Millipore). After protein transfer, the membranes were blocked and then immunostained with primary antibodies and HRP-conjugated secondary antibodies.

**Author contributions**—X. W., Y. Z., and X. Z. conceptualization; X. W. and Y. Z. formal analysis; X. W., Y. Z., K.-H. C., X. C., and H. A. methodology; X. W., Y. Z., and X. Z. writing-original draft; X. W., Y. Z., and X. Z. writing-review and editing; X. Z. supervision; X. Z. funding acquisition.

**Acknowledgments**—We thank I. Guerrero, W. Wojtowicz, L. Zipursky, S. Zhu, and the Iowa Developmental Studies Hybridoma Bank for reagents and members of the Zheng laboratory for comments on the manuscript. We thank D. Leahy, J. McLellan, P.-T. Chuang, S. Blair, and N. R. Gough for helpful discussions.

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