Supporting Information

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SI Materials and Methods

Cell Culture and Hh Reporter Assays. Control dsRNAs targeted the coding regions of *yfp*, *ptc*, and *smo* at nucleotides 1–498, 1–600, and 1–610, respectively. dsRNAs were included at 0.03 μg per transfection. Unless otherwise noted, expression constructs being tested were transfected at 0.01 μg per transfection for *dlp* UTR dsRNA rescue and at 0.1 μg per transfection for *trans*-dominant effects. To test pUAST construct activity, cells were cotransfected with 0.05 μg of pAct5C *GAL4* per transfection.

Expression Plasmid Construction. Constructs for gene expression in *Drosophila* cells were made by inserting PCR-generated coding fragments lacking the endogenous untranslated regions into the pAcSV vector. The *dally* gene was PCR-amplified from a construct kindly provided by Lawrence Lum (University of Texas Southwestern Medical Center, Dallas, TX) and altered by site-directed mutagenesis (Quikchange II XL Site-Directed Mutagenesis Kit; Stratagene) to match the *dally* coding sequence from cl-8 cells. The *dlp* constructs were derived from an untagged *dlp* expression construct created previously (1). A 3× FLAG epitope tag consisting of the amino acid sequence DYKDHDG-DYKDHDIDYKDDDDK was inserted following residues 26 and 47 in Dally and Dlp, respectively, and in all the following derivatives. DallyΔGAG was generated by mutation of Ser codons to Ala for residues 549, 569, and 573, and DlpΔGAG was generated by mutation of residues 463, 504, 625, 629, 631, and 643. Chimeras were derived from these 3× FLAG-tagged ΔGAG variants to create the fusions defined in Fig. 2*A* based on an amino acid alignment that aligned all the conserved Cys residues and the predicted α-helical content. −GPI variants were generated by replacement of codons 601–626 and 733–765 of Dally and Dlp, respectively, with a stop codon. Dlp:Sdc fusions were generated by replacement of codons 733–765 of Dlp with codons 339–364 (of 399) of Sdc and a C-terminal 1× HA epitope tag (YPYDVPDYA). The furin cleavage site RERR at residues 438–441 of Dlp and DlpΔGAG was mutated to GEGG. Derivatives for transgenesis were made by subcloning 3× FLAG-*dlp* and *dlpΔGAG* from pAcSV into pUAST. Fc fusion proteins in pCDNA3 were generated by fusing a human Ig Fc domain to residue 732 of Dlp and DlpΔGAG.

Mammalian glypican cDNAs were obtained as follows: mouse *GPC1* and *GPC3* from Open Biosystems, rat *GPC2* kindly provided by Art Lander (University of California at Irvine), mouse *GPC4* kindly provided by Yu Yamaguchi (The Burnham Institute), and human *GPC5* and mouse *GPC6* kindly provided by Scott Saunders (Washington University School of Medicine). Mammalian glypicans, and Dlp for comparison, were cloned for expression in *Drosophila* cells by replacement of the endogenous signal peptide with the signal peptide in residues 2–19 from the mouse glycoprotein BM-40/osteonectin/SPARC (RAWIFFLL-CLAGRALAAP), a dipeptide linker (LA), and 1× HA epitope tag (YPYDVPDYA). This BM40 + HA domain replaced the following residues in each protein: 2–47 in Dlp, 2–23 in mouse GPC1, 2–23 in rat GPC2, 2–24 in mouse GPC3, 2–22 in mouse GPC4, 2–24 in human GPC5, and 2–23 in mouse GPC6. To generate the glypican ΔGAG expression constructs, serine residues within the consensus GAG-chain attachment motif Ser-Gly-X-Gly (2) were altered to alanine by site-directed mutagenesis as for Dlp. mGPC1 ΔGAG was constructed by alteration of S485, S487, and S489; mGPC4 ΔGAG was constructed by alteration of S493, S494, S498, and S500; and mGPC6 ΔGAG was constructed by alteration of S502, S503, S505, S507, and S509.

Cloning, Expression, and Purification of HhN, Ihog FN1, and Dlp. Essentially as previously described (3), DNA fragments encoding *Drosophila melanogaster* Hh residues 85–248 (HhN) and *D. melanogaster* Ihog residues 446–557 (Ihog FN1) were amplified by PCR and cloned into the pT7HMT bacterial expression vector (4). Proteins were expressed in *Escherichia coli* strain B834(DE3) and purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity, cation exchange, and size-exclusion chromatographies. β-mercaptoethanol was added during HhN purification to prevent aggregation. $Dlp_{\Delta NCF}$ was subcloned into the pSGHVO mammalian expression vector (5), which directs expression of the inserted gene as a C-terminal fusion to human growth hormone, and transfected into CHO cells by electroporation. Stable transfectants were selected as previously described (5). The Dlp fusion protein was purified by Ni-NTA chromatography and cleaved by tobacco etch virus protease to release Dlp, which was then rechromatographed on a Ni-NTA column followed by further purification by size-exclusion chromatography.

Preparation of HhN-Coupled Resin and Dlp Pull-Down Assays. Purified HhN was incubated with CNBr-activated sepharose resin (Amersham), which had been swelled in activation buffer, 1 mM HCl, and washed with coupling buffer, 0.1 M Na/K phosphate/0.5 M NaCl /0.5 mM EDTA, according to the manufacturer's instructions. After termination of the coupling reaction by washing with 0.2 M Tris-HCl (pH 8.0), the resin was stored in 0.1 M Tris-HCl (pH 8.0)/0.5 M NaCl /0.5 mM EDTA. Purified Dlp (25 μM) or Ihog FN1 (25 μM) was added to 100 μL of binding buffer of 20 mM Tris-HCl (pH 8.0)/0.1 M NaCl with 30 μL of HhN-immobilized resin with or without 2 mM $CaCl_2$ or 50 μM decaheparin (Neoparin) and incubated overnight at 4 °C. The resin was then washed two times with binding buffer. Bound proteins were eluted with 20 mM Tris-HCl (pH 8.0)/0.5 M NaCl, resolved by SDS/PAGE, and stained with Coomassie blue.

Fly Strains and Genetic Analyses. Fly lines containing chromosome 2 *arm-GAL4*, chromosome 3 *P[Ubi-GFP.S65T]PAD2* (*Ubi-GFP*), and chromosome 3 *P[ovoD1-18]3L*, *P[FRT(w^{hs})]2A* (*ovoD1 FRT^{2A}*) were obtained from the Bloomington Drosophila Stock Center. The *dlp^{A187} FRT^{2A}* flies were kindly provided by Xinhua Lin (Cincinnati Children's Hospital Medical Center, Cincinnati, OH) (6). A balanced line containing X chromosome *flp122* was kindly provided by Duojia Pan (The Johns Hopkins University School of Medicine, Baltimore, MD). Transgenic *UAS-dlp* and *UAS-dlpΔGAG* lines were generated by Genetics Services, Inc. by means of injection of pUAST-derived plasmids into w^{1118} embryos.

Rescue of zygotic lethality of *dlp^{A187}* flies was quantified by counting the number of *$Antp^{Hu}$* and non-*$Antp^{Hu}$* progeny viable at the start of eclosion after intercrossing flies of the following genotype: *arm-Gal4/"A;" dlp^{A187} FRT^{2A}/TM6B ($Antp^{Hu}$)*, where *"A"* is *CyO*, *UAS-dlp*, or *UAS-dlpΔGAG*. In the total progeny of this cross, one-fourth will be homozygous for the *TM6B* third chromosome balancer and will die, one-fourth will be homozygous for *dlp^{A187}* (lacking *$Antp^{Hu}$*, the dominant marker of the *TM6B* balancer) and will die unless rescued, and one-half will be *dlp^{A187} FRT^{2A}/TM6B ($Antp^{Hu}$)* and will survive regardless of rescue. Of the *dlp^{A187}* homozygotes, one-half will be *arm-Gal4/"A"*, and if rescue of *dlp^{A187}* homozygosity by *arm-Gal4/"A"* is fully efficient, one-eighth of the total progeny of the cross will be *dlp^{A187}* homozygotes (non-*$Antp^{Hu}$*) that are rescued. Thus, with completely efficient rescue, five-eighths of the total progeny should survive, of which one-eighth, or 20%, should be non-

$Antp^{Hu}$. Flies were analyzed from two independent crosses involving the balancer line and three independent crosses involving each of the transgene-containing lines. In total, ~1,300, ~3,100, and ~2,400 progeny were analyzed for the *CyO*, *UAS-dlp*, and *UAS-dlpΔGAG* crosses, respectively.

GLC embryos were generated by Flp Recognition Target-FLP recombination (7–9) in larval progeny of the following parental flies: *w*; "B;" dlp^{A187} FRT^{2A}/TM6B × *y w flp122*/Y; "B;" *ovoD1* FRT^{2A}/TM2, where "B" is Sp or Sco/CyO, homozygous *UAS-dlp*, or homozygous *UAS-dlpΔGAG*. GLC female flies then were crossed to *w*/Y; *arm-GAL4*; dlp^{A187} FRT^{2A}/*P[Ubi-GFP]*, TM6B ($Antp^{Hu}$) male flies. Progeny were zygotically ~50% $dlp^{A187/+}$ and 50% homozygous dlp^{A187}. Progeny carrying the paternal dlp^{+} allele were identified by GFP fluorescence or anti-GFP antibody immunoreactivity. Viability at the wandering larval stage for GLC dlp^{A187} progeny was quantified by counting the number of larvae having and lacking GFP fluorescence from the *Ubi-GFP* transgene on the paternal dlp^{+} chromosome. Larvae were analyzed at 0–60 h after egg laying to account for any possible developmental delay in mutant lines. In total, ~250, ~2,100, and ~1,300 larvae were analyzed for the control, *UAS-dlp*, and *UAS-dlpΔGAG* crosses, respectively, over two distinct rounds of generating GLCs. Viability at the adult stage for GLC dlp^{A187} progeny was quantified by counting the number of $Antp^{Hu}$ and non-$Antp^{Hu}$ adult progeny. In total, ~100, ~1,100, and ~750 adults were analyzed for the control, *UAS-dlp*, and *UAS-dlpΔGAG* crosses, respectively, over two distinct rounds of generating GLCs.

In Situ Hybridization, Cuticle Analysis, and Wing Analysis. In situ probe sequences were PCR-amplified and subcloned into the vector provided with the DIG RNA Labeling Kit (Roche Applied Science) to include the following coding sequences: 250–650 of *bap*, 430–830 of *hh*, and 315–720 of *wg*. A *bap* cDNA used for making the *bap* probe was kindly provided by Manfred Frasch (University of Erlangen-Nürnberg, Erlangen, Germany). Riboprobes were reverse-transcribed and labeled with DIG-UTP according to the provided protocol. Control sense probes were generated in parallel and found to yield no significant labeling of WT embryos. In situ hybridization was performed in parallel with anti-GFP labeling as follows. Briefly, embryos 3–7.5 h old were dechorionated, fixed, devitellinized, and rehydrated. Embryos then were incubated overnight at 4 °C with anti-GFP antibody, washed, incubated overnight at 4 °C with biotinylated anti-rabbit IgG antibody (Vectastain Elite ABC Kit; Vector Laboratories), washed, bound to streptavidin-HRP complex (Vectastain Elite ABC Kit), and developed with diaminobenzidine (DAB) with hydrogen peroxide (DAB Substrate Kit for Peroxidase; Vector Laboratories). Embryos then were subjected to in situ hybridization and development of anti-DIG-AP labeling with NBT/BCIP substrates (Roche Applied Science) essentially as described (10). For cuticle preparations, embryos/larvae 0–60 h old were dechorionated, fixed, devitellinized, mounted in 1:1 Hoyer's solution/lactic acid, and cleared by incubation overnight at 60 °C. To distinguish embryos/larvae bearing the paternal dlp^{+} allele, embryos/larvae were sorted before fixation based on GFP fluorescence from a *Ubi-GFP* transgene on the same chromosome.

Immunoblotting and Antibodies. Protein expression analyses in cultured cells were performed by transfection of $S2R^{+}$ cells with expression plasmids and with empty vector or vector containing copper-inducible *hhN* (11), replacement of the medium with medium containing copper at 24 h after transfection, and harvest of cells and/or conditioned medium for SDS/PAGE at 24 h after copper addition. Cells were lysed with FLAG lysis buffer [50 mM Tris (pH 8.0)/150 mM NaCl/1 mM EDTA/1% Triton X-100] containing protease and phosphatase inhibitors [1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 15 μg/mL aprotinin, 100 μg/mL Pefabloc SC (Roche Applied Science), 1 mM Na_3VO_4, 1 mM NaF]. Soluble forms of the glypicans were detected by harvest of the conditioned medium before cell body lysis, centrifugation to remove cell debris, and dilution of the cleared conditioned medium with sample buffer. To detect HA-tagged Dlp and mammalian glypicans, cell lysates were incubated with anti-HA affinity matrix (Roche Applied Science) overnight, followed by washing and elution with sample buffer. To analyze transgene expression in vivo, embryos 0–24 h old were collected, dechorionated, flash-frozen in liquid nitrogen, thawed on ice in FLAG lysis buffer, and sonicated briefly. Lysates were cleared by centrifugation, and total protein was quantified using the Coomassie Plus protein assay (Thermo Fisher Scientific, Inc.). All embryos were F1 progeny from crosses between a strain containing *arm-GAL4* and control (w^{1118}) or transgene-containing strains (*UAS-dlp* or *UAS-dlpΔGAG*). All SDS/PAGE was performed under nonreducing conditions unless otherwise noted. Antibodies used were as follows: antilamin rabbit polyclonal R836 (gift from P. Fisher, State University of New York at Stony Brook, NY), anti-FLAG M2 monoclonal (Sigma Aldrich), mouse anti-human D-heparan sulfate 3G10 (Northstar Bioproducts, Associates of Cape Cod, Inc.), anti-HA rat monoclonal 3F10 (Roche Diagnostics), and rabbit anti-human IgG Fc fragment (Thermo Fisher Scientific, Inc.).

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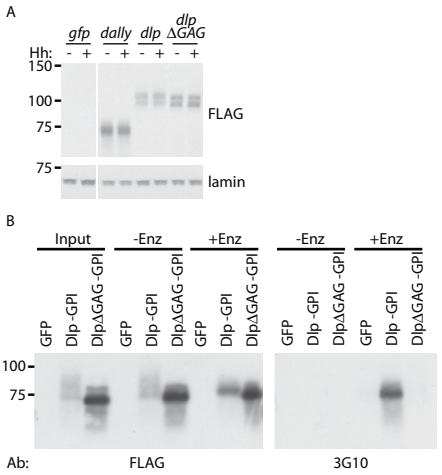

Fig. S1. (*A*) Dally, Dlp, and DlpΔGAG accumulate to similar levels. Whole-cell lysates of S2R$^+$ cells transfected with the expression constructs indicated were analyzed by SDS/PAGE and Western blotting with anti-FLAG and antilamin (loading control) antibodies. Transfection with *gfp* served as a control for antibody specificity. Molecular weight markers migrated as indicated. (*B*) DlpΔGAG-GPI shows no detectable heparan sulfate (HS) modification. −GPI variants or control GFP expressed in S2R$^+$ cells was incubated with (+Enz) or without (−Enz) heparitinase I and III and chondroitinase ABC and analyzed by SDS/PAGE and Western blotting to detect the 3× FLAG epitope (*Left*, FLAG) or the HS core fragment produced by enzymatic treatment (*Right*, 3G10). Molecular weight markers are indicated.

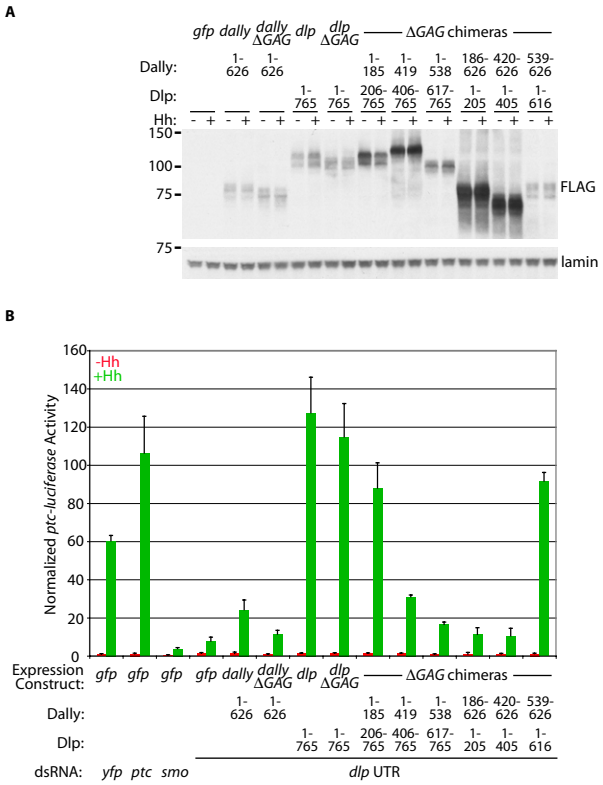

Fig. S2. Dally-Dlp chimeras have variable levels of protein accumulation and activities in Hh response. (*A*) Whole-cell lysates of S2R$^+$ cells transfected with the expression constructs indicated were analyzed by SDS/PAGE and Western blotting with anti-FLAG and antilamin (loading control) antibodies. Transfection with *gfp* served as a control for antibody specificity. Molecular weight markers migrated as indicated. (*B*) A *ptc*-luciferase Hh reporter assay shows the relative activities of the Dally-Dlp chimeras in rescuing Hh response in the presence of *dlp* UTR dsRNA with 0.01 μg of expression construct DNA transfected. Reporter assay details are presented in Fig. 1.

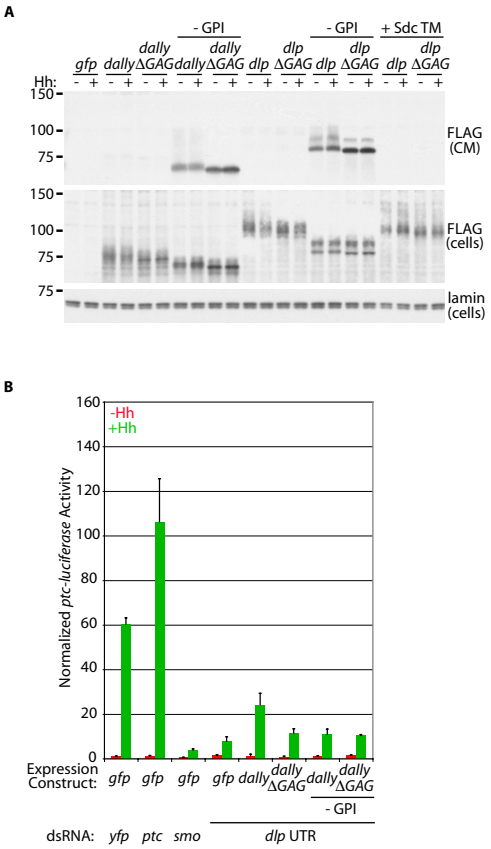

Fig. S3. Dally and Dlp derivatives lacking the GPI anchor have similar levels of protein accumulation but variable activities in Hh response. (*A*) Whole-cell lysates (cells) and conditioned media (CM) of S2R$^+$ cells transfected with the expression constructs indicated were analyzed by SDS/PAGE and Western blotting with anti-FLAG and antilamin (loading control) antibodies. Transfection with *gfp* served as a control for antibody specificity. Molecular weight markers migrated as indicated. (*B*) A *ptc*-luciferase Hh reporter assay shows the relative activities of Dally derivatives in rescuing Hh response in the presence of *dlp* UTR dsRNA. −GPI, deletion of the GPI attachment domain. Reporter assay details are presented in Fig. 1.

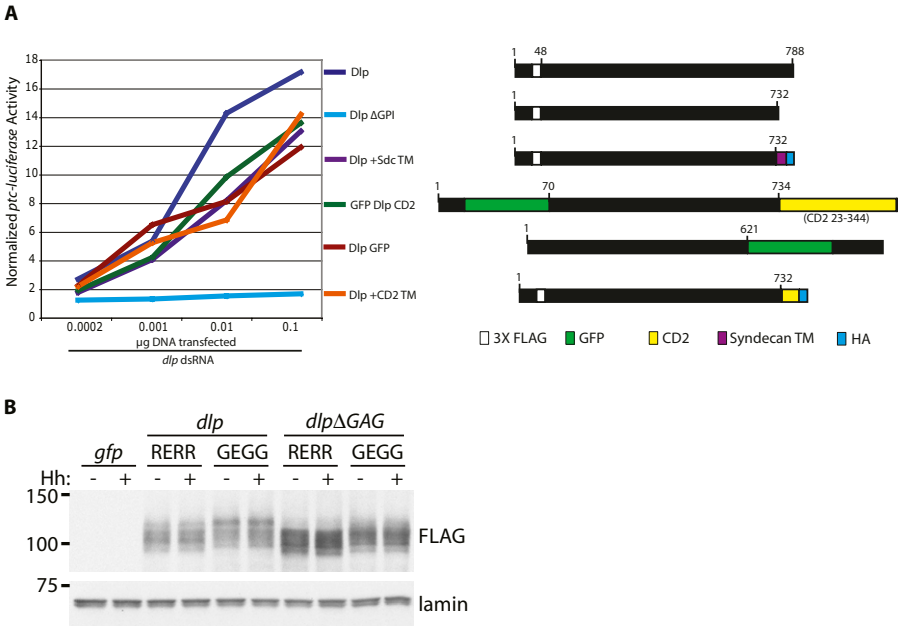

Fig. S4. (*A*) Dlp-mediated Hh response requires membrane tethering but not necessarily a GPI anchor. (*Left*) A *ptc*-luciferase Hh reporter assay shows the relative activity in rescuing Hh response in the presence of *dlp* UTR dsRNA of a variety of Dlp variants over a 500-fold range of transfected expression construct. The values shown are normalized *ptc*-luciferase levels in the presence of HhN-conditioned medium. (*Right*) Schematic diagram shows the relative scale of corresponding constructs with different forms of membrane anchoring. Black, Dlp; numbers and vertical bars indicate the relative positions and Dlp residues at the points of insertion; ΔGPI, Dlp with the C-terminal GPI attachment site deleted; +Sdc TM, Dlp with replacement of the GPI attachment domain with a Syndecan TM domain; GFP Dlp CD2, a fusion construct containing GFP, Dlp, and a CD2 TM domain (1); Dlp GFP, a different GFP fusion to Dlp provided by Xinhua Lin (2); Dlp + CD2 TM, Dlp with replacement of the GPI attachment domain with the CD2 TM domain. (*B*) Mutation of the Dlp furin cleavage site does not significantly alter protein accumulation. Whole-cell lysates of S2R$^+$ cells transfected with the expression constructs indicated were analyzed by SDS/PAGE and Western blotting with anti-FLAG and antilamin (loading control) antibodies. Transfection with *gfp* served as a control for antibody specificity. Molecular weight markers migrated as indicated.

1. Gallet A, Staccini-Lavenant L, Thérond PP (2008) Cellular trafficking of the glypican Dally-like is required for full-strength Hedgehog signaling and wingless transcytosis. *Dev Cell* 14: 712–725.
2. Han C, Belenkaya TY, Wang B, Lin X (2004) Drosophila glypicans control the cell-to-cell movement of Hedgehog by a dynamin-independent process. *Development* 131:601–611.

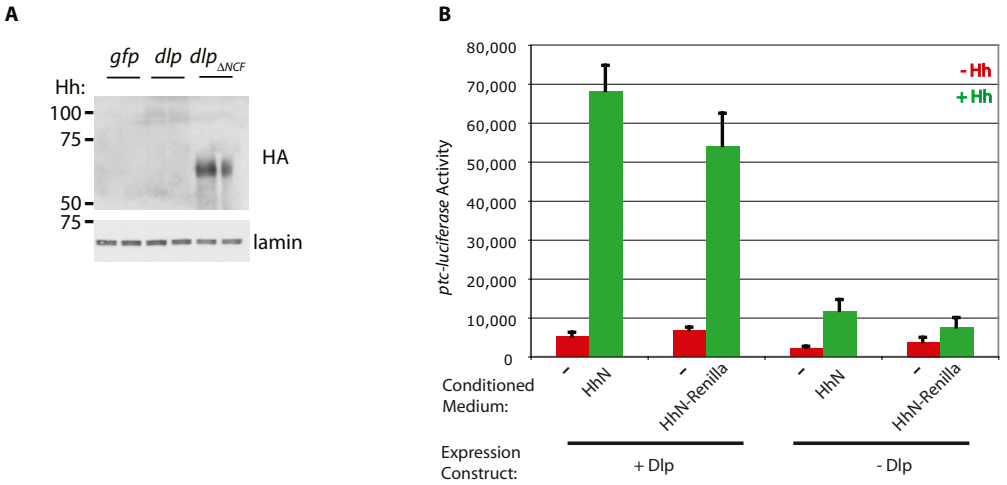

Fig. S5. Dlp$_{\Delta NCF}$::Dally(539–626) is functional in cell-autonomous Hh response but does not directly bind Hh. (*A*) Dlp$_{\Delta NCF}$::Dally(539–626) is expressed abundantly in S2R$^+$ cells. Whole-cell lysates of S2R$^+$ cells transfected with the expression constructs indicated (where *dlp*$_{\Delta NCF}$ = *dlp*$_{\Delta NCF}$*::dally ΔGAG*) were analyzed by SDS/PAGE and Western blotting with anti-HA and antilamin (loading control) antibodies. Transfection with *gfp* served as a control for antibody specificity. Molecular weight markers migrated as indicated. Dlp is weakly detectable as a smear migrating just above the 100-kDa marker. (*B*) HhN-Ren is functional for Dlp-mediated potentiation of Hh response. The cl-8 cells were treated with either HhN- or HhN-Ren-conditioned medium and tested for signaling activity using a *ptc*-luciferase reporter assay as described in Fig. 1. Green bars represent stimulated cells and red bars represent cells treated with control medium. Error bars represent standard deviation of three independent transfections.

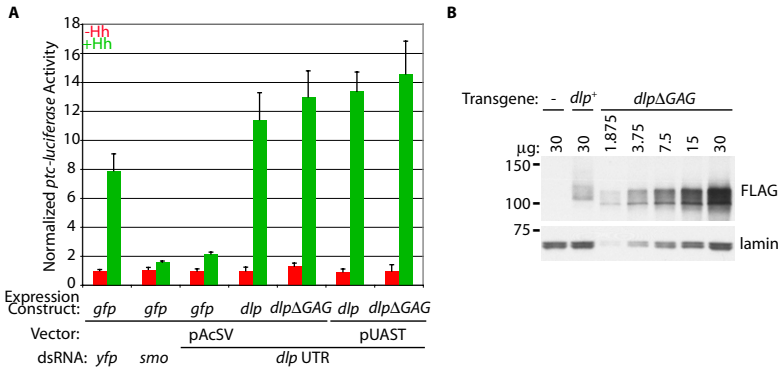

Fig. S6. Transgenes for expression of Dlp and DlpΔGAG are functional in cl-8 cells and are expressed in vivo. (*A*) A *ptc*-luciferase Hh reporter assay shows equivalent activities of the pAcSv and pUAST derivatives of *dlp* and *dlpΔGAG* in rescuing Hh response in the presence of *dlp* UTR dsRNA. Reporter assay details are presented in Fig. 1. (*B*) Lysates from *arm-GAL4/+* (–), *arm-GAL4/UAS-dlp*$^{+}$ (*dlp*$^{+}$), or *arm-GAL4/UAS-dlpΔGAG* (*dlpΔGAG*) embryos aged 0–24 h were prepared, and the protein was quantified and analyzed by SDS/PAGE and Western blotting with anti-FLAG antibody and antilamin antibody as a loading control. Molecular weight markers migrated as indicated.

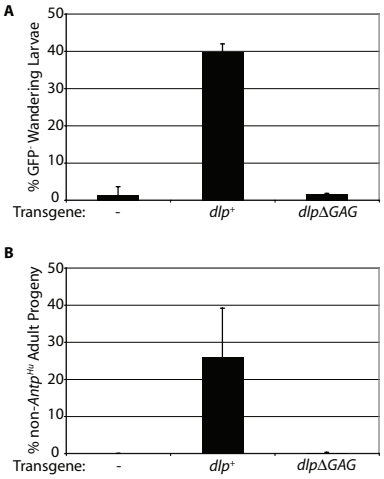

Fig. S7. *UAS*-driven expression of *dlp*$^{+}$ partially rescues viability of *dlp*^{A187} GLC embryos to larval and adult stages. (*A*) Larval viability of *dlp*^{A187} GLC embryos was restored by *arm-GAL4*-driven expression of *dlp*$^{+}$ but not of *dlpΔGAG*. Rescue was quantified as the percentage present of GFP^{-} wandering larvae, which represent larvae lacking the paternal *dlp*$^{+}$, *Ubi-GFP* chromosome, over two distinct rounds of generating GLC embryos. Full rescue by transgene expression is expected to generate 50% GFP^{-} larvae. (*B*) Adult viability of *dlp*^{A187} GLC embryos was partially restored by *arm-GAL4*-driven expression of *dlp*$^{+}$ but not of *dlpΔGAG*. Rescue was quantified as the percentage present of non-*Antp*^{Hu} adults, which represent flies lacking the paternal *dlp*$^{+}$, TM6B (*Antp*^{Hu}) chromosome, over two distinct rounds of generating GLC embryos. Full rescue by transgene expression is expected to generate 50% non-*Antp*^{Hu} adults.

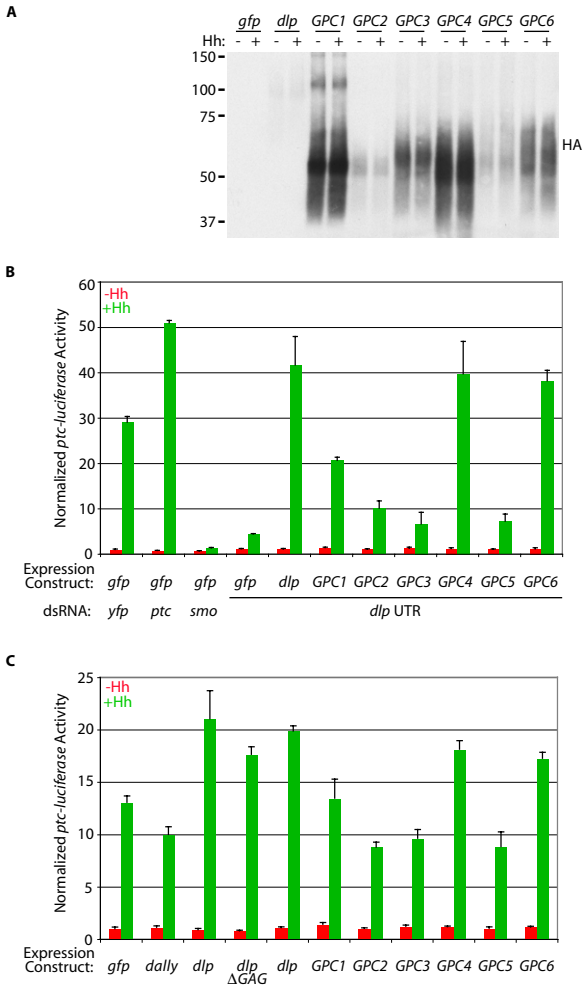

Fig. S8. *Drosophila* and mammalian glypicans have variable levels of protein accumulation and variable activities toward Hh response in *Drosophila* cells. (*A*) Whole-cell lysates of S2R$^+$ cells transfected with the expression constructs indicated were immunoprecipitated with anti-HA antibody matrix, and the immunoprecipitates were analyzed by SDS/PAGE and Western blotting with anti-HA antibody. Transfection with *gfp* served as a control for antibody specificity. Molecular weight markers migrated as indicated. HA-Dlp is very weakly detectable as a smear migrating just above the 100-kDa marker. (*B*) A *ptc*-luciferase Hh reporter assay using 0.01 μg of expression construct transfected shows that expression of *dlp*, *GPC4*, or *GPC6* rescues Hh response; expression of *GPC1* partially rescues Hh response; and expression of *GPC2*, *GPC3*, or *GPC5* does not rescue Hh response. (*C*) A *ptc*-luciferase Hh reporter assay with 0.1-μg DNA of expression construct transfected shows that expression of *dlp* has *trans*-dominant positive activity toward Hh response in the presence of endogenous Dlp, whereas expression of *dally*, *GPC2*, *GPC3*, and *GPC5* has *trans*-dominant negative activity. The *dally* construct is tagged with 3× FLAG, whereas *dlp* and the glypicans have a BM40 signal peptide and 1× HA tag. Reporter assay details are presented in Fig. 1.

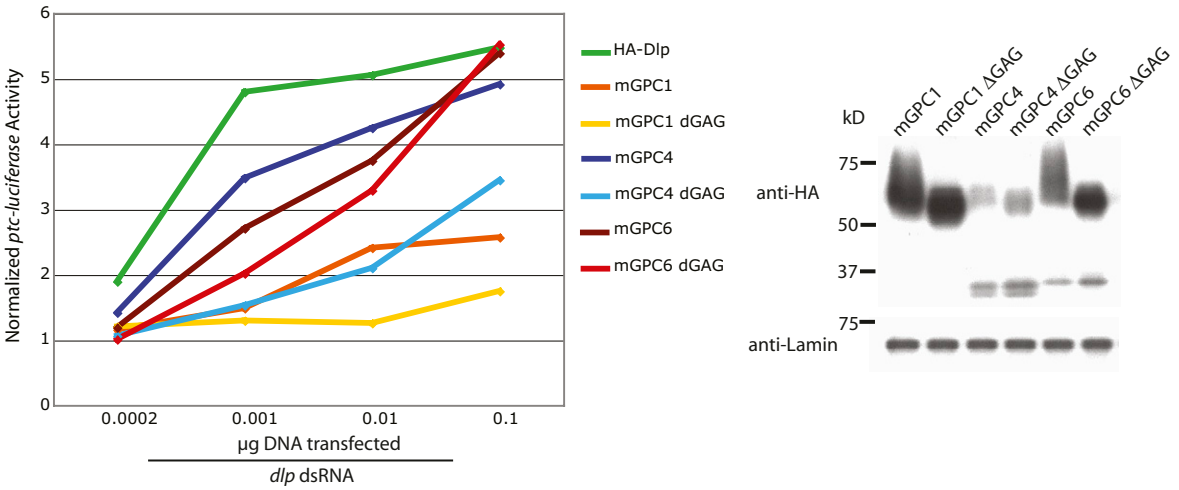

Fig. S9. Mammalian glypican core proteins rescue Dlp-mediated Hh response. Mouse *GPC1*, *GPC4*, and *GPC6* were mutated to remove GAG-chain attachment sites at consensus Ser-Gly-X-Gly motifs (1). (*Left*) A *ptc*-luciferase Hh reporter assay shows the relative activities of the glypican core proteins in rescuing Hh response in the presence of *dlp* UTR dsRNA over a 500-fold range of amount of transfected DNA. Values shown are normalized *ptc*-luciferase levels in the presence of HhN-conditioned medium. GPC4 and GPC6 core proteins both exhibited dose-dependent rescue of *dlp* RNAi over the range of expression construct transfected and were capable of moderate or full rescue of activity at higher levels of DNA transfected. (*Right*) Western blot shows the relative expression levels of *GPC1*, *GPC4*, and *GPC6*, with and without GAG modification, when transfected into *Drosophila* S2R$^+$ cells.

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