Lipoprotein particles are required for Hedgehog and Wingless signalling

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Wnt and Hedgehog family proteins are secreted signalling molecules (morphogens) that act at both long and short range to control growth and patterning during development. Both proteins are covalently modified by lipid, and the mechanism by which such hydrophobic molecules might spread over long distances is unknown. Here we show that Wingless, Hedgehog and glycophosphatidylinositol-linked proteins copurify with lipoprotein particles, and co-localize with them in the developing wing epithelium of *Drosophila*. In larvae with reduced lipoprotein levels, Hedgehog accumulates near its site of production, and fails to signal over its normal range. Similarly, the range of Wingless signalling is narrowed. We propose a novel function for lipoprotein particles, in which they act as vehicles for the movement of lipid-linked morphogens and glycophosphatidylinositol-linked proteins.

In the developing wing of *Drosophila*, Hedgehog activates short-range target gene expression up to five cells away from its source of production, and longer-range targets over more than twelve cell diameters[1]. Wingless can signal through a range of over 30 cell diameters[2]. These morphogens are anchored to the membrane via covalent lipid modification[3–9]. The mechanisms that allow long-range movement of molecules with such strong membrane affinity are unclear.

Like Wingless and Hedgehog, glycophosphatidylinositol (gpi)-linked proteins transfer between cells with their lipid anchor intact[10–12]. We observed that gpi-linked green fluorescent protein (GFP) expressed in Wingless-producing cells spreads into receiving tissue at the same rate as Wingless, where it co-localizes with Wingless in endosomes. Thus, we proposed that these proteins travel together on a membranous particle, which we called an argosome[13]. How might argosomes form? One possibility is that argosomes are membranous exovesicles. Such particles could be generated by plasma membrane vesiculation, or by an exosome-related mechanism[14]. Alternatively, argosomes might resemble lipoprotein particles like low-density lipoprotein (LDL). Vertebrate lipoprotein particles are scaffolded by apolipoproteins and comprise a phospholipid monolayer surrounding a core of esterified cholesterol and triglyceride. Insects construct similar particles called lipophorins[15,16]. Lipid-modified proteins of the exoplasmic face of the membrane (such as GFPgpi, Wingless or Hedgehog) might insert into the outer phospholipid monolayer of such a particle via their attached lipid moieties. Here, we use biochemical fractionation to determine the sort of particle with which lipid-linked proteins associate, and genetic means to address its function.

Lipid-linked proteins copurify with lipophorin

We compared sedimentation of Wingless, Hedgehog and gpi-linked proteins to that of transmembrane proteins, exosomes and lipophorin particles. To mark exosomes, we used flies expressing a vertebrate CD63:GFP fusion construct. CD63 is a tetraspanin that localizes to internal vesicles of multivesicular endosomes, and is released on exosomes[17,18]. In *Drosophila* imaginal discs, CD63:GFP localizes to late endosomes in producing cells, consistent with vertebrate studies (Supplementary Fig. S1A–D). It is released and endocytosed by neighbouring cells between one and three cell diameters away (Supplementary Fig. S1A,E), indicating that it is present on exosomes.

To mark lipoprotein particles, we made antibodies to *Drosophila* apolipophorins I and II (ApoLI and ApoLII); these proteins are generated by cleavage of the precursor pro-Apolipophorin[19,20]. Lipophorin is produced in the fat body[20]; consistent with this, we cannot detect *apolipophorin* transcripts in imaginal discs (data not shown). Nevertheless, the ApoLI and ApoLII proteins are as abundant in discs as in the fat body (Supplementary Fig. S1F and G).

Plasma membrane and exosomal markers are completely pelleted after centrifugation for 3 h at 120,000*g*, whereas most ApoLII remains in the supernatant (Fig. 1a). Most Wingless:GFP and Hedgehog is present in the pellet, as are the gpi-linked proteins Fasciclin I[21], Connectin[22], Klingon[23] and Acetylcholineasterase[24] (Fig. 1b); this is not unexpected, because these proteins localize to the plasma membrane and internal membrane compartments. Surprisingly, however, some Wingless:GFP (6%), Hedgehog (2%) and gpi-linked proteins (14–22%) remain in the supernatant (Fig. 1b).

The 120,000*g* supernatant (S120) contains both free soluble proteins and lipoprotein particles. To separate them, we performed isopycnic density centrifugation. In these gradients, lipophorin moves to the top low-density fraction whereas soluble proteins are present in higher-density fractions (top two panels of Fig. 1c). Gpi-linked proteins are found almost entirely in the top fraction with lipophorin. Treating the S120 with Phosphatidylinositol-specific phospholipase C (PI-PLC) before density centrifugation shifts their migration to higher-density fractions (Fig. 1c). This suggests that gpi-linked proteins associate with low-density particles via their gpi anchor.

Similarly, when S120s from larvae that express Wingless:GFP or Hedgehog:HA in imaginal discs are subjected to isopycnic density centrifugation, these proteins are found in the lowest-density fraction with ApoLII, as is endogenous Hedgehog (Fig. 1d). Antibodies to endogenous Wingless detect a doublet in the top fraction and a band of somewhat higher mobility in high-density fractions. These data indicate that non-membrane-bound Wingless and Hedgehog associate with low-density particles in imaginal discs *in vivo*; other larval tissues may secrete Wingless in a non-lipophorin-associated form.

We worried that lipophorin particles in the haemolymph might extract proteins from discs during larval homogenization, so we repeated these experiments using dissected discs. All Wingless, Hedgehog and ApoLII in the imaginal disc S120s are present on low-density particles (Fig. 1e), suggesting that their association is not an artefact of homogenization. Consistent with this, incubating

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pelleted imaginal disc membranes with an excess of purified lipoprotein particles does not extract Hedgehog:HA from membranes under the conditions used for homogenization (Fig. 1f). This suggests that association of lipid-linked morphogens with lipophorin depends on active cellular processes and does not occur during extract preparation.

To ask whether lipid-linked proteins associated with lipophorin, or with some other low-density particle, we immunoprecipitated ApoLII from larval S120s and probed precipitates for Wingless, Hedgehog or GFPgpi. These proteins are immunoprecipitated by anti-ApoLII, but not pre-immune serum (Fig. 1g). Furthermore, anti-ApoLII is unable to precipitate secreted GFP that does not contain a gpi anchor (Fig. 1h). Hedgehog and Fas-1 also immunoprecipitated with ApoLII from the more purified top fraction of KBr gradients (Supplementary Fig. S1). Thus, lipid-linked morphogens and gpi-linked proteins associate directly with lipophorin particles.

Morphogens colocalize with lipophorin

These experiments do not exclude the possibility that some Wingless or Hedgehog in the 120,000*g* pellet (P120) might be present on exosomes. To investigate this, we expressed CD63:GFP in either Wingless- or Hedgehog- producing cells and looked for co-localization with CD63:GFP-labelled exosomes in receiving tissue. No significant co-localization is detected (Fig. 2d–f, j–l). Thus, it seems unlikely that imaginal disc cells release Wingless or Hedgehog on exosomes, although the mechanism remains a possibility for transmembrane ligands such as Boss or Notch[25,26].

To test whether Wingless or Hedgehog co-localized with lipoprotein particles, we incubated imaginal discs with purified lipophorin particles fluorescently labelled with Alexa488; although they work for western blotting, neither anti-ApoLI nor ApoLII antibodies detect endogenous lipophorin by immunofluorescence. Immunostaining reveals that Wingless and Hedgehog are found in the same endosomes as Alexa488lipophorin (Fig. 2a–c, g–i). Unsurprisingly, lipophorin uptake is not limited to areas where these morphogens are abundant; lipophorin has a nutritional function as well and many potential receptors are encoded in the genome[27]. Strong co-localization between lipophorin and lipid-linked morphogens is predicted if Wingless and Hedgehog are endocytosed with lipophorin. Nevertheless, we cannot exclude the possibility that these proteins were internalized separately and converged in the same endosomes.

Lipophorin–RNAi perturbs lipid transport

To assess the role of lipophorin in larval growth and development, we reduced the levels of ApoLI and II by RNA interference directed against two different regions of the *apolipophorin* messenger RNA. Similar phenotypes were produced by each construct. To express double-stranded (ds)RNA, we used a modified GAL4:UAS system in which expression of inverted repeats can be temporally controlled by heat-shock-dependent excision of an intervening HcRed cassette by the flippase (FLP) recombinase. We tested extracts from wild-type larvae or larvae harbouring *hs-flp*, GAL4 driver and UAS dsRNA constructs at various times after heat shock to see how fast lipophorin levels were reduced (Fig. 3a). Larvae of the latter genotype made only 50% of the wild-type level of ApoLII, even in the absence of heat shock; basal activity of the heat-shock promoter in the fat body causes HcRed excision in approximately 50% of fat-body cells, although excision strictly depends on heat shock in other larval tissues (data not shown). Although they survive less frequently, these flies have no obvious phenotype.

After heat shock, all fat-body cells excise the HcRed cassette and ApoLII levels decrease further. After four days, ApoLII is reduced to 5% of wild-type levels. ApoLI levels are reduced with similar kinetics (Supplementary Fig. S3). These animals prolong the third larval instar and rarely pupariate. We performed all the experiments described below on third-instar larvae 4–6 days after heat shock.

To investigate the requirement for lipophorin in lipid transport, we assessed the accumulation of neutral lipids in larval tissues by staining them with Nile Red[28]. Cells of the posterior midgut

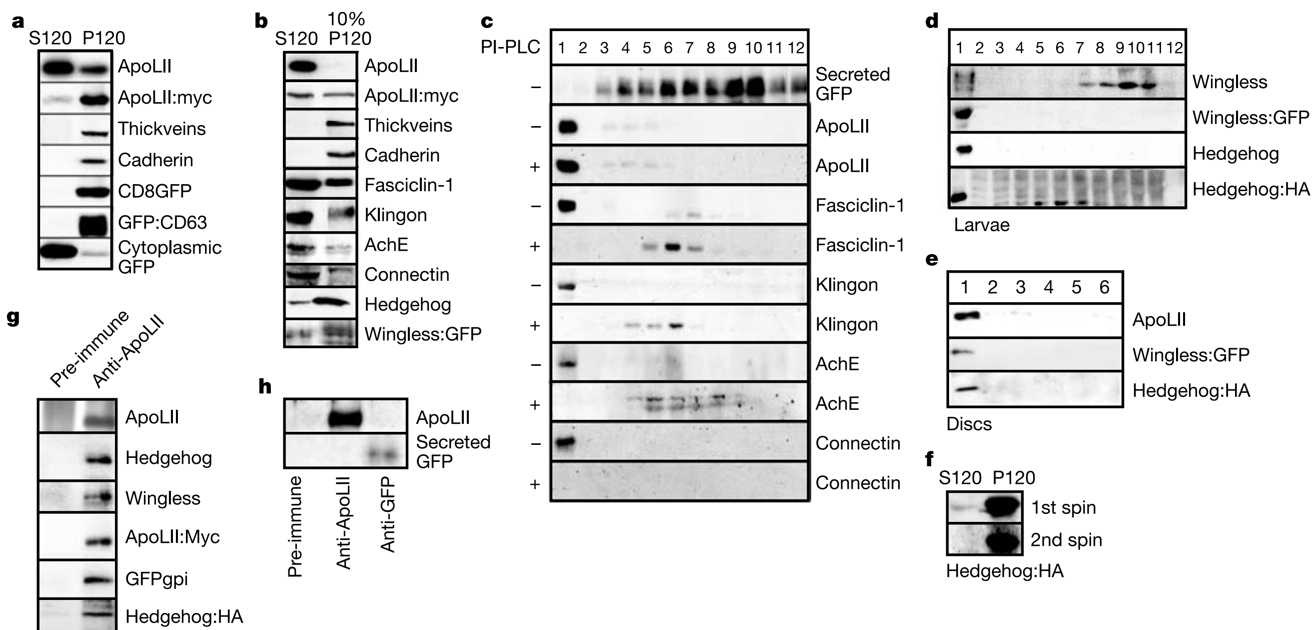

Figure 1 Lipid-linked proteins co-fractionate with lipophorin. Western blots of fractionated extracts probed with antibodies to indicated proteins. **a**, **b**, Larval S120s and indicated proportions of larval P120s. AchE, acetylcholinesterase. **c**–**e**, KBr isopycnic density gradient fractions made from larval (**c**, **d**) or disc (**e**) S120s. Top fraction, 1.14 g cm^{-3}; bottom fraction, 1.4 g cm^{-3}. +, PI-PLC-treated, −, mock-treated. **f**, Top panel: P120 and S120 from Hedgehog:haemaglutinin (HA)-expressing discs, probed with anti-HA. Lower panel: P120 and S120 after incubating P120 at 4 °C with fivefold excess of purified lipoprotein particles and recentrifuging. **g**, **h**, S120s from wild-type or fusion-protein-expressing larvae immunoprecipitated with pre-immune, anti-ApoLII or anti-GFP serum.

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normally contain many small lipid droplets (Fig. 3b). Lipophorin reduction causes a dramatic expansion of these droplets (Fig. 3c), suggesting that lipophorin is required for the efficient extraction of lipid from the midgut.

The wild-type fat body contains both small and large lipid droplets (Fig. 3d). Fat bodies of lipophorin–RNAi larvae are reduced in size and have fewer small lipid droplets (Fig. 3e), although larger droplets appeared normal. These data suggest that lipophorin delivers lipid to the fat body.

Lipid droplets in discs from lipophorin–RNAi larvae are fewer and smaller than in the wild type (compare Fig. 3f and g). Their discs are also reduced in size, particularly in the wing pouch (data not shown). Thus, discs require lipophorin for accumulation of lipid droplets and for growth. Neither Caspase3 activation nor membrane phosphatidylinositol 3,4,5-phosphate (PIP_3) accumulation is altered in lipophorin–RNAi discs (Supplementary Fig. S4), suggesting that their small size is not due to cell death or reduced insulin signalling[29].

Hedgehog function requires lipophorin

To test whether lipophorin association was required for Hedgehog function, we examined Hedgeghog distribution and signalling in lipophorin–RNAi larval discs. In wild-type discs, Hedgehog expressed in the posterior compartment moves across the anterior–posterior (AP) compartment boundary and activates transcription of short and long-range target genes. Cells closest to the source respond by activating the transcription of *collier* (Fig. 4b, c) and *patched* (Fig. 4h, i). Further away, Hedgehog activates transcription of *decapentaplegic* (Fig. 4a, b)[1,30,31]. We monitored levels of Collier and a *decapentaplegic* reporter construct (*dpplacZ*) in wild-type and lipophorin–RNAi discs stained in parallel and imaged under identical conditions. Discs from lipophorin–RNAi larvae activate *collier* at least as efficiently as those of the wild type (compare Fig. 4c and f). In contrast, the range of activation of *dppLacZ* is significantly narrowed in *lipophorin* RNAi discs. *dppLacZ* is expressed up to 11 cells away from the AP boundary in wild-type discs (Fig. 4a, b), but only up to six cells away in lipophorin–RNAi larvae (Fig. 4d, e, m). These data suggest that lipophorin knockdown decreases the range of Hedgehog signalling.

To discover whether Hedgehog trafficking was altered, we stained discs for Hedgehog and Patched. In wild-type discs, Hedgehog moves into the anterior compartment, where it is found in endosomes, often with Patched[32,33] (Fig. 4g–i). Patched-mediated endocytosis is thought to sequester Hedgehog and limit its spread[32,34]. Hedgehog is most abundant up to five cell rows away from the AP

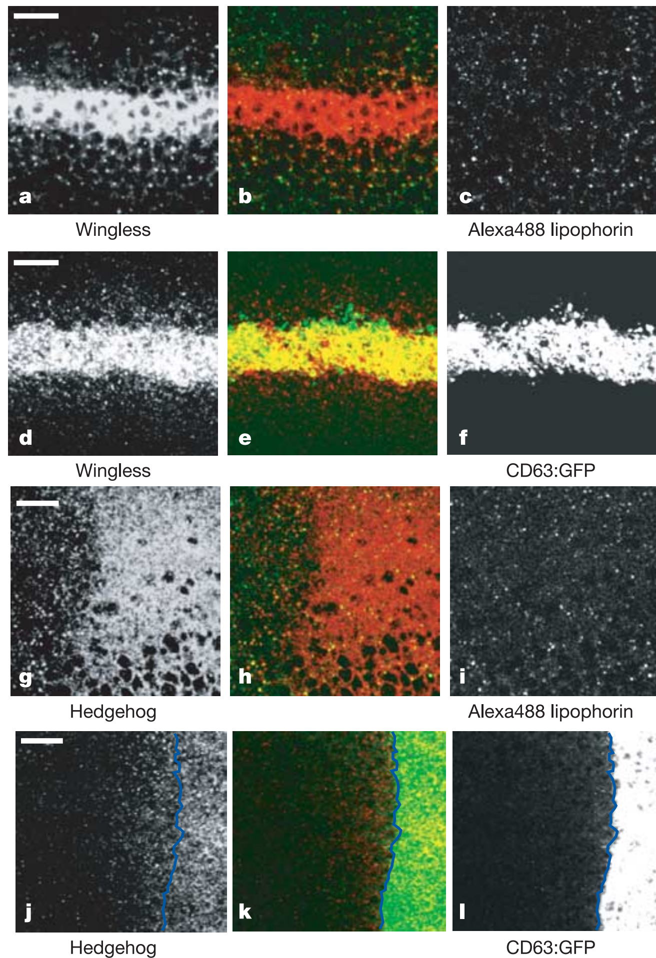

Figure 2 Wingless and Hedgehog co-localize with Alexa488lipophorin. Scale bars = 10 μm. Blue lines indicate AP boundaries. **a**–**c**, Disc stained with anti-Wingless (**a**, and red in **b**) after 20-min incubation with Alexa488lipophorin (green in **b**, and **c**). **d**–**f**, Disc expressing CD63:GFP (green in **b**, and **f**) in Wingless-producing cells stained with anti-Wingless (**d**, and red in **e**). **g**–**i**, Disc stained with anti-Hedgehog (**g**, and red in **h**) after 20-min incubation with Alexa488lipophorin (green in **h**, and **i**). **j**–**l**, Disc expressing CD63:GFP (green in **k**, and **l**) in Hedgehog-producing cells stained with anti-Hedgehog (**j**, and red in **k**).

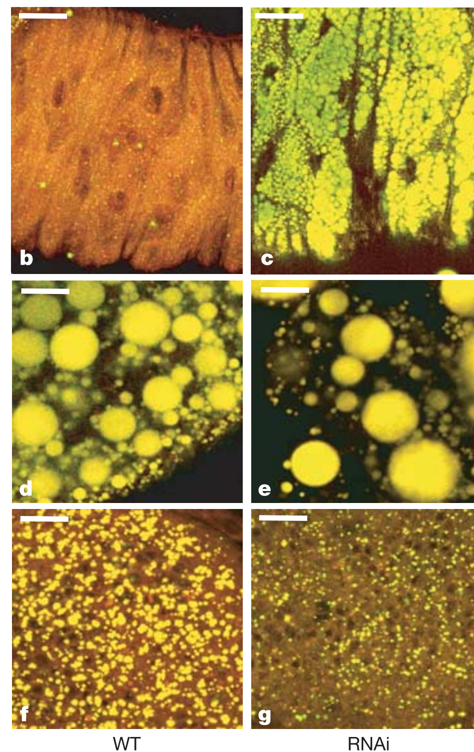

Figure 3 Lipophorin–RNAi perturbs lipid transport. **a**, Extracts from equal numbers of wild-type (WT) or *hs-flippase*/+; *UAS dsRNA/Tubulin:GAL4* larvae probed for ApoLII at indicated hours after heat shock. **b**–**g**, Posterior midgut (**b**, **c**), fat body (**d**, **e**) or imaginal discs (**f**, **g**) from wild-type larvae (**b**, **d**, **f**) or *hs-flippase*/+; *Adh:GAL4*/+; *UAS dsRNA*/+ larvae (**c**, **e**, **g**) 5 days after heat shock. Yellow, neutral lipid; red, plasma membrane. Scale bar = 40 μm (**b**, **c**) or 10 μm (**d**–**g**).

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boundary; although Hedgehog signals over a wider range, specific staining there cannot be distinguished from background. In lipophorin–RNAi discs, Hedgehog (Fig. 4j, k) accumulates to abnormally high levels in the first five rows of anterior cells. We counted 380 Hedgehog spots in the most apical 10 μm of the wild-type disc shown in Fig. 4g. The lipophorin–RNAi disc shown in Fig. 4j contains 1,208 Hedgehog spots in the same region. Most accumulated Hedgehog colocalizes with Patched (Fig. 4k, l) in endosomes (Supplementary Fig. S5). Furthermore, Patched co-accumulates more extensively with Hedgehog in endosomes than it does in wild-type (Fig. 4h, k). These data indicate that lipophorin RNAi either increases the susceptibility of Hedgehog to Patched-mediated endocytosis, or prevents subsequent degradation of the protein.

We wondered whether lipophorin depletion might affect Hedgehog trafficking indirectly by preventing release of a needed co-factor from some other larval tissue. To investigate this, we added purified lipophorin particles to explanted lipophorin–RNAi discs and examined Hedgehog and Patched distribution. Abnormal Hedgehog and Patched accumulation was strongly reduced by a two-hour incubation of dissected discs with lipophorin particles (Supplementary Fig. S7). Thus lipophorin acts directly in imaginal discs to control Hedgehog trafficking, although it is still possible that its effects on signalling are indirect.

Drosophila cannot synthesize sterols and relies on dietary sources. To assess whether reduced uptake of sterols or other lipids might cause the changes we see, we explored the effects of lipid deprivation on larval development. Larvae were allowed to hatch and feed on sucrose/agarose plates supplemented with yeast for 2–3 days, then transferred to plates containing chloroform-extracted yeast autolysate, rather than yeast. These larvae are developmentally delayed; after 7 days of lipid deprivation, their discs are much smaller than those of younger late-third-instar larvae (compare Fig. 5a, b). In contrast, yeast-fed siblings pupariate and begin to eclose by this time. Those flies that infrequently eclose after larval lipid depletion are small (35–60% of normal body weight) but normally patterned (Fig. 5c, d). Thus, lipid depletion stalls imaginal growth.

To discover whether lipid starvation affected Hedgehog trafficking or signalling, we deprived larvae of lipid 2 days after hatching and stained their discs 6 days later (Fig. 5h–j). No changes in Hedgehog or Patched distribution are apparent in these discs compared with younger yeast-fed discs of similar size (Fig. 5e–g). Furthermore, the range of *dpp* and *collier* expression does not differ in lipid-starved and yeast-fed discs (Fig. 5k–p). Thus, lipid starvation does not mimic the effects of lipophorin knockdown. We speculate that lipid-starvation-induced growth arrest prevents membrane sterol from dropping to levels that would interfere with the Hedgehog pathway. Thus, lipophorin does not indirectly affect the Hedgehog pathway via lipid deprivation.

Wingless function requires lipophorin

To discover whether lipophorin RNAi perturbed Wingless trafficking, we examined Wingless distribution. In lipophorin–RNAi discs, extracellular Wingless is less abundant on both the apical and basolateral epithelial surfaces and spreads over shorter distances

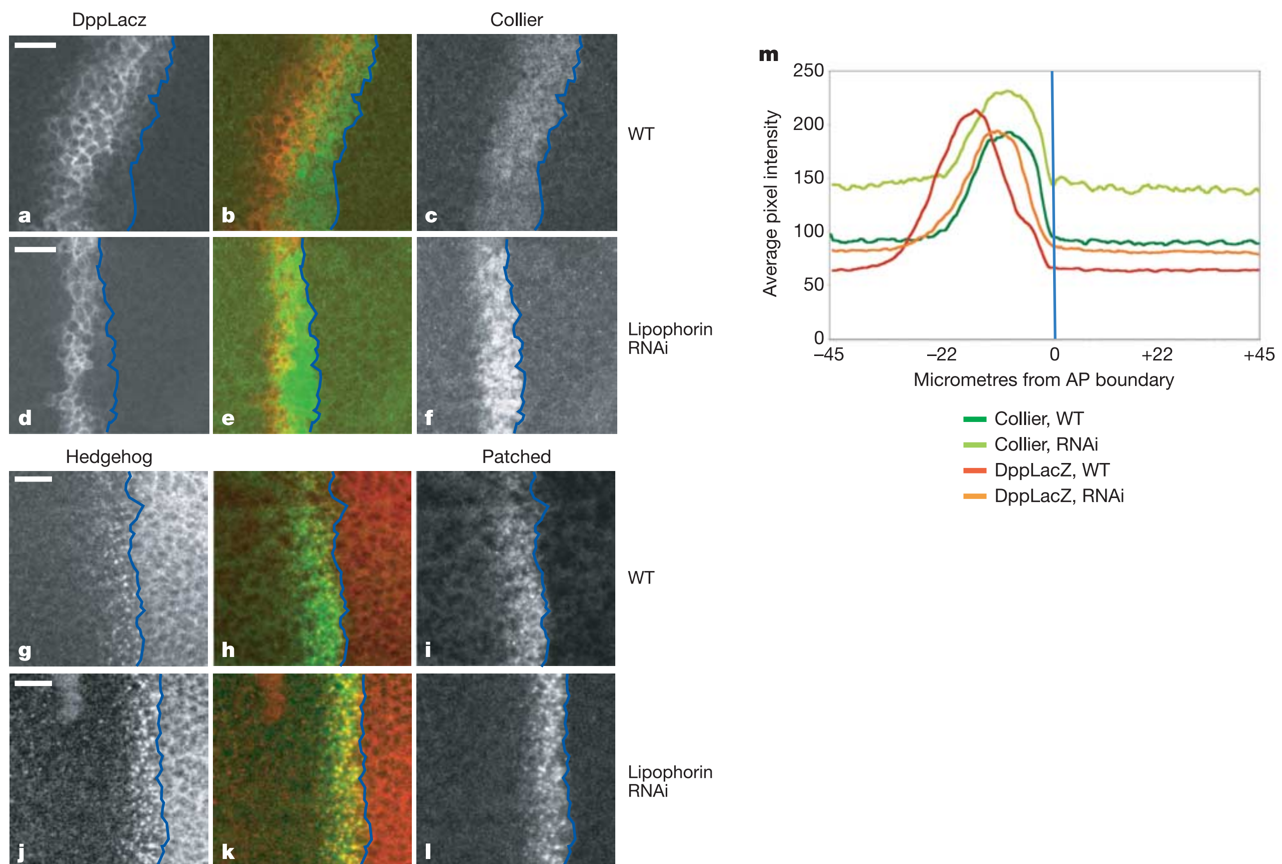

Figure 4 Lipophorin–RNAi alters Hedgehog distribution and signalling. Blue lines = AP boundary. Scale bar = 10 μm. **a**–**c**, *dpplacZ/+* disc 4 days after heat shock, stained for LacZ (**a**, and red in **b**) and Collier (green in **b**, and **c**). **d**–**f**, *hs-flippase/+;dpplacZ/+; Tubulin:GAL4/UAS:dsRNA* disc 4 days after heat shock, stained for LacZ (**d**, and red in **e**) and Collier (green in **e**, and **f**). **g**–**i**, Wild-type disc 4 days after heat shock, stained for Hedgehog (**g**, and red in **h**) and Patched (green in **h**, and **i**). **j**–**l**, *hs-flippase/+;UAS:dsRNA/Tubulin:GAL4* wing disc 4 days after heat shock, stained for Hedgehog (**j**, and red in **k**) and Patched (green in **k**, and **l**). **m**, Average Collier and DppLacZ staining intensities for four wild-type and four lipophorin–RNAi discs. Blue line indicates AP boundary. Average distance from AP boundary of peak LacZ staining was 16.6 ± 2.7 μm for the wild type, and 11.1 ± 1.5 μm for lipophorin–RNAi.

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(Fig. 6a–d). However, no consistent alterations in intracellular Wingless are detected (not shown). Thus, lipophorin promotes accumulation of extracellular Wingless.

To investigate whether Wingless signalling required lipophorin, we examined the activation of two target genes. Senseless is produced only in cells near the Wingless source and its expression is unaffected by lipophorin RNAi (not shown). Distalless is normally produced in a gradient throughout most of the wing pouch. In lipophorin–RNAi discs, the Distalless gradient is abnormally narrow (Fig. 6e–g). This suggests that lipophorin knockdown specifically perturbs long-range Wingless signalling.

Here, we establish the principle that lipid-linked proteins of the exoplasmic face of the membrane associate with lipoproteins. These include many gpi-linked proteins with diverse functions, as well as the lipid-linked morphogens Wingless and Hedgehog. The mechanism allowing long-range dispersal of lipid-linked proteins is not yet understood. The finding that these proteins exist in both membrane-associated and lipoprotein-associated forms suggests reversible binding to lipoprotein particles as a plausible mechanism for intercellular transfer, and the consequences of lowering lipoprotein levels in *Drosophila* larvae supports this idea.

Lipophorin knockdown narrows the range of both Wingless and Hedgehog signalling. Hedgehog accumulates to an abnormally high level in cells near the source of production and long-range signalling is inhibited; short-range target genes, however, are expressed normally. These data suggest that Hedgehog does not move as far when lipophorin levels are low. The range over which Hedgehog moves is normally restricted by Patched-mediated endocytosis. In discs from lipophorin RNAi larvae, accumulated Hedgehog co-localizes with Patched in endosomes, suggesting that it is more efficiently sequestered by Patched. How might lipophorin antagonize Patched-mediated sequestration and promote long-range movement?

Our data are consistent with the idea that lipophorin is continuously needed for movement, rather than required only for the release of morphogens. If lipophorin were important only for Hedgehog secretion, we would expect lipophorin RNAi to decrease the amount of Hedgehog found in receiving tissue; this seems not to

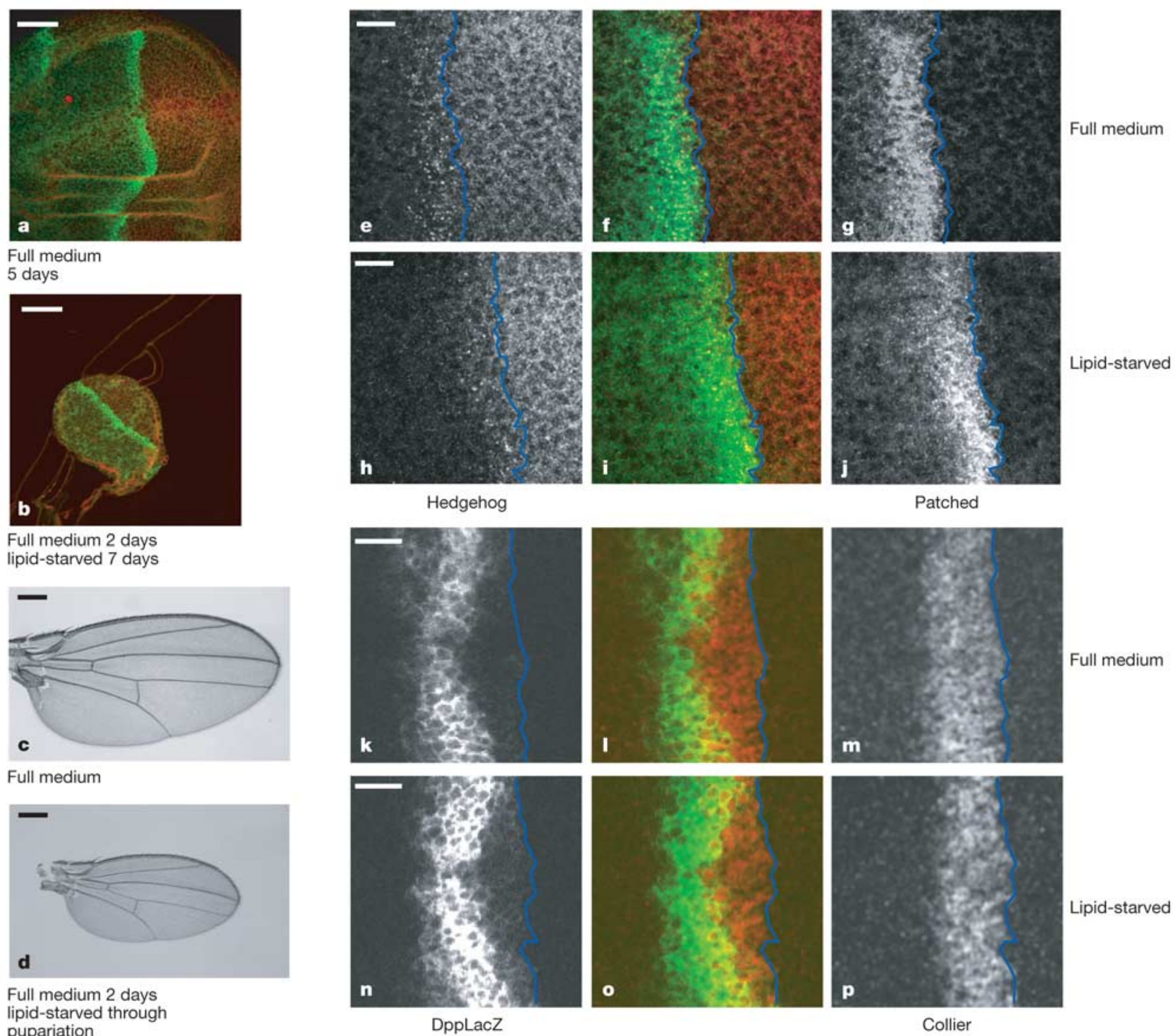

Figure 5 Hedgehog signalling is unaffected by lipid-depletion. **a**, **b**, Discs from fully fed (**a**) or lipid-starved (**b**) larvae. **c**, **d**, Wings from fully fed (**c**) or lipid-starved (**d**) flies. Scale bar = 250 μm. **e**–**j**, Discs from fully fed (**e**–**g**) or lipid-starved (**h**–**j**) larvae stained for Hedgehog (**e**, **h**, and red in **f**, **i**) and Patched (green in **f**, **i**; and **g**, **j**). Scale bar = 30 μm. **k**–**p**, Disc from fully fed (**k**–**m**) or lipid-starved (**n**–**p**) *dpplacZ*/+ larvae stained for LacZ (**k**, **n**, and green in **l**, **o**) and Collier (red in **l**, **o**; and **m**, **p**). Blue lines indicate AP boundary. Scale bars = 10 μm.

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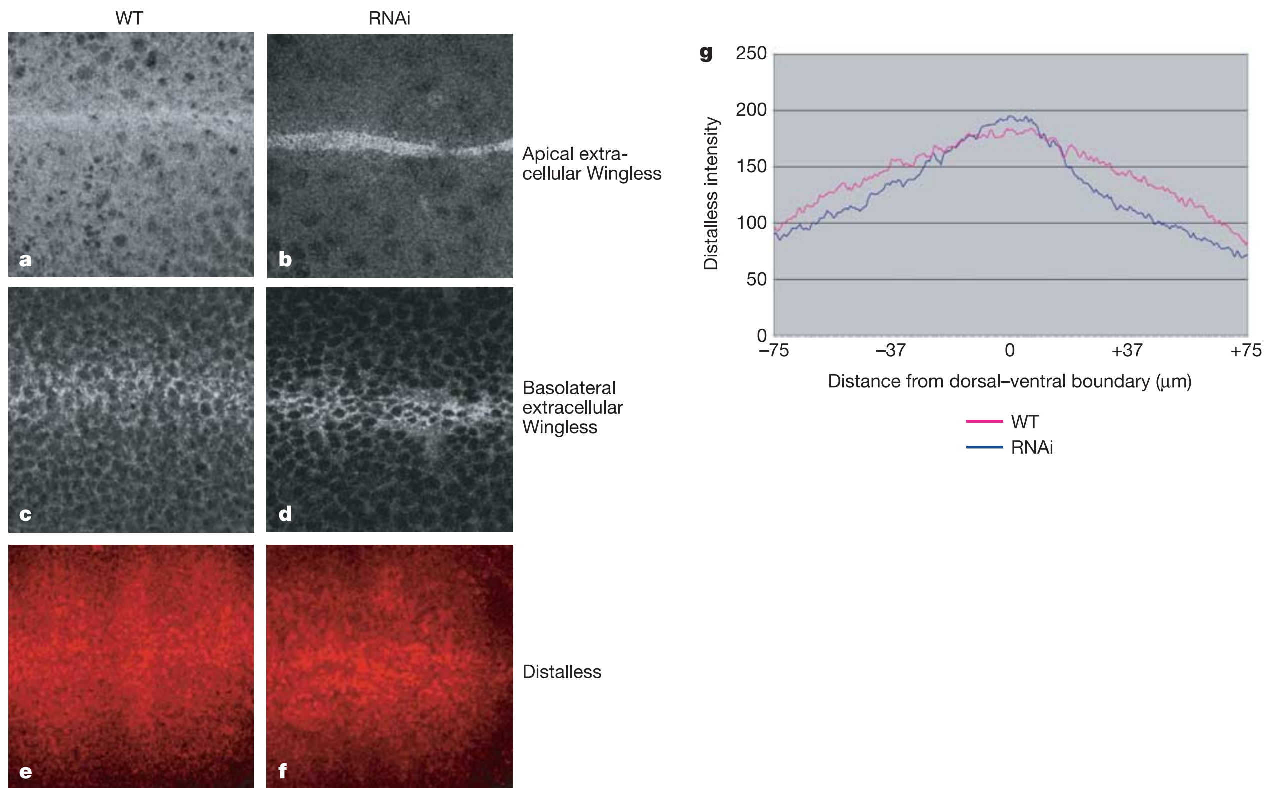

Figure 6 Lipophorin–RNAi narrows the range of Wingless signalling. **a**–**d**, Apical (**a**, **b**) and basolateral (**c**, **d**) sections of wild-type (**a**, **c**) and *Adh:GAL4/+; UASdsRNA/+* (**b**, **d**) wing discs 5 days after heat shock, stained for extracellular Wingless. **e**, **f**, Distalless protein accumulation in wild-type (**e**) and *hs-flippase/+;UAS dsRNA/ TubulinGAL4* (**f**) wing discs 5 days after heat shock. **g**, Average Distalless staining intensity with distance from dorsal–ventral boundary of five wild-type (pink) and five *hs-flippase/+;UAS dsRNA/ TubulinGAL4* (blue) wing discs. For plots of individual discs, see Supplementary Fig. S6.

be the case. Furthermore, altered Hedgehog trafficking in receiving tissue is consistent with a model in which lipophorin is required at each step of intercellular transfer. We favour the idea that reversible association of Hedgehog with lipophorin particles facilitates its transfer from the plasma membrane of one cell to that of the next. This model predicts that lowering lipophorin levels should increase the length of time that Hedgehog spends in the plasma membrane before becoming associated with lipophorin. This would slow its rate of transfer and increase the probability of Patched endocytosing Hedgehog before it moved to the next cell. Hedgehog would then signal efficiently in the short range, but be so efficiently sequestered by Patched that very little protein would travel far enough to activate long-range target genes. These predictions are completely consistent with our observations.

This model differs significantly from our original concept of argosome function. We initially speculated that argosomes were exosome-like particles with an intact membrane bilayer, and that lipid-linked morphogens needed to be assembled on these particles to be secreted by producing cells. Instead, we find that argosomes are exogenously derived lipoproteins that facilitate the movement of morphogens through the epithelium. Many questions remain as to how morphogens become associated with argosomes, and how the spread and cell-interactions of these particles are regulated. Clearly, heparan sulphate proteoglycans are essential for the movement of Hedgehog and Wingless into receiving tissue[35,36]. Because heparan sulphate binds to vertebrate lipoprotein particles[37,38], one might speculate that heparan sulphate proteoglygans (HSPGs) facilitate morphogen movement through lipoprotein binding. Conversely, we find many gpi-linked proteins, including the HSPG's Dally and Dally-like (unpublished data), on lipoprotein particles themselves. These associated proteins have the potential to modulate the cellular affinities or trafficking properties of lipoproteins and the morphogens they carry.

Our data suggest that lipophorin particles not only mediate intercellular transfer of Hedgehog, but may also be endocytosed together with the morphogen. Interestingly, LDL-receptor-related proteins Arrow and Megalin have demonstrated roles in Wingless signalling and Hedgehog endocytosis, respectively[39–41]. It is intriguing to speculate that these receptors might be important for interaction with the lipoprotein-associated form of the morphogen.

Cholesterol has the potential to modulate the activity of the Hedgehog pathway at many different points[3,42–44]. Whether changes in the level of cellular cholesterol normally play a role in regulating the activity of the pathway is unclear. Here we show that Hedgehog interacts with the particle that delivers sterol to cells. This observation raises the possibility that internalization of Hedgehog is linked to sterol uptake, and suggests new mechanisms to link nutrition, growth and signalling during development. □

Methods

Fractionation

Five millilitres of larvae were homogenized with 5 ml of 150 mM NaCl, 50 mM Tris-Cl pH 7.4, 2 mM EGTA plus protease inhibitors on ice. The 1,000*g* supernatant was centrifuged for 3 h at 33,600 r.p.m. (120,000*g*) at 4 °C in a SW40Ti rotor generating a pellet (P120) and supernatant (S120).

For isopycnic density centrifugation, we added 0.33 g ml^{-1} KBr to the S120, and centrifuged for 2 days at 40,000 r.p.m. (285,000*g*) at 10 °C in an SW40Ti rotor.

Immunoprecipitation

S120 pre-cleared 2 h with protein A-sephacryl CL4B beads was incubated with beads linked to different sera. Beads were washed with PBS, 1% BSA, then PBS, and eluted using Laemmli sample buffer or 150 mM NaCl, 2 mM EDTA, 100 mM Tris-Cl pH 8.3, 0.5% Nonidet-P40, 0.5% sodiumdeoxycholate and 0.1% SDS.

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Antisera

Rabbits were immunized with synthetic peptide LEGVIRRDSPKFKDL (Hedgehog amino acids 123–138), conjugated to keyhole limpet haemocyanin (Eurogentec). Antibody was affinity-purified on peptide-conjugated affigel-15 columns (Biorad).

DNA encoding amino acids 195–509 or 891–1070 (parts of ApoLII and ApoLI, respectively) was amplified from GH18004 (Resgen) and cloned into pQE30. His-tagged fusion proteins (Qiagen) were used to immunize rats or rabbits.

Expression construct

CD63:EGFP amplified from pEGFP-C1-bos (gift from G. Griffiths) was cloned into pUAST[45].

RNA interference

RNA interference was induced by expressing inverted repeats derived from two different regions of the Pro-apolipophorin cDNA (607 bp ending 47 bp from stop codon, and 500 bp starting at ATG). The first was amplified and inserted into pENTR2B (Invitrogen). Using the Gateway system, we inserted it twice in inverted orientation into pFRIPE. pFRIPE is derived from pUAST; downstream of the UAS are two Gateway insertion sites flanking an FLP cassette containing the HcRed gene and a transcription termination sequence.

The second fragment was amplified and cloned as an inverted repeat into pUhr. pUhr was derived from pUAST by inserting an HcRed-containing FLP cassette between the UAS and the multiple cloning site.

Flies containing lipophorin–RNAi constructs were crossed with others harbouring heat-shock-inducible FLP and one of several GAL4 drivers. After 5 days at 25 °C, larvae were heat-shocked for 90 min at 37 °C; this causes excision in all cells as determined by HcRed fluorescence. No excision occurs without heat shock in any larval tissue except the fat body (not shown).

dsRNA expressed under the control of either TubulinGAL4 (ubiquitous), AdhGAL4 (fat body and part of the gut) or C765GAL4 (disc-specific) was semi-lethal and produced identical larval phenotypes. No phenotype was ever observed when *lipophorin* dsRNA was expressed in imaginal discs.

Immunohistochemistry

Imaginal discs were fixed and stained as described[13]. Antibodies were diluted as follows: anti-Wg[46], 1:200; anti-Hh[47], 1:500; 1:100; anti-Ptc[48] 1:50; anti-βgal (Promega Z378A) 1:100; anti-Col[31] 1:200. To compare wild type and lipophorin–RNAi animals, tissues were stained in parallel and imaged under identical conditions with an LSMZeiss or Leica confocal microscope.

Image analysis

Hedgehog-positive spots in wild-type and lipophorin–RNAi discs were quantified in ten confocal sections 1 μm apart. The signal threshold was adjusted to 130 and images were despeckled using ImageJ (http://rsb.info.nih.gov/ij/). Grids were overlaid on the processed image and spots were counted manually.

We used ImageJ to quantify the Hedgehog signalling range in five projected apical sections of Col- and Dpp-stained discs. For each image, we determined pixel intensity along ten lines centred at the AP boundary using the Plot Profilefunction of ImageJ and averaged them to obtain a plot for each disc. Average plots from four discs of each type were generated using Microsoft Excel. Distalless range in Fig. 6 and Patched staining in Supplementary Fig. S7 were quantified similarly.

Lipid starvation

Eggs were collected on apple juice/agar plates + yeast for 24 h, then allowed to develop for 2–3 days on the same yeast-containing plates. Larvae were rinsed with PBS + 0.05% TritonX100, treated for 10 s with 50% Na hypochlorite, and rinsed with sterile H_2O. Larvae were transferred with sterile forceps to 10-cm plates containing 2% chloroform extracted agarose, 2.5% sucrose and 0.15% Nipagen, supplemented with either 0.3 g chloroform extracted yeast autolysate (for lipid starvation), or 0.3 g yeast (for lipid-fed controls).

Labelling lipophorin with Alexa488

Lipophorin particles were fluorescently labelled with AlexaFluor 488 (Molecular Probes) according to the manufacturer's instructions. Conjugate was separated from un-reacted label using Sephadex G-25 PD-10 columns (Amersham Pharmacia Biotech) and eluted with 100-mM Na-phosphate, pH 7.4, 100 mM NaCl, 10% sucrose.

Incubation of dissected discs with lipophorin particles

For experiments shown in Fig. 2 and Supplementary Fig. 7, imaginal discs were incubated at 29 °C with 50 $\mu g\, ml^{-1}$ lipophorin particles for 20 min and 2 h, respectively. On the basis of the starting volume of larvae and the final volume in which lipophorin was eluted, we estimate that this represents approximately 1/10 of the concentration of lipoprotein particles present in the haemolymph.

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