Lipoprotein particles associate with lipid-linked proteins and are required for long-range Wingless and Hedgehog signaling

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The thesis work was conducted from 15.9.2001 to 4.5.2005 under the supervision of Dr.Suzanne Eaton at Max Planck Institute of Molecular Cell Biology and Genetecis, Research group of Suzanne Eaton.

Dresden, 4.5.2005.

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signature

Im Kosmos wimmelte es überall von Ignoranz, und der Wissenschaftler verhielt sich wie ein Goldsucher, der im Bach der Unwissenheit nach den Nuggets der Erkenntnis fischte. Gelegentlich fand er einen kleinen gelben Klumpen im Kies der Unvernunft und im Sand der Ungewissheit, zwischen den haarigen, achtbeinigen und schwimmenden Dingen des Aberglaubens. In solchen Fällen richtete er sich auf und rief zum Beispiel: "Hurra, ich habe Boyles Drittes Gesetz entdeckt!" Dann fühlten sich alle viel besser…….

Terry Pratchett

Table of contents

SUMMARY

Morphogens of the Wnt and Hedgehog families are secreted signaling molecules that coordinate growth and patterning of many different tissues. Both, Wingless and Hedgehog spread across long distances in the developing wing of *Drosophila melanogaster*. However, both proteins are covalently modified with lipid moieties. The mechanisms that allow long-range movement of such hydrophobic molecules are unclear.

Like Wingless and Hedgehog, glycosylphosphatidylinositol (gpi)-linked proteins also transfer between cells with their lipid anchor intact. It has been speculated that gpi-linked proteins and lipid-linked morphogens travel together on a membranous particle, which was termed argosome. As yet however, no functional link between argosome production and dispersal of lipid-linked proteins has been established.

The topic of this thesis is to understand the cell biological nature of the argosome and thus contribute to understanding of morphogen gradient formation. To address the question of argosome biosynthesis, at least two models have been proposed. One possibility is that argosomes are membranous exovesicles with a complete membrane bilayer. Alternatively, argosomes might resemble lipoprotein particles that comprise one of a family of apolipoproteins scaffolded around a phospholipid monolayer that surrounds a core of esterified cholesterol and triglyceride. Lipid-modified proteins of the exoplasmic face of the membrane (like GFPgpi, Wingless or Hedgehog) might fit well into the outer phospholipid monolayer of such a particle.

Here, I utilize biochemical fractionation to determine the sort of particle that lipid-linked proteins associate with. I show that Wingless, Hedgehog and gpi-linked proteins bind *Drosophila* lipoprotein particles *in vitro*, and colocalize with them in wing imaginal discs. Next, I use genetic means to address the functional importance of this association. I demonstrate that reducing Lipophorin levels in Drosophila larvae perturbs long-range but not short-range Wingless and Hedgehog signaling, and increases the sequestration of Hedgehog by Patched. I propose that Lipophorin particles are vehicles for the long-range movement of lipid-linked morphogens and gpi linked proteins.

ABBREVATIONS

A	anterior
A/P	anterior/posterior
AChE	acetylcholinesterase
Adh	alcoholdehydrogenase
ADP	adenosine diphosphate
ap	apterous
apo	apolipoprotein
ApoLI	apolipophorin I
ApoLII	apolipophorin II
ART	ADP-ribosyltransferase
CD	cellular determinant
Cys	cysteine
D	dorsal
D/V	dorso/ventral
DAF	decay accelerating factor
Dpp	Decapantaplegic
ECM	extracellular matrix
en	engrailed
Fas-1	Fasciclin 1
GAG	glycosoaminoglycan
GFP	green fluorescent protein
GlcA	glucuronic acid
GlcN	glucosamine
Gly	glycine
GPC	glypican
GPI (gpi)	glycosylphosphatidylinositol
GPI-APs	Gpi-anchored proteins
HDL	high-density lipoprotein
Hh	Hedgehog
Hh-C	Hedgehog C terminal fragment
Hh-N	Hedgehog N terminal fragment
HS	heparan sulfate
HS-GAG	heparan sulfate glycosoaminoglycan
HSPGs	heparan sulfate proteoglycans
IDL	intermediate density lipoprotein
kDa	kilo Dalton
LDL	low-density lipoprotein

Man	mannose
MTP	Microsomal Triglyceride Transfer Protein
MVB	multi vesicular bodies
P	posterior
Phe	phenylalanine
PI-PLC (D)	phosphatidylinositol phospholipase C (D)
PrPC	cellular prion protein
Ptc	Patched
RNA	ribonucleic acid
RNAi	RNA interference
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	Serine
Shh	Sonic Hedgehog
Smo	Smoothened
Tub	Tubulin
V	ventral
VLDL	very low density lipoprotein
VSG	variant surface glycoprotein
Wg	Wingless
Wnt-1	*Drosophila* Wingless integration site 1
WT	wild type

NOTE: the terms, lipidic particle and lipoprotein particle are used interchangeably

1. INTRODUCTION

In the developing wing of *Drosophila*, the morphogens Wingless and Hedgehog control growth and cell fate determination during development. Both proteins act at both short and long distances to activate target gene expression. Despite their ability to travel many cell diameters away from the cells that produce them, both morphogens are covalently modified by lipid and exhibit strong affinity to membranes. By which mechanism such hydrophobic molecules might spread across long distances is unknown.

Like Wingless and Hedgehog, glycosylphosphatidylinositol (GPI)-linked proteins are released from cells by various means. How intracellular transfer of these proteins with their lipid anchor intact might occur?

It has been observed that gpi-linked 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, it has been proposed that these proteins travel together on a membranous particle, which was named an argosome. How might argosomes form? One possibility is that argosomes are membranous exovesicles. Alternatively, argosomes might resemble lipidic particles.

Chapter 1.1. Morphogens of the wing imaginal discs

In the course of development cells differentiate within a tissue or multicellular field according to their spatial position. This is achieved by interactions between neighboring cells or by long-range signaling events controlled by morphogens (Kerszberg, M. *et al.* 1998). By definition, morphogens are secreted signaling molecules that are produced in a subset of cells, and are subsequently released to spread through the field of cells in a graded manner. The receiving cells are provided with positional information as they are exposed to varying concentrations of morphogen at different distances from the morphogen source. Thus, as a function of their position in the field of cells, they respond to the morphogen concentration differently to induce short-,

medium- or long-distance target gene expression (Figure 1) (Strigini, M. *et al.* 1999; Tabata, T. 2001; Teleman, A.A. *et al.* 2001).

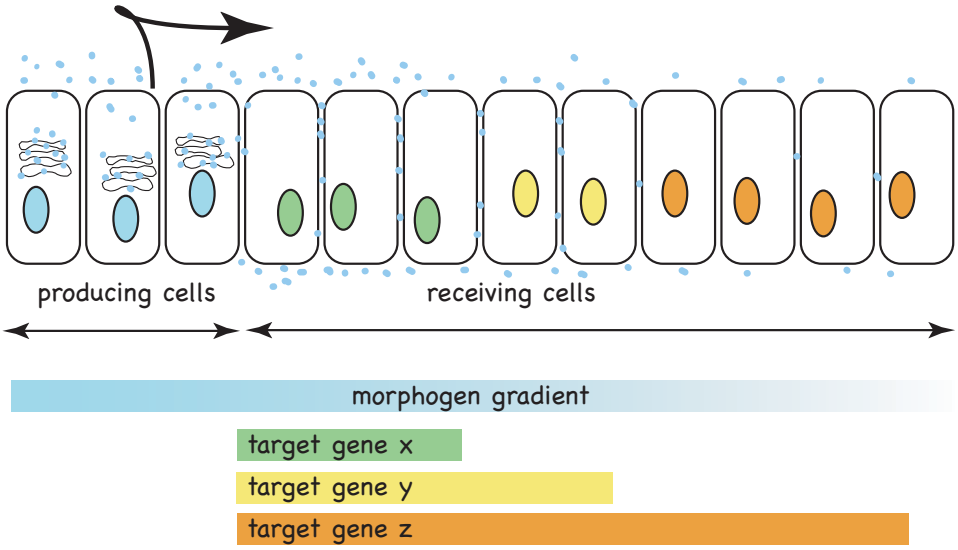


Figure 1: **Morphogen theory.** Morphogens are produced and released from a subset of cells and set positional information of a cell by forming concentration gradient across the developmental field in which the cell resides. Above different concentration thresholds, different genes are activated at different distances from the source (adapted from Tabata, T., 2001).

The developing wing of *Drosophila melanogaster* has been used as a favourable model system to study the morphogens and their gradient formation. An adult wing of *Drosophila* derives from the wing imaginal disc. By the end of the third instar larval stage, the wing imaginal disc is an invaginated epithelial sac composed of a single-layered polarized pseudostratified epithelium that is continuous with the squamous epithelium of the peripodial membrane. It is subdivided into specific compartments: anterior (A) and posterior (P) along the A/P axis, dorsal (D) and ventral (V) along the D/V axis (Figure 2). The wing imaginal disc is patterned by morphogens that spread and form a gradient in the cells of pseudostratified epithelium.

At least three criteria must be met to qualify a signaling molecule as a morphogen: secretion and diffusion from a source, the concentration dependent induction of discrete cellular states and direct instructive action from a distance (Teleman, A.A. *et al.* 2001; Martinez Arias, A. 2003). Three proteins of *Drosophila* wing imaginal disc

fulfill these conditions: Wingless (Wg), Hedgehog (Hh) and Decapentaplegic (Dpp) (Nellen, D. *et al.* 1996; Zecca, M. *et al.* 1996; Lecuit, T. *et al.* 1997; Neumann, C.J. *et al.* 1997; Strigini, M. *et al.* 1997). Wingless is released by the cells straddling the D/V boundary and is distributed along its both sides (Figure 2). At a short distance, Wingless induces expression of genes like *achaete scute* and at longer distances, genes such as *vestigial* or *Distal-less.* It is required to specify the wing pouch and in later stages to pattern the wing blade and the wing marginal structures. Hedgehog is secreted by P compartment cells and forms a gradient in A compartment cells (Figure 2), inducing short-distance target genes like *patched* and long-distance targets like *decapentaplegic*. In addition, it controls the patterning of the central domain of the wing primordium. Decapentaplegic forms a symmetric gradient in both A and P compartments (Figure 2). Here, in a concentration dependent manner, it induces genes like *spalt* or *optomotorblind*. Decapentaplegic controls the patterning of the wing disc beyond the central domain and is required for cell growth (reviewed in Tabata, T. 2001).

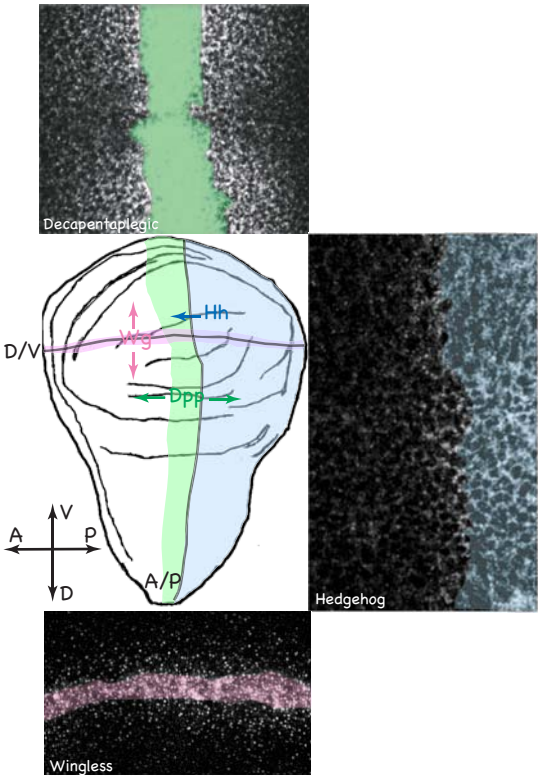


Figure 2: *Drosophila* wing imaginal disc as a model to study morphogen gradients. The wing imaginal disc consists of two epithelial layers. It is subdivided into the compartments; dorsal (D), ventral (V), anterior (A) and posterior (P) and patterned by three morphogens; Decapentaplegic, Hedgehog and Wingless.

Wingless is a lipid modified protein

Wingless, the *Drosophila* homologue of Wnt-1 (*Drosophila* Wingless integration site-1) is one of the best-characterized Wnt family members. Wnt proteins share several characteristic motifs, including 23 or 24 conserved cysteine residues, many highly charged amino acid residues and glycosylation sites. The changes in the protein structure can lead to misfolding and impaired secretion. Indeed, several *wingless* mutations cause retention of mutant Wingless protein in the producing cells, suggesting that the overall conformation of the molecule may control distinct functions (van den Heuvel, M. *et al.* 1993; Bejsovec, A. *et al.* 1995). *N*-glycosylation also appears to be a common modification of Wnts and might contribute to their proper folding, secretion, and biological activity (Smolich, B.D. *et al.* 1993).

In spite of the fact that Wingless is a secreted glycoprotein, it associates tightly with the cell surface or with the proteins of the extracellular matrix (Smolich, B.D. *et al.* 1993; Reichsman, F. *et al.* 1996). Strikingly, it has been shown that mammalian Wnt-3a is palmitoylated at a conserved cysteine residue (Cys77) (Willert, K. *et al.* 2003). The idea that Wingless also undergoes palmitoylation, is supported by the following observations; in *Drosophila* embryos mutant for a gene called *porcupine*, Wingless protein is confined to its producing cells (van den Heuvel, M. *et al.* 1993; Kadowaki, T. *et al.* 1996). Molecular cloning of *porcupine* revealed that it encodes an endoplasmic reticulum multi-transmembrane protein that has homology to *O*-acyltransferases, indicating that it might facilitate Wingless acylation (Hofmann, K. 2000). It has been recently reported that Wingless is indeed palmitoylated (Figure 3A). The lipid modification occurs in the endoplasmic reticulum and is porcupine dependent (Zhai, L. *et al.* 2004).

Noteworthy, *S*-palmitoylation is one of the most common posttranslational modifications. It increases protein hydrophobicity and promotes membrane association (Linder, M.E. *et al.* 2003). In addition, palmitoylation can serve as a targeting signal that tethers protein to specific organelles as well as to lipid rafts (Ikonen, E. *et al.* 1998; Patterson, S.I. 2002). Interestingly, Zhai *et al*. (Zhai, L. *et al.* 2004) have shown that

lipid-modified Wingless partitions to lipid rafts before secretion and that this targeting is mediated by porcupine activity. In addition, Wingless protein has high affinity to molecules of the extracellular matrix, namely heparan sulfate proteoglycans (HSPGs). Their role in Wingless membrane association and signaling is described later (see Chapter 1.2.).

Lipid modifications of Hedgehog

Hedgehog family proteins are another class of secreted signaling molecules that control the growth and patterning of developing organisms. Despite their ability to induce expression of target genes at a distance, they undergo two posttranslational lipid modifications that confer stable membrane association (Figure 3B) (Ingham, P. 2001).

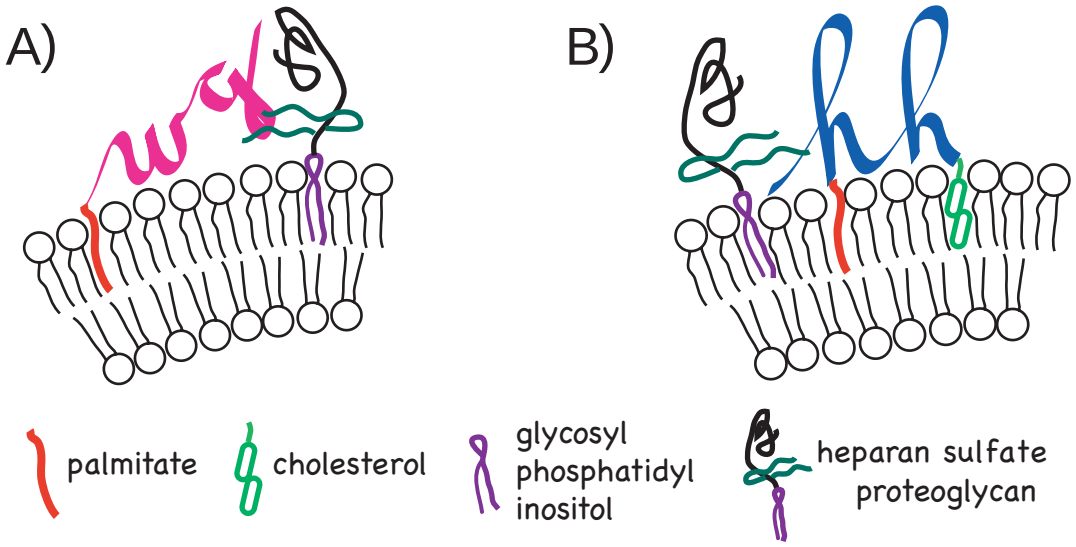


Figure 3: Lipid modifications of Wingless and Hedgehog. Both Wingless and Hedgehog have high affinity for membranes. Wingless is palmitoylated, Hedgehog is palmitoylated and cholesterol modified. The membrane affinity is further facilitated by their interactions with heparan sulfate proteoglycans.

Hedgehog is synthesized as a 46-kDa precursor protein in the secretory pathway. In addition to the cleavage of a signal peptide, the Hedgehog molecule undergoes an intramolecular cleavage between Gly and Cys residues within the conserved tripeptide Gly-Cys-Phe (Lee, J.J. *et al.* 1994; Porter, J.A. *et al.* 1995). The autoprocessing reaction

is catalysed by a 25-kDa C-terminal fragment (Hh-C) that yields an active 19-kDa N-terminal fragment (Hh-N) involved in signaling (Mann, R.K. *et al.* 2000; Jeong, J. *et al.* 2002). As a result of this autoproteolytic cleavage, the C terminus Hh-N is covalently bound to cholesterol (Lee, J.J. *et al.* 1994; Porter, J.A. *et al.* 1996). The second lipid modification of the Hedgehog protein is, like in the case of Wingless, palmitoylation (Pepinsky, R.B. *et al.* 1998). Palmitoylation occurs at the most N terminal cysteine (Cys 28 in murine Shh and Cys 85 in fruitfly Hh) of Hh-N and is mediated by *O*-acyltransferase named variably *sightless, central missing, skinny hedgehog*, and *rasp* that shares high homology to *porcupine* (Amanai, K. *et al.* 2001; Chamoun, Z. *et al.* 2001; Lee, J.D. *et al.* 2001; Micchelli, C.A. *et al.* 2002). Interestingly, autoprocessing and cholesterol modification precede palmitoylation (Chamoun, Z. *et al.* 2001). Moreover, both of these modifications target Hedgehog to lipid rafts (Rietveld, A. *et al.* 1999; Jeong, J. *et al.* 2002).

Intriguingly, these modifications are important for proper Hedgehog distribution and signaling activity. When *Drosophila* Hedgehog lacking the cholesterol modification is expressed in the wing imaginal disc, it is efficiently secreted and causes a gain-of-function phenotype (Porter, J.A. *et al.* 1996; Burke, R. *et al.* 1999). On the contrary, performing the similar experiments in mice, have lead to the opposite observations; Sonic hedgehog (mice homologue of *Drosophila* Hedgehog) without cholesterol moiety have influenced the cells only near its source of production (Lewis, P.M. *et al.* 2001). It has been shown that palmitoylation is required for effective Hedgehog production and release but not for Hedgehog expression. The absence of palmitoyl chain caused a similar effect as the absence of cholesterol moiety in *Drosophila* tissue; the Hedgehog protein was more efficiently released and has reached the target cells (Chamoun, Z. *et al.* 2001).

Clearly, the lipid modifications are required for proper Hedgehog distribution in the tissue, either to influence its spatial mobility or to facilitate its interaction with other components of Hedgehog pathway. Indeed, cholesterol anchor is required to mediate the interaction between Hedgehog and Dispatched or heparan sulfate proteoglycans (see Chapter 1.2.) (Bellaiche, Y. *et al.* 1998; Burke, R. *et al.* 1999; The, I. *et al.* 1999).

Thus, the covalent attachment of palmitoyl to Winglesss and of cholesterol and palmitoyl to Hedgehog lead to the incorporation of both proteins into exoplasmic leaflet of the membrane bilayer (Pepinsky, R.B. *et al.* 1998; Ingham, P. 2001; Willert, K. *et al.* 2003; Peters, C. *et al.* 2004; Zhai, L. *et al.* 2004). Paradoxically, in the wing imaginal disc, Wingless can signal through a range of over 30 cell diameters (Neumann, C.J. *et al.* 1997) and Hedgehog over at least 12 cell diameters (Strigini, M. *et al.* 1997). What mechanisms allow these morphogens with such a strong membrane affinity to travel long distances in the developing epithelia?

Spread of morphogens through the developing tissue: models

Several mechanisms might account for the long-ranged and graded dispersal of morphogens through the developing multicellular field (Figure 4). In general, signaling molecules could diffuse passively through the extracellular space or the active process of planar transcytosis might control their dispersal. Alternatively, the transport through cellular protrusions or spread, of especially lipid-linked morphogens, on a carrier might be involved (reviewed in Strigini, M. *et al.* 1999; Christian, J.L. 2000; Tabata, T. 2001; Teleman, A.A. *et al.* 2001; Cadigan, K.M. 2002; Vincent, J.P. *et al.* 2002; Gonzalez-Gaitan, M. 2003).

In the first model, a passive diffusion of molecules through the extracellular matrix would permit the spread of the signaling molecules across long distances. In this model, the morphogen is diluted as it moves away from its source of production. Degradation of the protein within the extracellular space might also control the half-life of the morphogen (Lander, A.D. *et al.* 2002). Alternatively, the morphogen might travel extracellularly, but binding to its receptor at the plasma membrane restricts morphogen diffusion. In such a model, dispersal is controlled by the abundance of the receptor at the plasma membrane. If the internalization and recycling of the receptor is blocked, the receptor accumulates at the plasma membrane and limits the morphogen spread. Next, the morphogen diffuses in the extracellular space, but the rate of degradation in the

lysosomes of the receiving cells helps to shape the morphogen distribution (Strigini, M. *et al.* 1999; Vincent, J.P. *et al.* 2002).

If the diffusion through the extracellular space is restricted, the cell-to-cell movement of morphogens can be explained by active transport that involves planar transcytosis (Entchev, E.V. *et al.* 2000). In this model, the morphogen is internalized by endocytosis, transported intracellularly and subsequently released to be endocytosed by the adjacent cell. Subsequent steps of endocytocysis and recycling facilitate the spread of the morphogen through the epithelium. To form the gradient the ligand is targeted for degradation to the lysosome and the rate between recycling and degradation specifies the slope of such a gradient.

The next model involves the traveling of the signaling molecules through membranous protrusions. It has been shown that *Drosophila* wing imaginal disc cells make apical membrane extensions called cytonemes that are filapodia-like structures. It has been suggested that the receiving cells make these protrusions towards the producing cells to capture morphogen molecules (Ramirez-Weber, F.A. *et al.* 1999). Another type of membrane extensions, in the imaginal discs, are projected from the squamous peripodial membrane towards the underlying columnar epithelium. It has been suggested that these peripodial membrane extensions might be used by the morphogens for their spread throughout the whole epithelium (Gibson, M.C. *et al.* 2000).

Clearly, a combination of these models is possible. However, each of these models assumes that morphogens are secreted from the producing cells in their soluble form and do not take into account their linkage to the plasma membrane or strong affinity to the molecules of extracellular matrix. One option that has been suggested is that lipid moieties can promote the multimerization of lipid-linked morphogens by hiding the lipids inside the complex, and thus facilitate their solubility (Zeng, X. *et al.* 2001). On the other hand, membrane anchored morphogen could move through the epithelium also by cell-to-cell transfer. However, this possibility does not explain the long-range dispersal of such a ligand. Alternatively, it has been proposed that lipid-linked morphogens can travel on the hydrophobic carriers, named argosomes (see Chapter1.3) (Greco, V. *et al.* 2001). The work in this thesis addresses the latter possibility.

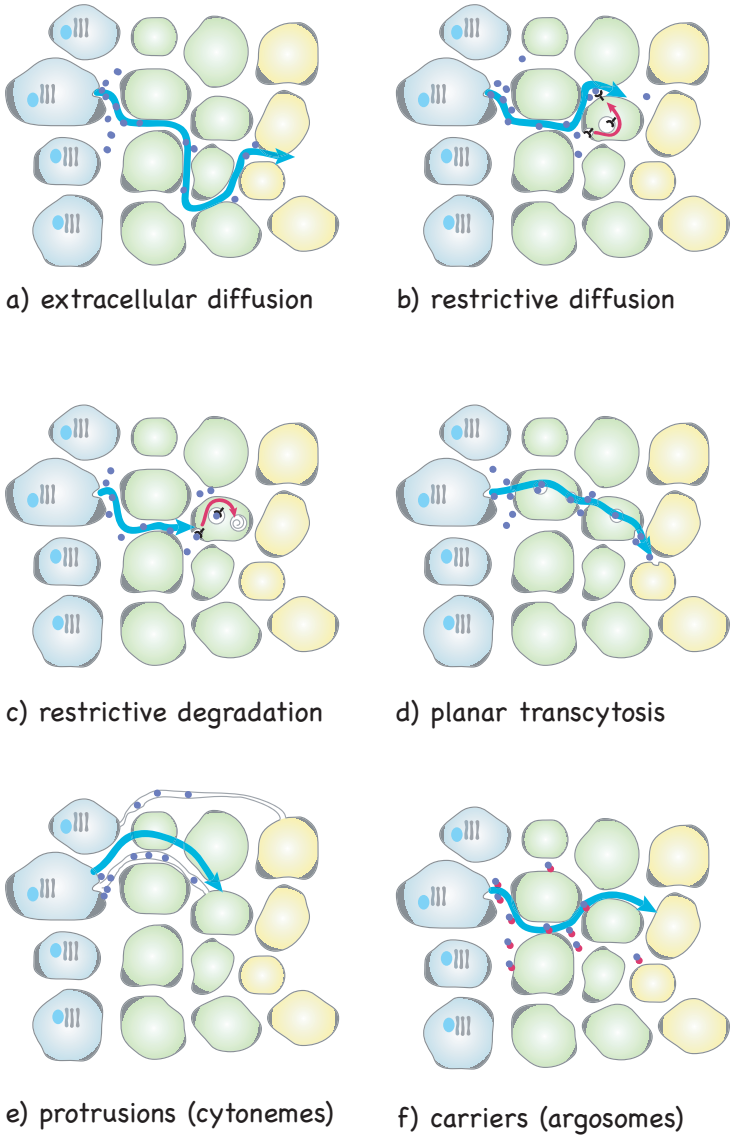


Figure 4: Models of morphogen trafficking. A) In the extracellular diffusion morphogen gets diluted as it moves away from its source. **B)** Restrictive diffusion. Morphogen's dispersal in the extracellular space is restricted by binding to its receptors. The rate of internalization and recycling of the receptors shapes the morphogen gradient. **C)** In the restrictive degradation model, morphogen also traffics by diffusion, but it is taken up cells and targeted to the lysosomes. The rate of its degradation shapes the gradient. **D)** Planar transcytosis is an active mechanism of transcellular transport by which morphogen is internalized by endocytosis, transports intracellularly and is released to signal in the adjacent cell. **E)** Morphogen is released and taken up by protrusions, like cytonemes coming from the receiving cells. **F)** Morphogen is associated with a carrier (e.g. argosome) that facilitates its intercellular spread. (adapted from González-Gaitán, M., 2003)

Chapter 1.2. GPI anchored proteins

Additionally to the acylation, the covalent attachment of the glycosyl-phosphatidylinositol (gpi) anchor is another common posttranslational lipid modification that tethers proteins to the exoplasmic leaflet of the membrane bilayer. Since the discovery of the gpi moiety (Ferguson, M.A. *et al.* 1988) a large number of eukaryotic proteins have been identified as gpi anchored, including protozoans surface coats, prion proteins, various adhesion molecules, enzymes, cell surface antigens and receptors (Sharom, F.J. *et al.* 2002).

The general structure of gpi anchor consists of glycan core Man(α1->2)Man(α1->6)Man(α1->4)GlcN that is glycosidically linked to the 6-hydroxylgroup of phosphatidylinositol. The terminal mannose is linked via phosphoethanolamine to the C terminal residue of the protein (Figure 5) (Ferguson, M.A. 1999; Sharom, F.J. *et al.* 2002). Interestingly, the specificity of the anchor can be facilitated by acylation of the inositol ring, usually with palmitate, or by glycosylation of mannosyl residues (Sharom, F.J. *et al.* 2002). Gpi anchored proteins (GPI-APs) are localized to the exoplasmic leaflet of the apical (Lisanti, M.P. *et al.* 1989) as well as to the basolateral plasma membranes (Zurzolo, C. *et al.* 1994; Greco, V. *et al.* 2001) Gpi-anchored proteins are well-established components of lipid rafts (reviewed in Sharom, F.J. *et al.* 2002) in mammalian systems as well as in *Drosophila*. In fact, the majority of the proteins associated with *Drosophila* lipid rafts are gpi-anchored (Rietveld, A. *et al.* 1999).

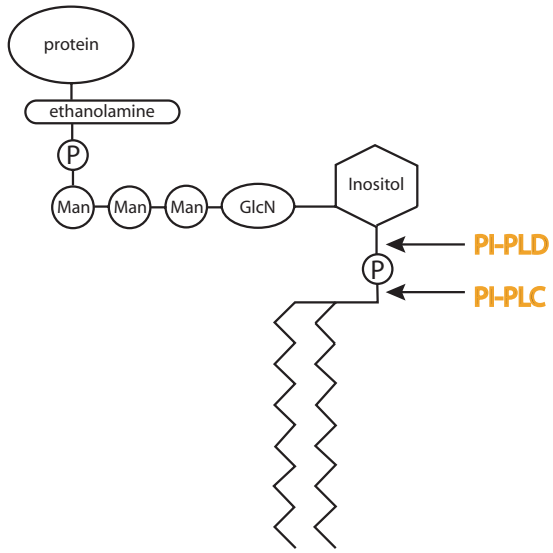


Figure 5: Conserved structure of GPI anchor. The glycan core is glycosidically linked to phosphatidyl inositol. The C terminus of the protein is linked to the glycan core via phosphoethanolamine. The sites of anchor cleavage by PI-PLC and PI-PLD are indicated (adapted from Sharom, F.J. and Lehto, M.T., 2002)

Glypicans form a subclass of GPI-anchored proteins

Glypicans are members of the heparan sulfate proteoglycans (HSPG) family that are linked to the cell surface via their gpi-anchor and are considered as a subclass of GPI-APs. The glypican family currently consists of six mammalian proteins (GPC1-6), one *C. elegans* glypican and two *Drosophila* glypicans (De Cat, B. *et al.* 2001; Filmus, J. 2001). The modular structure of glypicans is highly conserved across species; alignment of the protein sequences revealed more than 90% similarity (De Cat, B. *et al.* 2001). The structure comprises a N-terminal signal sequence that targets protein to the secretory pathway, a core protein, a glycosoaminoglycan (GAG) attachment site domain and a hydrophobic C-terminal signal sequence that is cleaved off during the transfer of the gpi

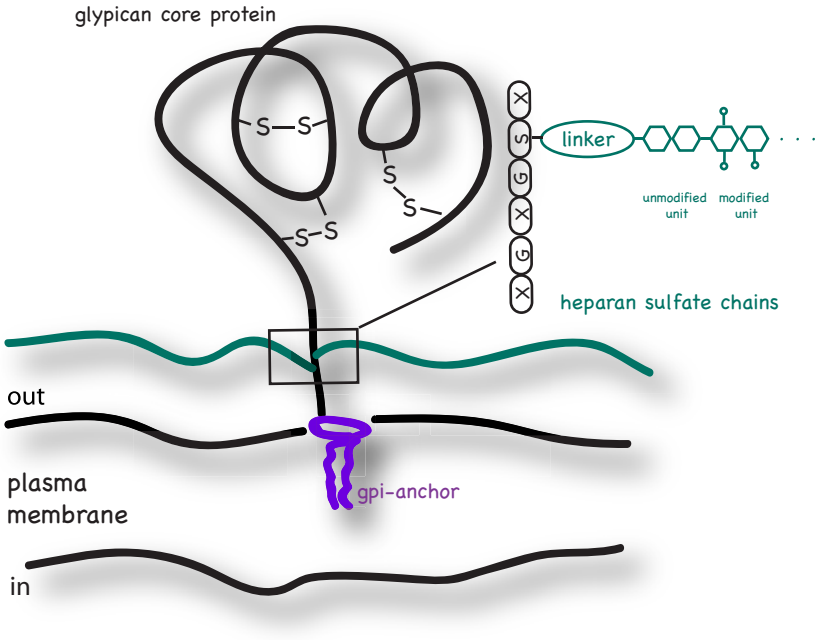


Figure 6: Schematic representation of glypicans. The glypican core protein locates completely to the extracellular space. Disulfide bridges organize it into a compact globular domain. HS chains are covalently bound through the sugar linker to the serine residues of Ser-Gly-X-Gly motifs in the polypeptide chain at the close vicinity to the plasma membrane. The protein is covalently linked to the plasma membrane at its carboxyterminus via a GPI anchor (adapted from De Cat, B. and David, G., 2001)

anchor (Figure 6) (Fransson, L.A. *et al.* 2000; Baeg, G.H. *et al.* 2001; De Cat, B. *et al.* 2001; Fransson, L.A. 2003). The core protein contains 14 conserved cysteine residues that maintain its globular structure by number of disulfide bridges (Fransson, L.A. *et al.* 2000).

The heparan sulfate glycosoaminoglycans (HS-GAG) side chains are covalently attached to serine residues in serine-glycine-x-glycine motifs in the domain close to the

plasma membrane. The heparan sulfate (HS) side chains are linear polysaccharide polymers of alternating *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) residues that are assembled in modified and unmodified units. The numerous modifications include among others deacetylation, sulfation and epimerization at various positions; at least 14 biochemical steps contribute to HS-GAG synthesis (Baeg, G.H. *et al.* 2000; Fransson, L.A. *et al.* 2000; Perrimon, N. *et al.* 2000; De Cat, B. *et al.* 2001; Fransson, L.A. 2003). The variability and the degree of these modifications create a high structural diversity of HS side chains.

Glypicans face the extracellular space due to the gpi anchorage and are part of extracellular matrix (ECM) where they associate with many ECM proteins. However, mainly their role in binding specifically to growth factors and morphogens has been extensively studied. Glypicans have been implicated in the regulation and fine-tuning of several transduction pathways that control the cell proliferation and differentiation. Mutations in the glypican genes or in the genes encoding glypican modifying enzymes result in abnormal morphogenesis, perturbed cell division and cell death and can lead to several pathological conditions such as Simpson-Golabi-Behmel syndrome (mutation in GPC3) or hereditary multiple exostoses (mutation in HS co-polymerase) (reviewed in De Cat, B. *et al.* 2001; Filmus, J. 2001; Princivalle, M. *et al.* 2002).

Dally and Dally-like

Studies of two *Drosophila* glypicans, Dally (division abnormally delayed) and Dally-like have provided invaluable knowledge about the function of these molecules in the development. Dally was identified in a genetic screen for mutants involved in cell division in the developing central nervous system (Nakato, H. *et al.* 1995). Searching for additional putative glypicans encoded in the *Drosophila* genome, Dally-like was isolated (Khare, N. *et al.* 2000; Baeg, G.H. *et al.* 2001). Since their identification, their role in Dpp, Hh and Wg pathways is established (Jackson, S.M. *et al.* 1997; Lin, X. *et al.* 1999; Tsuda, M. *et al.* 1999; Baeg, G.H. *et al.* 2001; Desbordes, S.C. *et al.* 2003; Fujise, M. *et al.* 2003; Bornemann, D.J. *et al.* 2004; Han, C. *et al.* 2004).

How are Dally and Dally-like implicated in Wingless and Hedgehog pathways? The initial observation that Wingless can be released from the cell surface by addition of exogenous sulfated glycosaminoglycan and that Wingless directly binds to heparin has suggested that it can be associated with HSPGs *in vivo* (Reichsman, F. *et al.* 1996). Studying the interaction between Dally and Frizzled 2, Wingless receptor, raise the possibility that HSPGs might sequester Wingless in order to restrict its diffusion or to act as a co-receptors (Lin, X. *et al.* 1999; Tsuda, M. *et al.* 1999). In addition, isolation of mutants in the biosynthesis of HS side chains: *sugarless* (Binari, R.C. *et al.* 1997; Hacker, U. *et al.* 1997; Haerry, T.E. *et al.* 1997), *fringe connection* (Goto, S. *et al.* 2001; Selva, E.M. *et al.* 2001) and *sulfateles*s (Baeg, G.H. *et al.* 2001) and the studies of their effect on Wingless pathway have supported the idea that HSPGs are involved in Wingless signaling or distribution. Finally, overexpression of Dally-like in the wing imaginal disc results in ectopic accumulation of extracellular Wingless (Baeg, G.H. *et al.* 2001). Based on these observations, it has been proposed that HSPGs might play a role as co-receptors or they might alter Wingless distribution by increasing its membrane affinity. Several new studies have addressed these two possibilities (Luders, F. *et al.* 2003; Bornemann, D.J. *et al.* 2004; Han, C. *et al.* 2004; Takei, Y. *et al.* 2004). These data have shown that perturbed synthesis of HSPGs results in defective Wingless trafficking and extracellular distribution and that this might be an effect of combined action of both Dally and Dally-like (Han, C. *et al.* 2005).

Similarly, Hedgehog distribution also requires heparan sulfate proteoglycans. Early studies implicated the activity of Tout velu, HS co-polymerase. Tout velu mutant cells are able to release Hedgehog normally, but they impair Hedgehog movement across the receiving cells. Interestingly, only cholesterol-modified Hh requires Tout velu activity (Bellaiche, Y. *et al.* 1998; The, I. *et al.* 1999). This observation suggested that there is a specific transport mechanism for lipid-linked Hedgehog mediated by HSPGs. Han and colleagues (Han, C. *et al.* 2004) have shown that Dally and Dally-like are direct substrates for Tout velu. Furthermore, by studying cells mutant for both Dally and Dally-like, the authors have shown that Dally and Dally-like play redundant roles in mediating Hedgehog cell-to-cell movement that is independent of dynamin-mediated endocytosis.

In contrast, other recent evidence that Hedgehog is reduced in the producing cells lacking HSPGs has indicated their role in the stabilization of Hedgehog ligand on the cell membranes (Bornemann, D.J. *et al.* 2004).

To summarize, these results suggest that Dally and Dally-like increase the membrane affinity of Wingless and Hedgehog by stabilizing them on the cell surface, by clustering both of them in lipid rafts and/or in the extracellular space. These data also show that they are actively involved in the trafficking of lipid-linked morphogens; they promote the spread of extracellular Wingless as well as cell-to-cell movement of Hedgehog. This idea that Dally and Dally-like play an active role in the context of lipid-linked morphogen spread, raises the possibility that they might be released from cells together with lipid-linked morphogens, perhaps on a carrier and thus regulate Wingless and Hedgehog intercellular movement.

GPI-APs are released from the cells by various mechanisms

Unlike transmembrane proteins that are stably inserted into the membrane bilayer, GPI-APs are exclusively associated with exoplasmic leaflet of the plasma membrane. This fact has led to the idea that GPI-APs might detach from membranes and be released in the extracellular space or reintegrated into the host membranes. Indeed, many gpi-anchored proteins are released from their donor membranes and many studies have attempted to assign a function to this intriguing phenomenon (Ferguson, M.A. 1992; Ilangumaran, S. *et al.* 1996; Sharom, F.J. *et al.* 2002).

There exist several possible mechanisms for the release of GPI-APs. First, GPI-APs can be released from membranes in their soluble form without the gpi moiety. The early studies of gpi anchorage had lead to the discovery of gpi-specific phospholipase C (PI-PLC) from *Trypanosoma brucei.* To invade successfully the host immune system, the parasite cleaves off the old coat of VSG (variant surface glycoprotein) with PI-PLC and replaces it with new antigenic variants (reviewed in Sharom, F.J. *et al.* 2002). The sensitivity to PI-PLC has become a common diagnostic test of GPI-APs (Ploug, M. *et al.*

1991; Theveniau, M. *et al.* 1991). Interestingly, mammalian GPI-APs are released from the plasma membranes as well. Mammalian serum contains a gpi-specific phospholipase D (PI-PLD) that is synthesized in the pancreas and released to the blood stream (Metz, C.N. *et al.* 1991). It has been suggested that by the cleavage with phospholipases, GPI-APs can be downregulated in the expressing tissues. By the same means, their concentration in the plasma can be increased or second messengers can be produced, allowing for the regulation of distinct signaling pathways. (Sharom, F.J. *et al.* 2002).

In addition to phospholipases, it has also been shown that gpi-anchored ADP-ribosyltransferase (ART) or cellular prion protein (PrP^C) can be shed to the medium by the proteolytic action of metalloproteases (Kahl, S. *et al.* 2000; Parkin, E.T. *et al.* 2004). Recently, it has been demonstrated that an enzyme called Notum, belonging to the family of α/β hydrolases, induces cleavage of Dally-like from its anchor and facilitates the shedding of Dally-like to the extracellular space (Kirkpatrick, C.A. *et al.* 2004; Kreuger, J. *et al.* 2004). Furthermore, to release GPI-APs from membranes as soluble proteins without lipid moiety, the possibilities of alternative splicing or secretion by independent biosynthetic pathways have been suggested (Nonaka, M. *et al.* 1995; Seidenbecher, C.I. *et al.* 1995; Wang, J. *et al.* 1997).

Another option is that GPI-APs are transferred between cell surfaces with their gpi anchor intact. The structural features of the gpi moiety can provide the possibility for spontaneous exchange of GPI-APs from donor membranes to recipient ones. Several studies have described this ability *in vitro* as well as *in vivo* (reviewed in Ilangumaran, S. *et al.* 1996). Purified GPI-APs insert into the cell membranes and are fully functional when added to cells *in vitro*. Thus, virtually any cell surface can be "painted" by exogenous gpi-anchored proteins (Medof, M.E. *et al.* 1996). To study whether GPI-APs move from cell to cell, cells stably expressing CD4-gpi fusion protein or PrP^C have been co-cultured with non-expressing cells. In these cases, proteins were transferred between the cells in a gpi-dependent manner. While transfer of PrP^C required cell-to-cell contact, CD4-gpi was released in the supernatant on some kind of particles (Anderson, S.M. *et al.* 1996; Liu, T. *et al.* 2002).

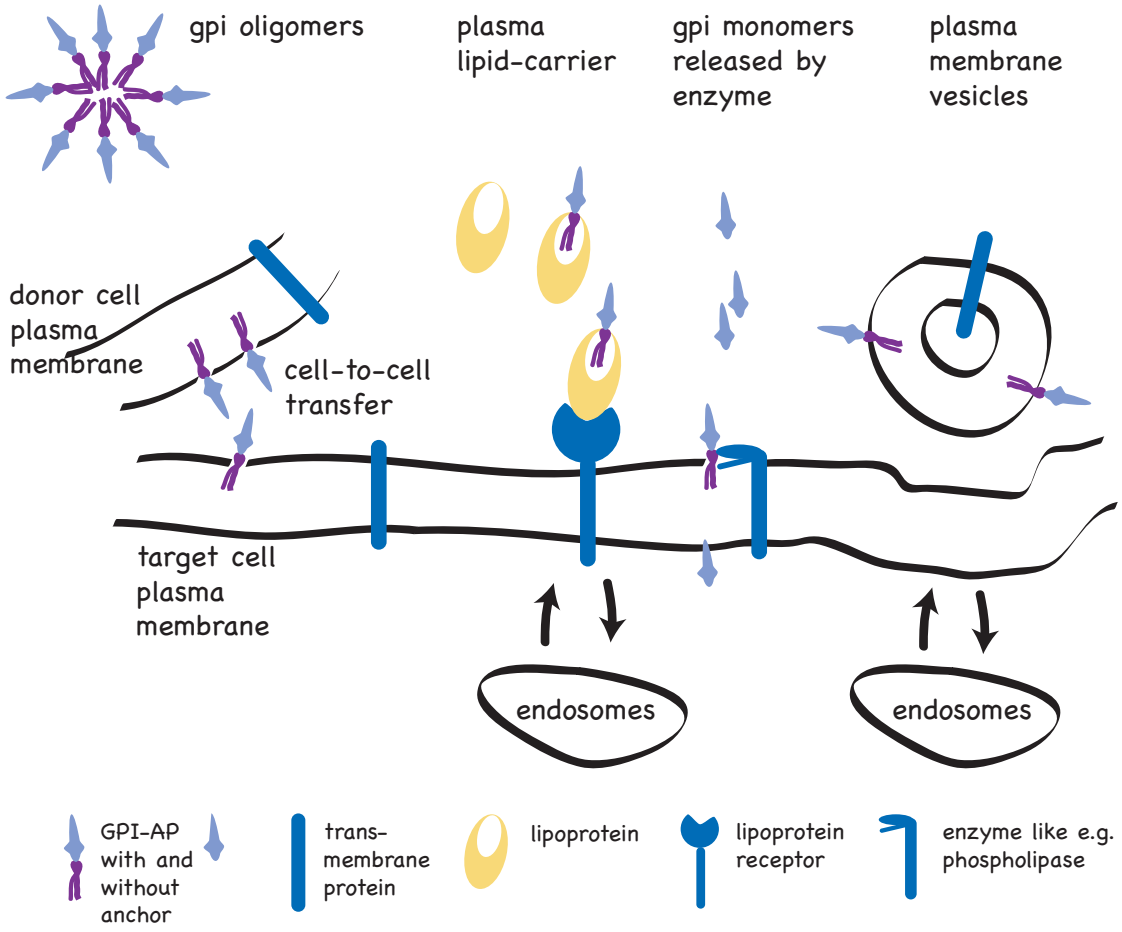


Figure 7: Models for the transfer of GPI-APs. GPI-linked proteins can be released to the extracellular space by the action of various enzymes, e.g. PI-PLC, without their GPI anchor. On the other hand, they can be transferred between cells with their GPI anchor intact. They can flip over from donor to acceptor membranes at cell-cell contacts. They can form oligomer complexes that can facilitate their solubility and mediate their dispersal in the hydrophilic environment. Or GPI-APs can be sorted to membrane vesicles such as exosomes or their spread can be mediated by plasma lipid-carriers such as HDL (adapted from Ilangumaran, S. *et al.* 1996).

Furthermore, gpi-linked surface glycoprotein from *Trypanosoma cruzi* is spontaneously shed into the culture medium on plasma membrane vesicles, ranging from 20 to 80 nm in diameter (Goncalves, M.F. *et al.* 1991). Later reports have shown that a number of GPI-APs, including PrP^{C}, can be released on exosomes (Rabesandratana, H. *et al.* 1998; Fevrier, B. *et al.* 2004). Fewer studies describe the functional intermembrane transfer of gpi-anchored proteins *in vivo*. It has been reported that the parasite *Schistosoma mansoni* evades the host immune system by inserting host gpi-linked decay accelerating factor (DAF) protein into their surfaces (Pearce, E.J. *et al.* 1990). Additional studies have demonstrated that human CD55 and CD59, specifically expressed on mouse erythrocytes, were also found on membranes of vascular endothelial cells (Kooyman, D.L. *et al.* 1995; McCurry, K.R. *et al.* 1995). Finally, Vakeva and colleagues have shown that HDL (high density lipoprotein) binds to CD59 in normal human serum and so have speculated that HDL can shuttle CD59 between cell membranes (Vakeva, A. *et al.* 1994).

Thus, it seems to be a common feature of GPI-APs that they are released from membranes of their producing cells. They are released without their lipid moiety by the action of phospholipases, metalloproteases and hydrolases. Alternatively, they can be released with their gpi anchor intact. They can be shed either in the micellar form as hydrophobic aggregates, on the membrane vesicles such as exosomes, or in association with plasma lipid carriers (Figure 7).

Chapter 1.3. Argosomes: a novel system for the spread of lipid-linked proteins

Recently, Greco et al. have identified a novel cell biological mechanism whereby lipid-linked proteins are transported over large distances through the wing imaginal disc epithelium (Greco, V. *et al.* 2001). When gpi linked to GFP was expressed in different subsets of the wing disc, fluorescence was observed not only on the plasma membrane of producing cells, but also within endosomes in the non-expressing region. These fluorescent particles were named “argosomes” for they ability to travel. Argosomes could be produced by different subsets of cells of wing disc; their distribution in the receiving tissue is graded; they are endocytosed by the receiving cells and they travel through the epithelium with a speed of approximately 0.33 μm per second.

It has been suggested that argosomes might be involved in the spread of Wingless because of the following observations. First, Wingless producing cells are able to produce argosomes. Second, Wingless in the receiving cells colocalizes with argosomes derived from cells that synthesize Wingless. Third, argosome and wingless trafficking depend on dynamin in similar ways. Finally, the rate at which argosomes spread through the disc epithelium is broadly consistent with the rate at which Wingless gradient is formed.

These data have indicated that argosomes might act as vehicles for lipid-linked morphogens such as Wingless or Hedgehog in the wing disc epithelium. Furthermore, other lipid-linked proteins, gpi-anchored proteins in particular, might associate with them as well.

Models for argosome formation

How might argosomes form? Taking into consideration a number of different possibilities of release and transfer of proteins with their intact lipid anchor (see Chapter 1.2.), at least two models might account for argosome production. One possibility is that argosomes are membranous exovesicles. Such particles could be generated by plasma

membrane vesiculation, or by an exosome-related mechanism (Figure 8, model 1) (Denzer, K. *et al.* 2000). Alternatively, argosomes might be reminiscent of lipidic particles (Figure 8, model 2).

Model 1: Exovesicles

The release of membranous exovesicles forms the basis for signalling events in many different cell types. The uptake of entire regions of the plasma membrane has been considered as a mechanism for the endocytosis of transmembrane ligands (Cagan, R.L. *et al.* 1992; Klueg, K.M. *et al.* 1999; Parks, A.L. *et al.* 2000). Plasma membrane vesiculation of a donor cell and subsequent transendocytic event by a neighbouring cell is one possible way how exovesicles are produced. This mechanism could be similar to that used by *Listeria monocytogenes* as it spreads through the epithelial monolayer of host MDCK cells (Figure 8, 1a) (Robbins, J.R. *et al.* 1999). Another possibility is that

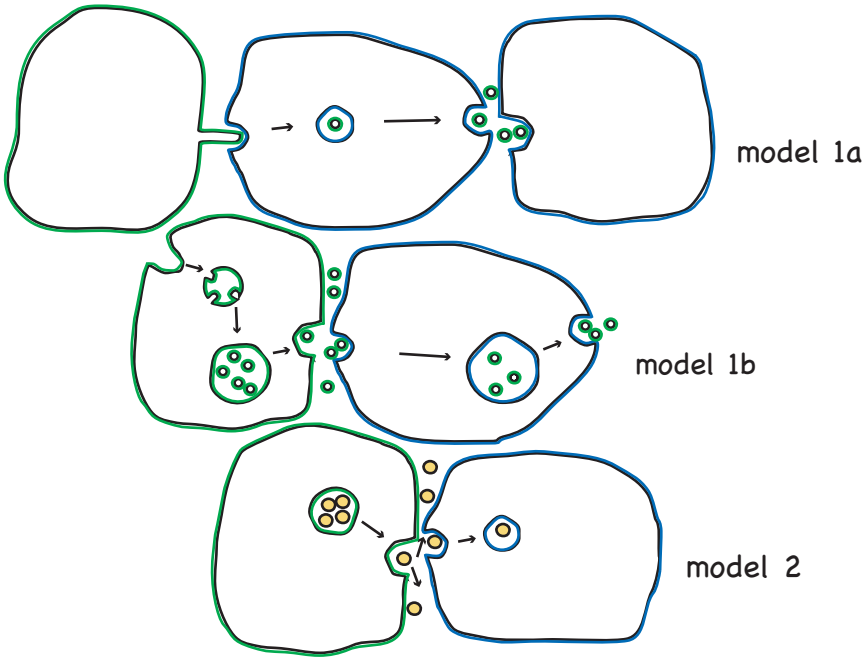


Figure 8: Models of argosome formation. Argosomes can be formed by plasma membrane vesiculation, model 1a; or by the mechanism that might resemble formation of exosomes in multi vesicular bodies, model 1b. In both cases, argosomes would represent membrane vesicles. Alternatively, argosomes might resemble plasma lipid-carriers i.e. lipidic particles, model 2

membrane exovesicles are generated through exosome production. Exosomes are formed within multi vesicular bodies (MVB) by the inward budding of the limiting membrane. Upon exocytic fusion of MVB with the cell surface they are released into the extracellular space. (Figure 8, 1b) The size of exosomes ranges between 40 to 100 nm. Many different cell types like the cells of the hematopoeitic lineage, epithelial cells and reticulocytes are capable of secreting exosomes (reviewed in Denzer, K. *et al.* 2000; Stoorvogel, W. *et al.* 2002; Fevrier, B. *et al.* 2004).

Using biochemical methods, it has been possible to obtain a highly purified fraction of exosomes that allowed identification of their protein composition. Proteomic analysis of exosomes has revealed many proteins involved in antigen presentation (MHC class I and II), in cell adhesion (integrins and tetraspanins), in membrane transport and fusion (e.g. Rab 7), and also raft-associated proteins (e.g. GPI-APs) (Thery, C. *et al.* 2001; Wubbolts, R. *et al.* 2003). These data have also indicated that protein sorting into exosomes is tightly regulated and it allows different cells to produce exosomes with highly specific functions and target cell specificities. Cytolytic T cells produce exosomes that deliver killing reagents specifically to their targets (Peters, P.J. *et al.* 1990). Exosomes derived from antigen-presenting cells carry peptide-MHC molecules and are capable of activating specific subsets of T-cells *in vitro* (Raposo, G. *et al.* 1996; Zitvogel, L. *et al.* 1998). Recently, it has been shown that PrP^{C} is released on exosomes and that these can be infectious (Fevrier, B. *et al.* 2004).

The fact that specific proteins including lipid-linked proteins can be sorted and concentrated in exosomes, (Figure 9A) and that they may play a part in intercellular communication, makes them an attractive vehicle for the spread of membrane bound morphogens.

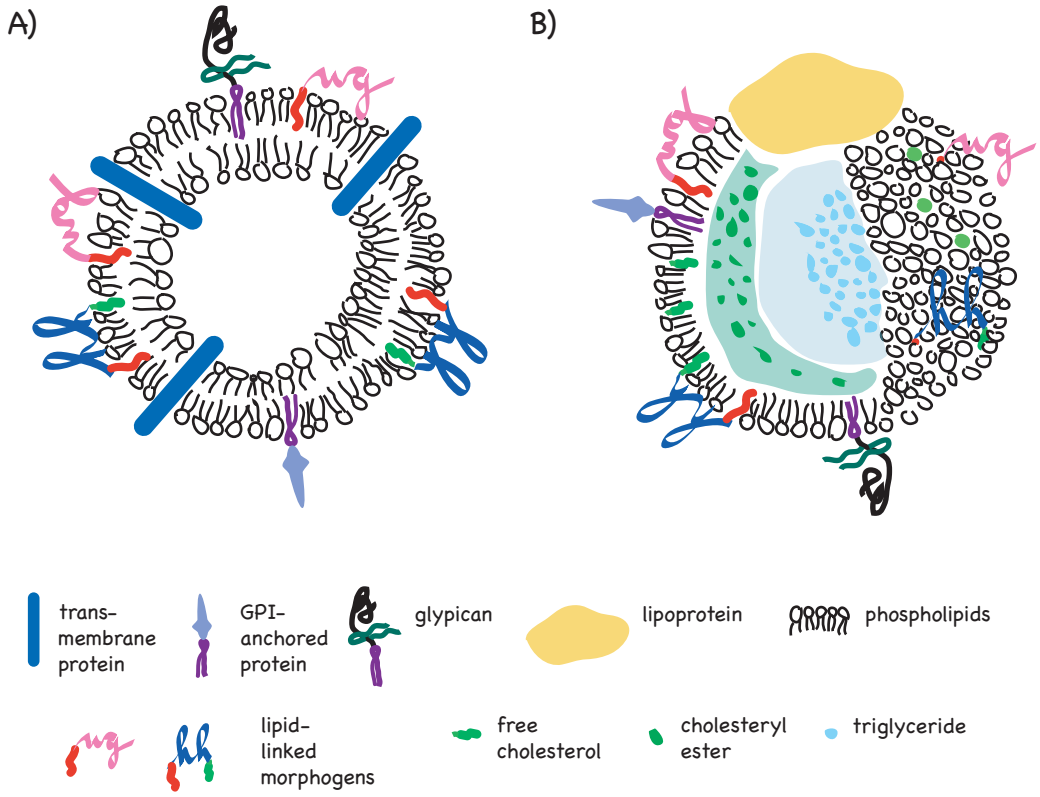


Figure 9: Exosome vs. Lipidic particle. If argosomes were produced like exosomes, they would be sorted on this vesicle, consisting of a membrane bilayer, with other transmembrane proteins. On the contrary, only proteins that carry lipid modifications of exoplasmic leaflet of the membrane would be able to be incorporated to the phospholipids monolayer of the lipidic particle. Transmembrane proteins would not be associated with such a particle.

Model 2: Lipidic particles

In order to utilize the nutrition derived from the dietary sources, lipids have to be metabolized and transported throughout the blood circulation as well as the aqueous compartments within the cell and tissue spaces. Most of these lipids as well as the lipids synthesized *de novo* are usually in the form of triacylglycerol (TAG), fatty acids and/or cholesterol. However, lipids are hydrophobic molecules and hence insoluble in the plasma. In order to be transported, they are combined with specific plasma carrier proteins, apolipoproteins in particular, to form lipoprotein/lipidic particles.

Lipidic particles consist of a core containing a droplet of triacylglycerols,

cholesteryl esters and a surface monolayer of phospholipids, cholesterol and apolipoproteins (Figure 9B). In mammalian systems, alteration in the content of proteins and lipids reflect different functions of different apolipoproteins (van der Horst, D.J. *et al*. 2002). The classification of apolipoproteins is based on their density. There are five types of apolipoproteins in the mammals: chylomicrons (they are the largest and the lowest in density due to high lipid/protein ratio), VLDL (very low density lipoprotein), IDL (intermediate density lipoprotein), LDL (low density lipoprotein, it has the highest content of cholesteryl esters), HDL (high density lipoprotein; it has the highest density due to high protein/lipid ratio). Apolipoproteins can associate with the lipidic particle permanently (i.e. non-exchangeable lipoproteins such as apoB-100), or they are exchangeable lipoproteins, able to detach from the lipoprotein particle and bind another one (e.g. apoE) (Shelness, G.S. *et al*. 2001). Generally, apolipoprotein possesses domains that interact with the hydrophobic lipid core, domains that interact with the hydrophilic environment and domains that interact with cell surface receptors like LDL receptor (Brown, M.S. *et al.* 1986).

In contrast to mammals, insects construct lipidic particles using just one apolipoprotein, Lipophorin (Van der Horst, D.J. 1990; Sundermeyer, K. *et al.* 1996). Lipophorin transports lipids throughout the insect hemolymph and has a higher protein/lipid ratio that resembles the density of HDL. The prevalent lipid is diacylglycerol, which is the major storage lipid in insects (Arrese, E.L. *et al.* 2001). One characteristic feature of Lipophorin is that it functions as a reusable shuttle; it is able to selectively load and unload lipid cargo at different target organs (reviewed in van der Horst, D.J. *et al.* 2002). In *Drosophila*, Lipophorin was first identified as retinoid- and fatty acid-binding protein. The gene encodes 3351-amino acid glycoprotein and is synthesized as a proapolipophorin precursor. The latter is then posttranslationally processed; the proteolytic reaction yields two subunits Apolipophorin I (220 kDa) and II (70 kDa) (Kutty, R.K. *et al.* 1996) and is furin dependent (Smolenaars, M.M. *et al.* 2004). The density of Lipophorin particle is 1.12 g/ml (Pho, D.B. *et al.* 1996) that resembles that of the HDL. Thus, even though the major role of Lipophorin particles is to transport lipids, their protein/lipid ratio is intriguingly high.

Interestingly, it has been reported that gpi-anchored CD59, when isolated from the human plasma, associates with HDL (Vakeva, A. *et al.* 1994). We propose that lipid- modified proteins of the exoplasmic face of the membrane (like GFPgpi, Wingless or Hedgehog) might fit well into the outer phospholipid monolayer of such a particle and thus associate with *Drosophila* Lipophorin particles (Figure 9B).

Chapter 1.4. Scope of the thesis

Despite the fact that morphogens Wingless and Hedgehog strongly associate with membranes, not just through their lipid anchorage but also via their interaction with gpi-linked heparan sulfate proteoglycans, they are able to induce the long-range target genes at many cell diameters away from their source. Thus, they are able to travel long distances in the wing disc epithelium. To comprehend this paradoxical phenomenon, a novel solution for lipid-linked protein dispersal has been proposed (Greco, V. *et al.* 2001). The observation that argosomes could spread through the epithelia and that they extensively colocalised with Wingless suggested their role in facilitating the intercellular movement of this molecule.

As yet however, no functional link between argosome production and dispersal of lipid-linked proteins has been established. The understanding of the cell biological nature of argosomes would provide this missing information. To address the question of argosome production, we have proposed at least two models. To test those models I first generated markers for exosomes and lipidic particles (Chapter 2.1.). Next, I utilized biochemical fractionation to determine particle with which lipid-linked proteins associate (Chapter 2.2.). In Chapter 2.3., I investigate the localization of the lipid-linked morphogens in respect to the markers. Finally, I use genetic means to address the functional importance of this association (Chapter 2.4.).

The work presented in this thesis was partially done in collaboration with Hein Sprong and Christoph Thiele. Sarah Bowman, Ali Mahmoud, Eric Marois, Christina Eugster and Suzanne Eaton have also contributed to this thesis as indicated in the text or figure legends. Results of these experiments are included for completeness.

2. RESULTS

Chapter 2.1. Generation of specific markers

To test whether argosomes represent very small membrane vesicles or lipoprotein particles, I have generated specific markers to label exosomes and lipidic particles, respectively.

CD63 is an exosomal marker

To test the exosome-like model of argosome formation, I have constructed transgenic flies expressing CD63:GFP fusion protein under the control of GAL4:UAS system. Human CD63 is a member of the tetraspanin TM4 superfamily (Figure 10) (Boucheix, C. *et al.* 2001). It is a well-established component of late endosomal and lysosomal membranes and unlike other tetraspanins, it is predominantly found intracellularly. It has been shown, in the studies of Weibel-Palade bodies of vascular endothelial cells, that CD63 is present within these specialized secretory organelles and can cycle between late endosomes and exocytic compartment of these cells (Kobayashi, T. *et al.* 2000). Furthermore, several studies suggest that it is present on exosomes released by many cell types. In B-lymphocytes, CD63 is localized to the internal vesicles of MHC class II compartments (MIICs), which resemble multivesicular bodies. MIICs can fuse with plasma membrane

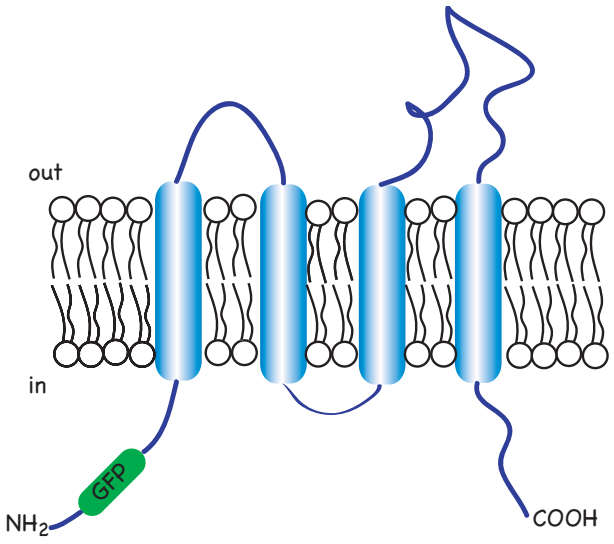


Figure 10: Schematic structure of CD63:GFP fusion construct. CD63 is a tetraspanin with four transmembrane domain and two extracellular loops; both C and N-termini face the cytoplasm. EGFP was inserted at the N terminus of the protein.

and release exosomes containing CD63 together with other proteins into the extracellular space (Escola, J.M. *et al.* 1998; Wubbolts, R. *et al.* 2003). In addition, when expressed in HeLa cells, CD63 is released on exosomes produced by these cells (J. Gruenberg, pers. Com.).

To ask whether CD63:GFP behaves similarly when expressed in *Drosophila* imaginal disc cells, I examined its subcellular localization in this tissue. In producing cells, it is found on the plasma membrane and in large internal structures (Figure 11A and B in green; arrowheads). To determine whether these were late endsomes, I stained CD63:GFP-expressing discs with lysotracker, a vital dye that is retained within acidic compartments. CD63:GFP colocalizes extensively with lysotracker in these discs, and even appears to enlarge the endocytic compartment marked by lysotracker (Figure 11D-F). These data suggest that CD63:GFP is targeted to late endosomes in *Drosophila* cells.

To ask whether CD63:GFP-labelled exosomes might be released by imaginal disc cells, I examined non-expressing tissue for GFP fluorescence. Expressing CD63:GFP in the dorsal compartment using *apterous*GAL4 driver, I detected CD63:GFP in ventral, non-expressing compartment from 1 to 3 cell diameters away from producing cells (Figure 11A and B in green; arrows). To determine which subsets of cells within the disc are capable of generating exosomes, I expressed CD63:GFP in a variety of different patterns using different GAL4 driver lines. In all cases, CD63:GFP is localized to the GFP positive particles up to 3-4 cell diameters in receiving tissue. To verify that GFP fluorescence in the receiving cells is not due to the cell death, I expressed CD63:GFP together with p35, a baculoviral protein that specifically inhibits apoptosis in *Drosophila* cells (Hay, B.A. *et al.* 1994). Again, CD63:GFP vesicles were detected in non-expressing cells, suggesting that CD63:GFP is taken up by receiving cells on exosomes rather than on membrane fragments that could be generated by apoptotic cells (data not shown). To ask whether released CD63:GFP was endocytosed by neighboring cells, transgenic flies has been constructed in whose early endosomes were labeled by ubiquitously expressed CFP:Rab5 fusion protein. Colocalization between CFP:Rab5 and CD63:GFP in receiving cells (Figure 11G) indicates that imaginal disc cells release exosomes that can be endocytosed by their neighbors.

In conclusion, these data suggest that the cells of *Drosophila* wing imaginal discs are capable of producing exosomes and that CD63 is a suitable marker for those exosomes.

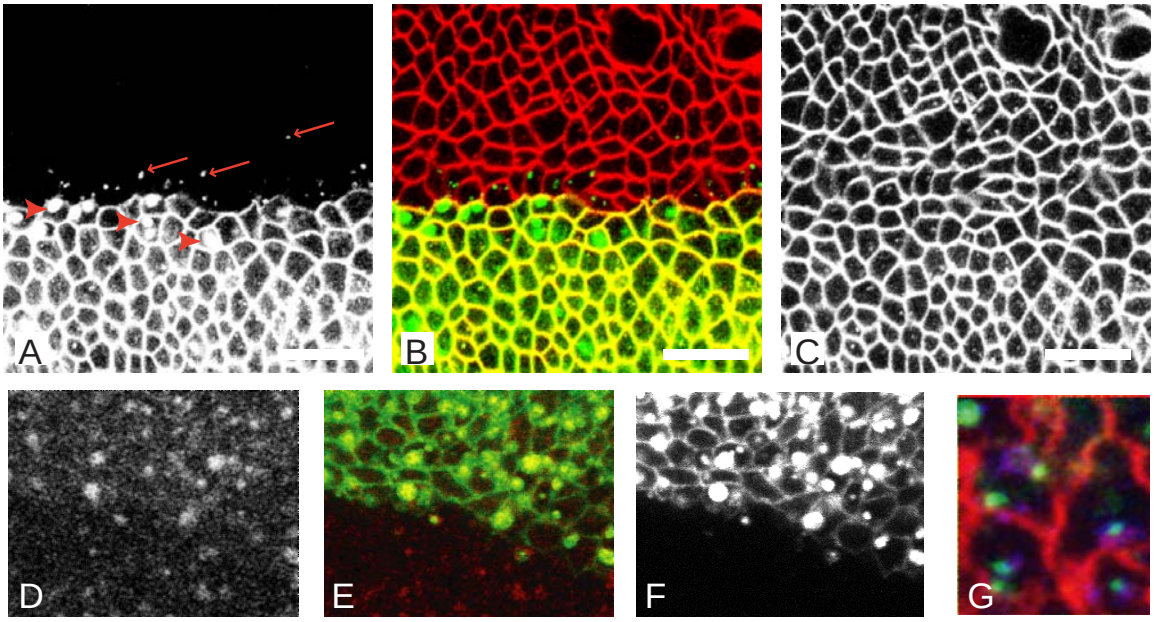


Figure 11: CD63:GFP localizes to exosomes in the imaginal discs. A-F) Wing imaginal discs expressing CD63:GFP fusion construct. **A)** Wing imaginal disc expressing CD63:GFP in the dorsal compartment under the control of *apterous*GAL4. CD63:GFP localizes to the plasma membrane of the producing cells and in the intracellular endocytic compartments; arrowheads. In the non-expressing tissue, CD63:GFP is present up to 3 cells away from the expressing region; arrows. **B)** merge of A and C; CD63:GFP is in green, FM4-64 in red. **C)** Plasma membrane and endosomes are stained with the vital dye FM4-64. **D)** Lysotracker staining of the wing imaginal disc expressing CD63:GFP in the dorsal compartment; upper half of the micrograph. **E)** merge of D and F; CD63:GFP is in green, lysotracker in red. CD63:GFP is present in the late endosomes in expressing cells. **F)** CD63:GFP expressed in the dorsal compartment. **G)** Wing imaginal disc expressing CD63:GFP and CFP:Rab5 fusion constructs. Released CD63:GFP (green) in the receiving tissue is present in the early endosomes that are marked by CFP:Rab5 (blue). The plasma membrane has been stained red with FM4-64 (experiment performed by S. Bowman).

Lipophorin as a marker for lipidic particles

As mentioned earlier, a lipidic particle consists of lipoproteins and phospholipid monolayered particle filled with triacylglycerol and cholesteryl esters. The main function of lipidic particles in insects is to shuttle endogenously synthesized or dietary lipids between gut and fat body and the peripheral tissues, such as wing imaginal discs (Arrese, E.L. *et al.* 2001; van der Horst, D.J. *et al.* 2002). Lipophorin is the single lipoprotein

encoded in the *Drosophila* genome. It is transcribed in the fat body as a Proapolipophorin precursor molecule that is postranslationally cleaved, yielding Apolipophorin I (ApoLI; 220 kDa) and Apolipophorin II (ApoLII; 70 kDa) (Figure 12A) (Kutty, R.K. *et al.* 1996; Sundermeyer, K. *et al.* 1996). Consistent with this, I could not detect *apolipophorin* transcripts in imaginal discs by *in situ* hybridization (data not shown). In collaboration with Hein Sprong and Christoph Thiele, we have generated polyclonal antibodies against ApoLI and ApoLII.

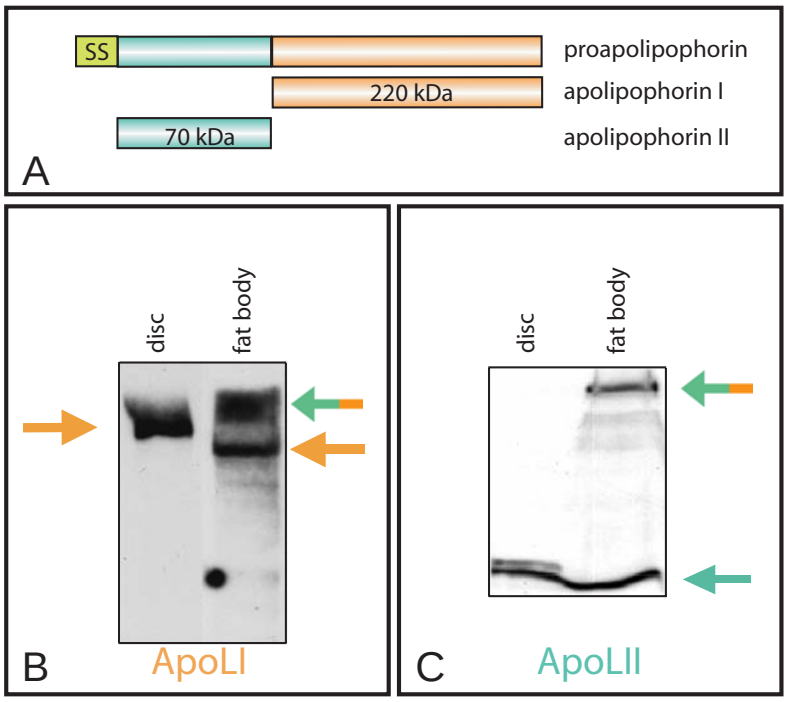


Figure 12: Lipophorin is detected by western blotting in the fat body and imaginal disc. A) Schematic picture of Lipophorin protein. Proapolipophorin is synthesized as more than 300 kDa precursor that is processed into Apolipophorin I (ApoLI) and Apolipophorin II (ApoLII). **B,C)** Discs and fat bodies from 4 larvae were collected in different tubes, dissolved in loading buffer and subjected to gel electrophoresis, followed by Western blotting with antibodies to either ApoLI **(B)** or ApoLII **(C)**. Blue/yellow arrows indicate Proapolipophorin, yellow arrows indicate ApoLI and blue arrows indicate ApoLII.

Since Lipophorin is produced mainly in the fat body, I wished to determine whether Lipophorin particles accumulated in imaginal discs at all. To address this question, I prepared both fat body and imaginal disc extracts from the same third instar larvae and probed Western blots of these extracts with anti-ApoLI and anti-ApoLII. Both antibodies detect the unprocessed Proapolipophorin only in fat body extracts, consistent with the idea that transcription and translation of Lipophorin occurs in the fat body (bi-colored arrows in Figure 12B and C). Anti-ApoLI recognizes a single band corresponding to the processed form in extracts from fat body and imaginal discs (yellow

arrows in Figure 12B). ApoLI from disc extracts migrates somewhat more slowly than its counterpart in the fat body. Similarly, anti-ApoLII detects a single processed form in fat body extracts, but a doublet in disc extracts (blue arrows in Figure 12C). This suggests that imaginal disc cells modify the Lipophorin they acquire from the hemolymph.

Although, Lipophorin is made by the fat body cells, the protein accumulates to an almost equivalent level in imaginal discs, therefore it can be used as a lipidic particle marker in these tissues.

Chapter 2.2. Cofractionation studies

To study argosome biosynthesis I have developed, together with Hein Sprong, a biochemical approach.

A fraction of gpi-linked proteins, Wingless and Hedgehog does not associate with membranes

To identify the cellular fraction containing argosomes, I compare the behaviour of Wingless, Hedgehog and gpi-linked proteins with that of exosomes, lipidic particles and free, soluble proteins in a differential centrifugation assay. I homogenized either whole 3rd instar wild type larvae, or larvae that expressed different GFP fusion proteins. Alternatively, I prepared homogenates of dissected wing imaginal discs. I then centrifuged these homogenates at 1000 *g* for 10 min and 120 000 *g* for 3 hrs, respectively; yielding pellet and supernatant, further referred to as P120 and S120. These experiments showed that plasma membrane and exosomal markers were completely pelleted and present solely in P120, whereas the majority of soluble cytoplasmicGFP and ApoLII remained in the S120 (Figure 13A). I found that most Wingles:GFP, Hedgehog and gpi-linked proteins, including Fasciclin-I (Hortsch, M. *et al.* 1990), Connectin (Nose, A. *et al.* 1992), Klingon (Butler, S.J. *et al.* 1997) and Acetylcholineasterase (AchE)

(Incardona, J.P. *et al.* 1996), were present in the P120. This was not unexpected, because a major pool of these proteins can be found on the plasma membrane or in internal membrane compartments like endosomes.

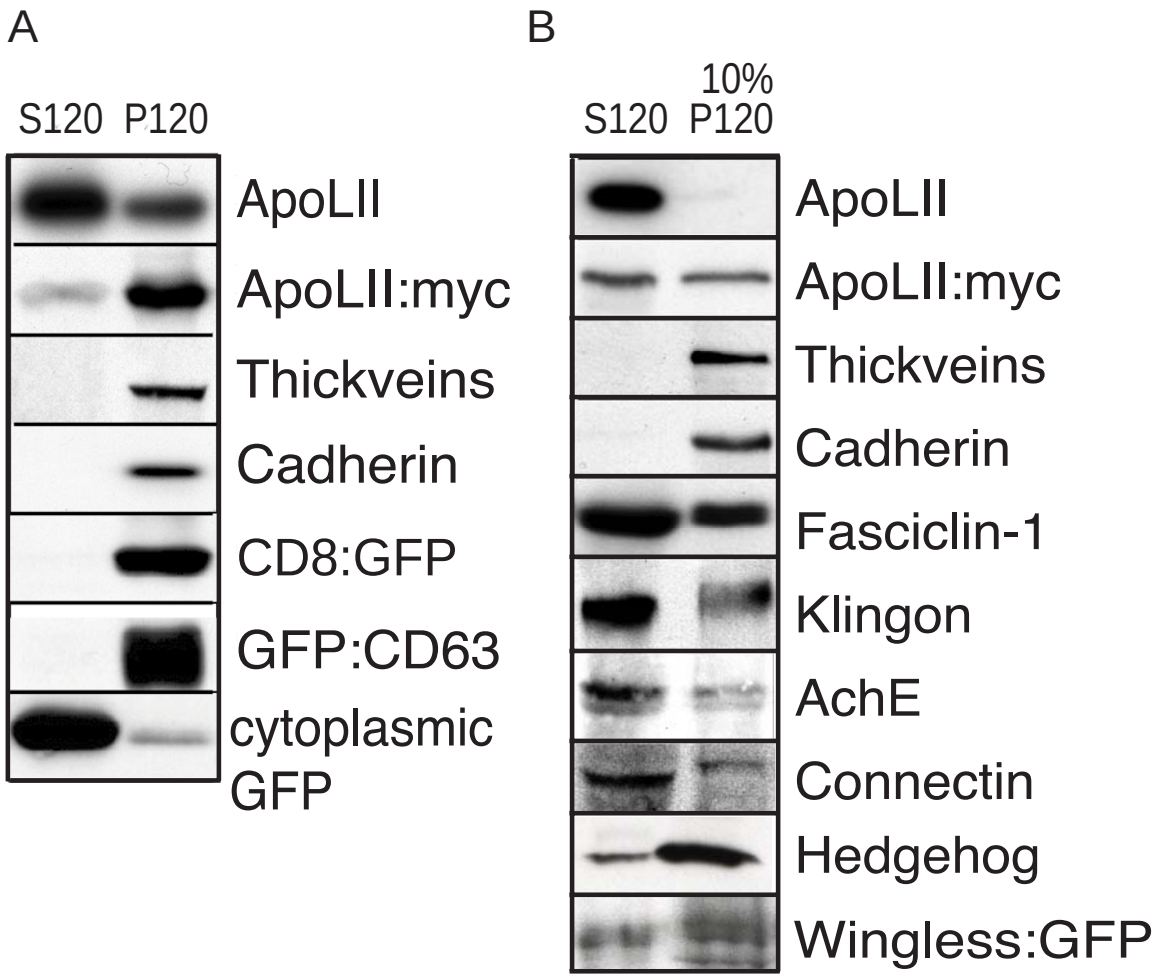


Figure 13: A fraction of Wingless, Hedgehog and GPI-anchored proteins does not pellet with the membranes. Post nuclear supernatants derived from whole third instar larvae were centrifuged at 120,000g for three hours. Western blots of supernatants and the indicated proportion of pellets were probed with antibodies to the indicated proteins. Where tagged proteins are specified, they were expressed in imaginal discs under the control of the GAL4:UAS system.
A) The plasma membrane proteins Thickvein, Cadherin, CD8:GFP and the late endosomal/exosomal protein CD63:GFP are completely pelleted. Cytoplasmic GFP is present mainly in the supernatant.
B) A fraction of the GPI-linked proteins Fasciclin I, Klingon, Acetylcholinesterase and Connectin remains in the supernatant after centrifugaton at 120,000g. Hedgehog and Wingless:GFP are also present in small but significant amounts in the supernatant. (Western blots against ApoLII, ApoLII:myc, Thickveins, Cadherin, Fasciclin-1, Klingon, AchE, Connectin and Hedgehog were performed by H. Sprong.)

Suprisingly however, a significant fraction of Wingless:GFP (6%), Hedgehog (2%) and gpi-linked proteins (14-22%) remained in the S120 and failed to pellet at 120 000 *g* (Figure 13B). These data are consistent with the idea that Wingless:GFP, Hh and gpi-linked proteins in this fraction exist either as free soluble proteins or on lipidic particles.

Non-membrane-associated Gpi-linked proteins, Wingless and Hedgehog are present on lipoprotein particles.

To distinguish free proteins from lipoprotein particles, we subjected proteins in the S120 to isopycnic density centrifugation in KBr. Figure 14A shows the Coomasie-stained proteins present in the fractions from such a gradient. Western blotting reveals that under these conditions, Lipophorin moves to the low-density fraction at the top of the gradient whereas soluble proteins like secreted GFP are present in higher density fractions at the bottom of the gradient (compare the first two panels of Figure 14B). Gpi-linked proteins are found almost entirely in the top, low-density fraction with Lipophorin, indicating that they are present on a low-density particle (Figure 14B). To ask whether the association of gpi-linked proteins with low-density particles is via their gpi anchor, we examined the effect of Phosphatidylinositol-specific Phospholipase C (PI-PLC). This enzyme specifically cleaves the glycosylphosphatidylinositol anchor and releases the gpi-anchored protein in its soluble state. Treatment of S120 with PI-PLC prior to density centrifugation causes previously gpi-linked proteins to shift from the low-density fraction to the higher density fractions that contain soluble proteins (Figure 14B). These data suggest that gpi-linked proteins associate with low-density particles and that this association is gpi anchor dependent.

We used the same approach to address the possibility that the Wingless and Hedgehog proteins, which fail to pellet with membranes, might also associate with low-density particles. To ask whether endogenous Wingless and Hedgehog or tagged Wingles:GFP and Hedgehog:HA co-migrate with Lipophorin, we prepared isopycnic

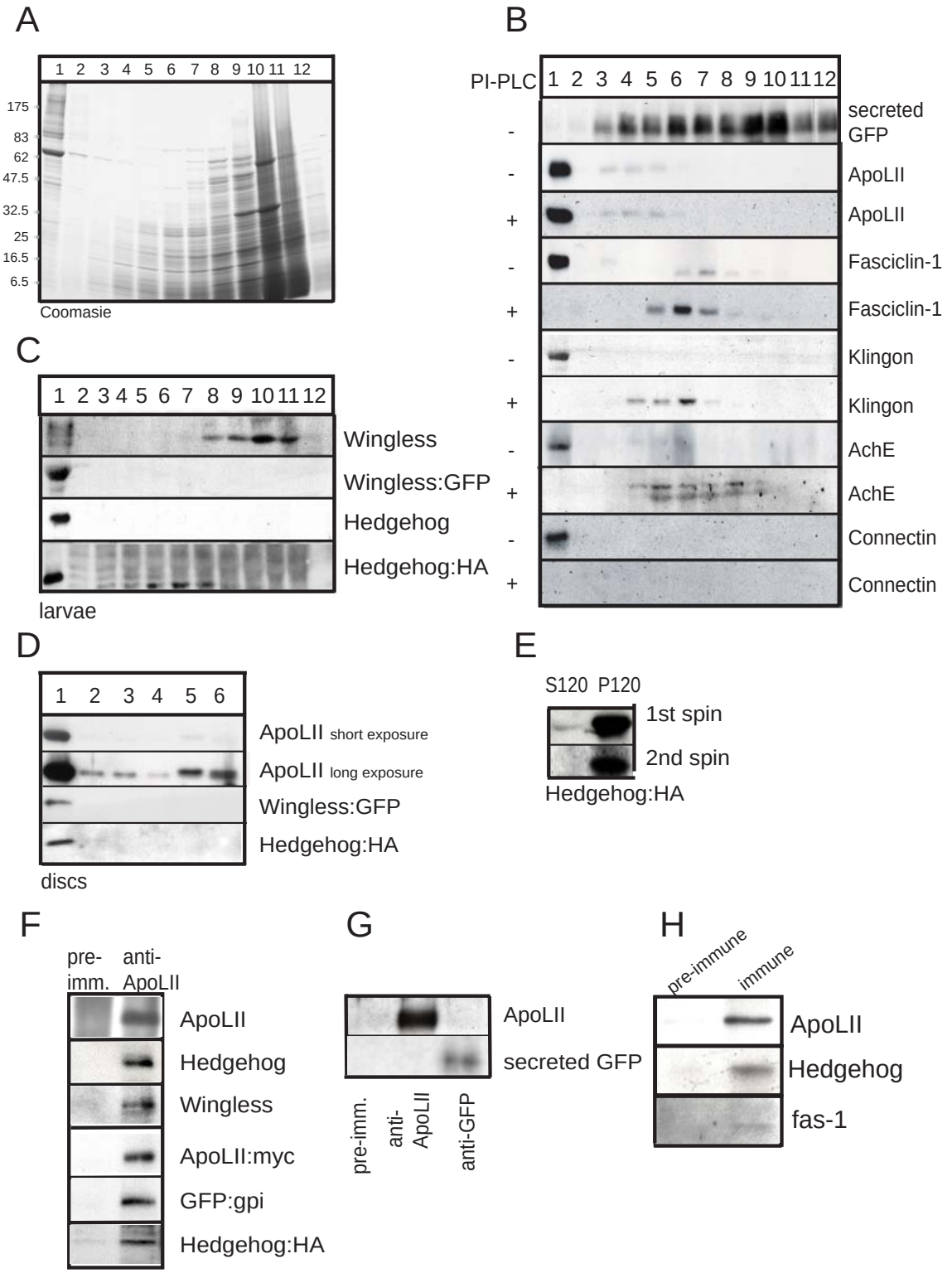


Figure 14: Wingless, Hedgehog and gpi-linked proteins co-purify and immunoprecipitate with Lipophorin. A,B,C,D). Isopycnic density centrifugation in KBr was performed on the 120,000g supernatants of either whole third instar larvae, or of dissected imaginal discs. **A)** Coomasie stained 5-25%

density gradients from S120 of whole larval extracts (Figure 14C). We found that virtually all Hedgehog present in the S120 floats with Lipophorin to the top fraction of isopycnic density gradients. We observed that Wingless:GFP co-migrate with Lipophorin as well. In contrast, we detected two different pools of endogenous Wingless: a doublet in the lowest density fraction and a band of somewhat higher mobility in soluble fractions. This may indicate that some larval tissues other than imaginal discs secrete Wingless in a non-Lipoprotein-associated form. To examine whether Wingless and Hedgehog that is present only in imaginal discs might be found on low-density particles, I prepared S120 from dissected imaginal discs that were specifically expressing Wingless:GFP or Hedgehog:HA. Figure 14D shows that 100% of non-membrane-bound Wingless:GFP and Hedgehog:HA synthesized in imaginal discs co-migrates with Lipophorin in the top fraction of isopycnic density gradients. These data indicate that Hedgehog and Wingless are associated with low-density particles. They further confirm that low-density Lipophorin particles exist in imaginal disc tissue.

I was worried, however, that Wingless and Hedgehog might be extracted from membranes during the homogenization procedure by high amount of Lipophorin particles present in the hemolymph. To address this issue, I further tested whether extraction of Hedgehog might occur during the fractionation procedure. I attempted to extract HA-tagged Hedgehog from imaginal disc membranes using an excess of Lipophorin particles

Figure 14, continued: gradient gel of 12 fractions from a KBr gradient from the whole larval extract. The top fraction has a density of 1.14 g/cm3 and the bottom fraction of 1.4 g/cm3. Most proteins are present in the higher density fractions 5-11. These represent soluble proteins. Significant amount of the proteins is present in the low density fraction. **B)** Western blots of KBr gradient fractions prepared from the whole larval extracts probed with antibodies to the indicated proteins. + indicates that extracts were treated with PI-PLC before fractionation, - indicates that they were mock-treated. **C)** Western blots of KBr gradient fractions prepared from the whole larval extracts probed with antibodies to endogenous Wingless, endogenous Hedgehog, or to tagged version of these proteins (Wg:GFP and Hh:Ha). **D)** Western blots of KBr gradient fractions from the dissected imaginal discs probed with antibodies to indicated proteins. **E)** 120,000g pellet and supernatant from dissected imaginal discs were probed with antibody to tagged version of Hedgehog. P120 was incubated with purified Lipoprotein fraction for 30 minutes at 4 ^{o}C, re-centrifuged at 120,000g for 3 hours and probed with antibody to tagged version of Hedgehog. No additional Hedgehog is extracted from the membrane fraction. **F)** 120,000g supernatants from either wild type larvae, or larvae expressing various fusion proteins were immunoprecipitated either with pre-immune serum, or with anti-ApoLII antiserum. Immunoprecipitates were probed with antibodies to the indicated proteins. **G)** 120,000g supernatant from the larvae expressing secreted version of GFP were immunoprecipitated with pre-immune serum, anti-ApoLII serum, or anti-GFP antibody. Immunoprecipitates were probed with antibodies to the indicated proteins. Anti-ApoLII cannot precipitate secreted form of GFP that does not contain any lipid modification. **H)** Purified Lipoprotein fraction from wild type larvae was immunoprecipitated either with pre-immune serum, or anti-ApoLII serum. Immunoprecipitates were probed with antibodies to the indicated proteins. (experiments B,C,F) were performed by H. Sprong)

under the same conditions that are normally followed during homogenization. The top panel of Figure 14E shows the amount of Hedgehog typically present in the P120 and S120 derived from 100 dissected larvae; the lower panel shows that no additional Hedgehog is extracted when the P120 is incubated with purified lipoprotein particles from 500 larvae and re-centrifuged. This confirms that association of Hedgehog with lipoproteins is not an artifact of the fractionation protocol.

To further determine whether lipid-linked proteins associated with Lipophorin particles, or with some other low-density particle, we immunoprecipitated ApoLII from the S120 and probed for Wingless, Hedgehog or GFPgpi in the precipitates. Figure 14F shows that all these proteins can be immunoprecipitated by anti-ApoLII, but not by pre-immune serum. Furthermore, anti-ApoLII is unable to precipitate secreted GFP that contains neither gpi anchor nor other lipid modification (Figure 14G). This suggests that Wingless, Hedgehog and gpi-linked proteins associate specifically with Lipophorin particles. To ask whether association of Hedgehog and Lipophorin is depended on non-Lipoprotein-associated factors in the S120, I asked whether Hedgehog and gpi-anchored Fasciclin-I could be precipitated from the purified Lipoprotein fraction by antibodies to ApoLII. All, Hedgehog, FasI and ApoLII were precipitated from the top fraction of KBr density gradient by anti-ApoLII serum, but not by pre-immune serum (Figure 14H). This indicates that the binding of Hedgehog to Lipophorin occurs even when most other proteins in the S120 are removed.

Taken together, these data suggest that gpi-anchored proteins and the lipid-linked morphogens Wingless and Hedgehog directly associate with low-density Lipophorin particles in imaginal discs.

Chapter 2.3. Colocalization studies

To determine whether Wingless or Hedgehog colocalize with either exosomes labeled by CD63:GFP or Lipophorin particles *in vivo,* I examined their distribution in wing imaginal discs.

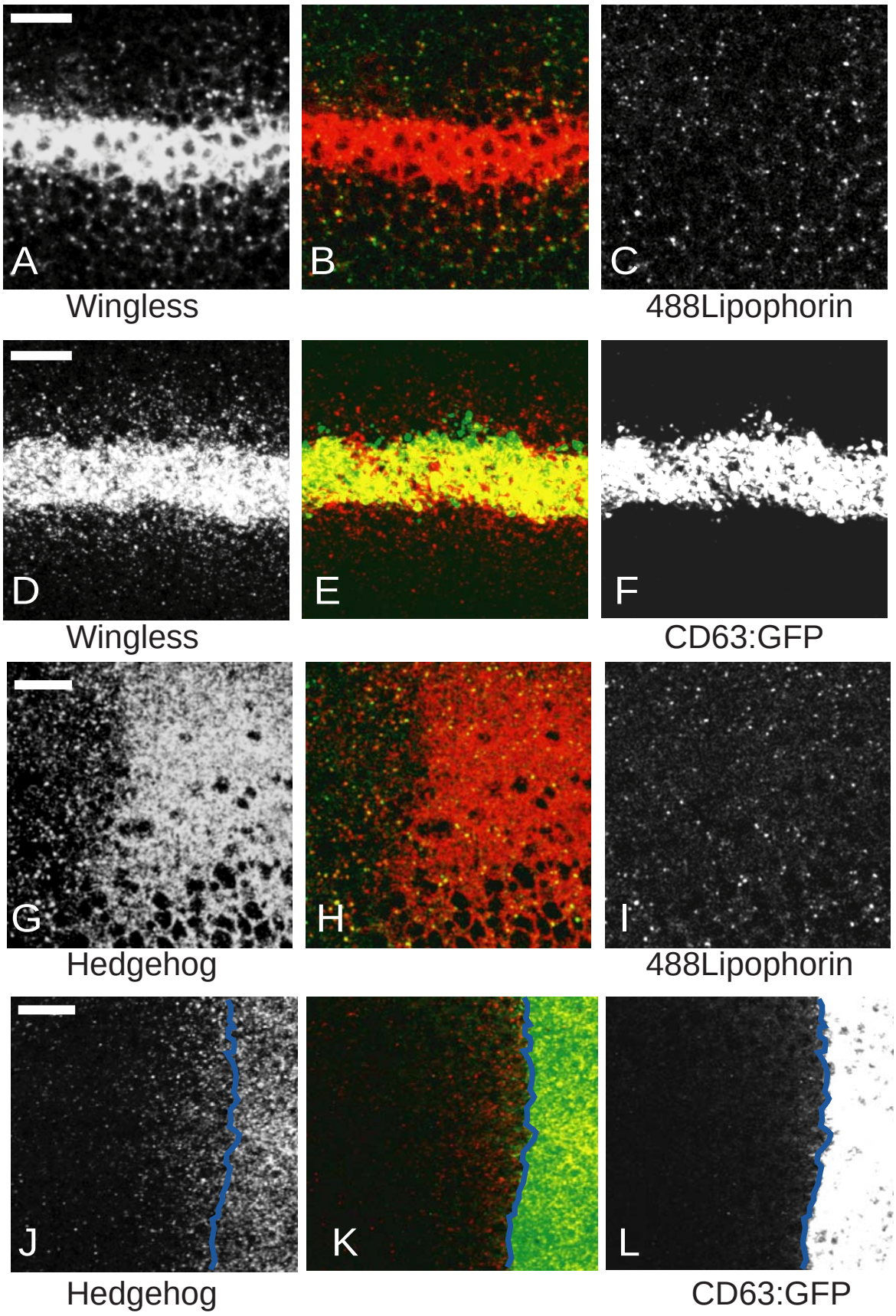
Neither Wingless nor Hedgehog colocalizes with exosomes

The distribution of CD63:GFP suggested that imaginal disc cells release and endocytose exosomes. Since the P120 contained all transmembrane proteins including exosomes, our biochemical experiments did not rule out the possibility that some Wingless or Hedgehog might still be present on these vesicles. To investigate this, I expressed CD63:GFP in either Wingless or Hedgehog expressing domains and looked for colocalization with CD63:GFP labeled exosomes. I observed that in the receiving tissue, CD63:GFP localizes to the particles that are distinct from Wingless or Hedgehog labeled particles. There was no significant colocalization between morphogens and released exosomes (Figure 15D-F and 15J-L). Furthermore, the range of movement of exosomes was more restricted than that of Wingless or Hedgehog (see also Figure 11A). Thus, it seems unlikely that any Wingless or Hedgehog is released on exosomes in imaginal disc cells, although the mechanism remains an intriguing possibility for transmembrane ligands such as Boss or Notch (Cagan, R.L. *et al.* 1992; Klueg, K.M. *et al.* 1999; Parks, A.L. *et al.* 2000)).

Wingless and Hedgehog colocalize with Lipophorin *in vivo*

Despite working well on Western blots, we could not get satisfying results by immunoflourescence using anti-ApoLI or II antibody. As an alternative approach, we purified Lipophorin particles from the top fraction of isopycnic density gradient and labeled them with Alexa 488 fluorophore. We used this system first to ask how Lipophorin was distributed in imaginal discs. Live confocal imaging of wing imaginal discs incubated with fluorescently labeled Lipophorin particles revealed punctate structures throughout the whole wing pouch that colocalized with internalized dye FM4-64 in endosomes (data not shown).

The data indicates that the Lipoprotein-Alexa488 particles are endocytosed by cells throughout the disc. Moreover, Lipophorin uptake is not limited to the expression


A
B
C
Wingless
488Lipophorin
D
E
F
Wingless
CD63:GFP
G
H
I
Hedgehog
488Lipophorin
J
K
L
Hedgehog
CD63:GFP

domains of Wingless or Hedgehog. This is not surprising since lipoproteins have a nutritional function in discs as well and many potential Lipophorin receptor related are encoded in the *Drosophila* genom (Culi, J. *et al.* 2003).

To test whether Wingless or Hedgehog was found with Lipophorin particles in the same endosomes, I incubated wing imaginal discs with Lipophorin-Alexa488 particles and immunostained with antibodies to these proteins. I observed that majority of endocytosed Wingless and Hedgehog is found in the same endosomes as Lipophorin-Alexa488 (Figure 15A-C and 15G-I). Strong colocalization between Lipophorin and lipid-linked morphogens in endosomes is predicted if Wingless and Hedgehog are endocytosed together with Lipophorin. Nevertheless, we cannot exclude the possibility that these proteins entered the cells separately and converged in the same endosomes.

Taken together, these data suggest that exosomes do not act as carriers of lipid modified proteins through the epithelium whereas lipidic particles are very good candidates for this task.

Figure 15: Wingless and Hedgehog colocalize with labeled Lipophorin-Alexa488 but not CD63:GFP. A-C) wing imaginal disc incubated for 20 minutes with fluorescently labeled Lipophorin-Alexa488 **(B** in green and **C)** and stained with anti-Wingless **(A** and **B** in red**)**. **D-F)** wing imaginal disc expressing CD63:GFP **(E** in green and **F)** in the Wingless producing cells stained with antibody to Wingless **(D** and **E** in red**)**. **G-I)** wing imaginal disc incubated for 20 minutes with fluorescently labeled Lipophorin-Alexa488 **(H** in green and **I)** and stained with anti-Hedgehog **(G** and **H** in red**)**. **J-L)** wing imaginal disc expressing CD63:GFP **(K** in green and **L)** in the Hedgehog producing cells stained with antibody to Hedgehog **(J** and **K** in red**)**. Blue lines indicate A/P compartment boundaries. Scale bars = 10 μ.

Chapter 2.4. Functional studies

To assess the role of Lipophorin in larval growth and development, I used RNA interference to reduce its levels. First, I examine Lipophorin role in lipid transport and its effect on larval growth. Second, I address whether trafficking of lipid-linked morphogens is dependent on Lipophorin particles.

Lipophorin protein levels are reduced by dsRNA expression

To reduce ApoLI and II levels, dsRNA was targeted against two distinct regions of the *apolipophorin* mRNA. Similar phenotypes were produced by each construct. To induce RNA interference in *Drosophila* tissues in temporally and spatially controlled manner, a novel system has been developed. Expression of the *lipophorin*-derived inverted repeat relied on a modified form of the GAL4:UAS system in which the dsRNA was separated from the promoter by a removable cassette containing the HcRed gene flanked by FRT sites. Upon heat shock-mediated excision of the HcRed cassette by FLP recombinase, *lipophorin* dsRNA was expressed at specific times under the control of different GAL4 drivers.

Because *lipophorin* is not expressed in the discs, it is not possible to restrict the depletion of Lipophorin to this tissue. Thus we are only able to study the requirement for Lipophorin in morphogen signaling in the context of systemic reduction of Lipophorin levels. Induction of dsRNA in the fat body of second instar larvae, the site of endogenous Lipophorin expression, under the control of *Adh*GAL4 caused a delay in larval development. Such larvae prolonged the late third instar at least by three days compared with the siblings that did not express *lipophorin* dsRNA, they only occasionally pupariated, and never gave rise to viable adults. Similar results were obtained when dsRNA was induced ubiquitously under the control of *Tub*GAL4. In contrast, no phenotype was ever observed in adult flies when *lipophorin* dsRNA was expressed in

imaginal discs. This observation also shows that *lipophorin* dsRNA expression does not cause non-specific degradation of any essential RNAs in this tissue.

To determine how efficiently dsRNA induction reduced Lipophorin protein levels, I tested extracts from wild type larvae or larvae harbouring *hs-flippase*, GAL4 driver and UAS dsRNA constructs at various times after induction (Figure 16). 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. Four days after heat shock, the lower band of ApoLII doublet gradually dropped to approximately 5% of wild type levels (Figure 16, top panel). However, the minor upper band appeared to be only slightly reduced. This protein band migrates to the higher density fractions of isopycnic density gradients, indicating its soluble properties (see Figure 14D, ApoLII long exposure). Thus, I conclude that the upper band of the doublet enriched in extracts from imaginal discs represents a protein that cross-reacts with the Lipophorin antibody. ApoLI levels are reduced with similar kinetics (Figure 16, lower panel,). I performed all the experiments described below on third instar larvae 4-6 days after RNAi induction.

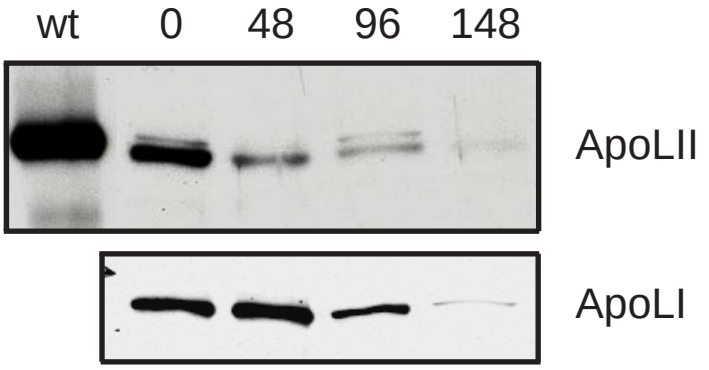


Figure 16: Reduction of Lipophorin protein levels by lipophorin dsRNA expression. Western blots of larval extracts from equal numbers of wild-type (wt) or *hs-flippase/+*; UAS:*lipophorin dsRNA/Tubulin*GAL4 larvae probed with anti-ApoLII serum (top panel) and anti-ApoLI serum (lower panel) at indicated hours after heat shock (0, 48, 96, 148 hours).

Lipophorin reduction interferes with lipid transport

The principal function of Lipophorin is transport of neutral lipids, triglyceride and esterified cholesterol, throughout the organism. Therefore we asked how reduction of Lipophorin levels affects neutral lipids distribution in various tissues. To address this question, we used Nile Red staining to visualise lipid droplets in Lipophorin RNAi larvae. This dye fluoresces red when present in cell membranes, but its emission shifts towards yellow in the more hydrophobic environment of esterified cholesterol or triglyceride (Greenspan, P. *et al.* 1985). Cells of the posterior midgut are thought to mediate nutrient uptake and they normally contain numerous small lipid droplets that are specifically apparent within this region. (Figure 17A). Lipophorin reduction causes massive overaccumulation and dramatic expansion of these lipid droplets (Figure 17B). This suggests that Lipophorin is required for the efficient extraction of dietary lipid from the cells of the posterior midgut.

We then asked whether reducing Lipophorin levels affected the amount of lipid stored in the fat body. Wild type fat body cells contain both small and large lipid droplets (Figure 17C). The fat bodies of Lipophorin RNAi larvae were slightly smaller and were somewhat depleted of small lipid droplets (Figure 17D), although the larger ones appeared normal in size and number. Even though the effect on fat body droplets was less severe, these data suggest that a significant amount of lipids present in fat body lipid droplets is derived from nutritional sources and delivered by Lipophorin particles.

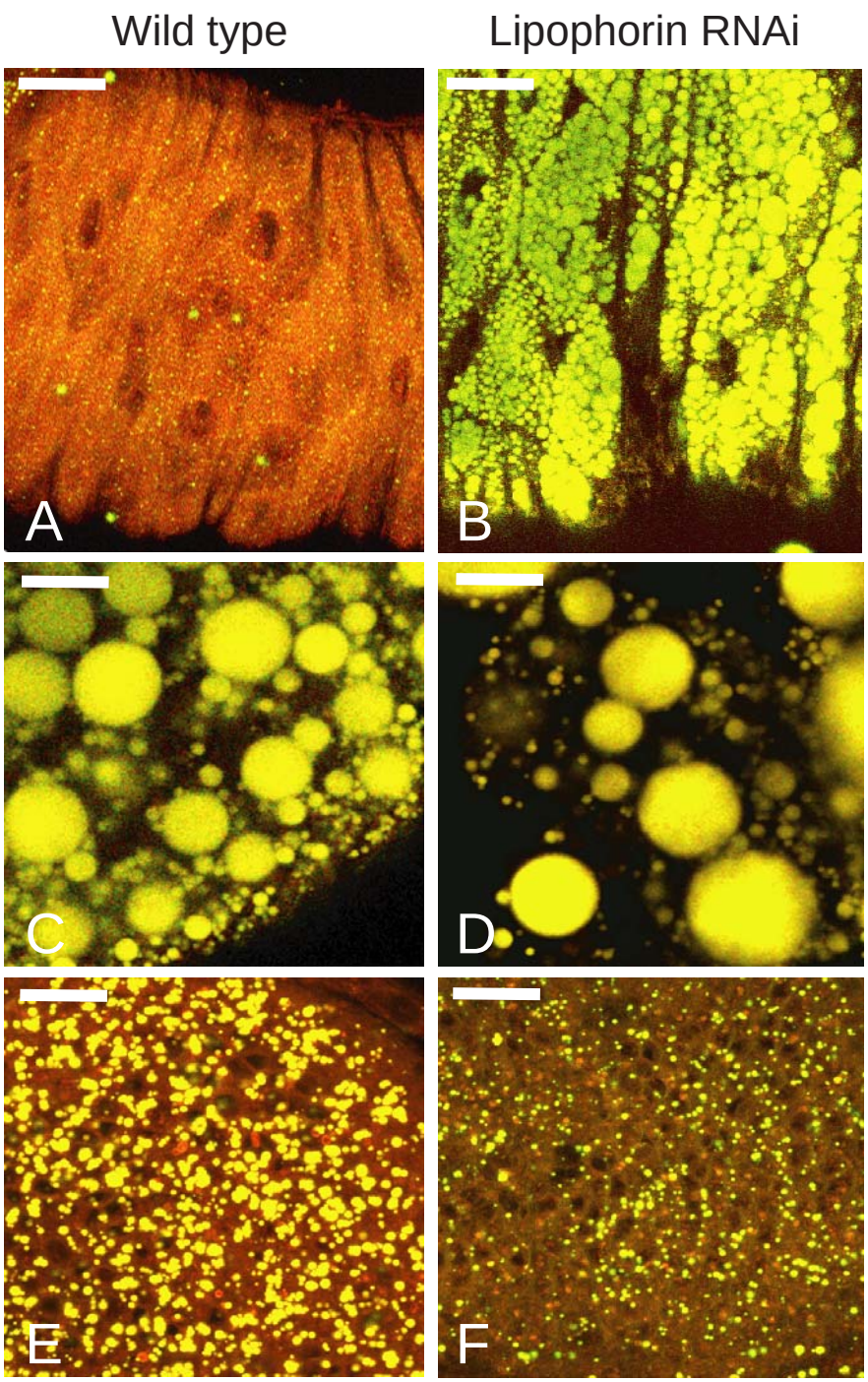


Figure 17: Expression of *lipophorin* dsRNA alters lipid trafficking. A-F) Nile red staining of posterior midgut **(A,B)**, fat body **(C,D)** or imaginal discs **(E,F)** from either wild-type **(A,C,E)** or *hs-flippase/+*; *Adh*GAL4/+; UAS:*lipophorin* dsRNA/+ larvae **(B,D,F)** 5 days after heat shock. Neutral lipids are stained yellow and plasma membrane is stained red by Nile Red. Scale bars = 40 μ in **(A, B)** or 10 μ in **(C-F)**. (performed by S. Eaton)

Lipophorin is needed for efficient lipid accumulation and growth in imaginal discs

Wing imaginal discs normally contain numerous large lipid droplets (Figure 17E). To investigate whether their formation or growth relied on Lipophorin-mediated lipid delivery, we examined Nile Red-stained imaginal discs from *lipophorin* dsRNA-expressing larvae. The lipid droplets from Lipophorin RNAi imaginal discs are decreased in number and they are significantly smaller than those of wild type discs (Figure 17F). This result supports the idea that deposition of neutral lipids in disc lipid droplets requires proper Lipophorin function.

I also observed that discs from *lipophorin* dsRNA-expressing larvae were smaller than those of wild type. This was particularly obvious in the wing pouch region, which averaged 50% of the normal wild type area (data not shown). Signaling via insulin-like growth factors is required for disc growth and is inhibited in response to starvation. I wondered whether lipid deprivation in Lipophorin RNAi larvae might restrict disc growth by inhibiting this pathway. Insulin signaling elevates the level of PIP_3 in the plasma membrane. Many proteins containing Plekstrin homology domains (PH) bind to PIP_3 and translocate to the plasma membrane (Britton, J.S. *et al.* 2002). To test whether insulin signaling is perturbed by reducing Lipophorin levels, I monitored the localization of GFP:PH in discs from Lipophorin RNAi larvae. This fusion protein binds to PIP_3 via a PH derived from AKT (Britton, J.S. *et al.* 2002). I observed no difference in membrane localization of GFP:PH in discs from wild type versus Lipophorin RNAi larvae (Figure 18A and B), and conclude that insulin signaling is normal, despite the reduced levels of lipid delivered to this tissue.

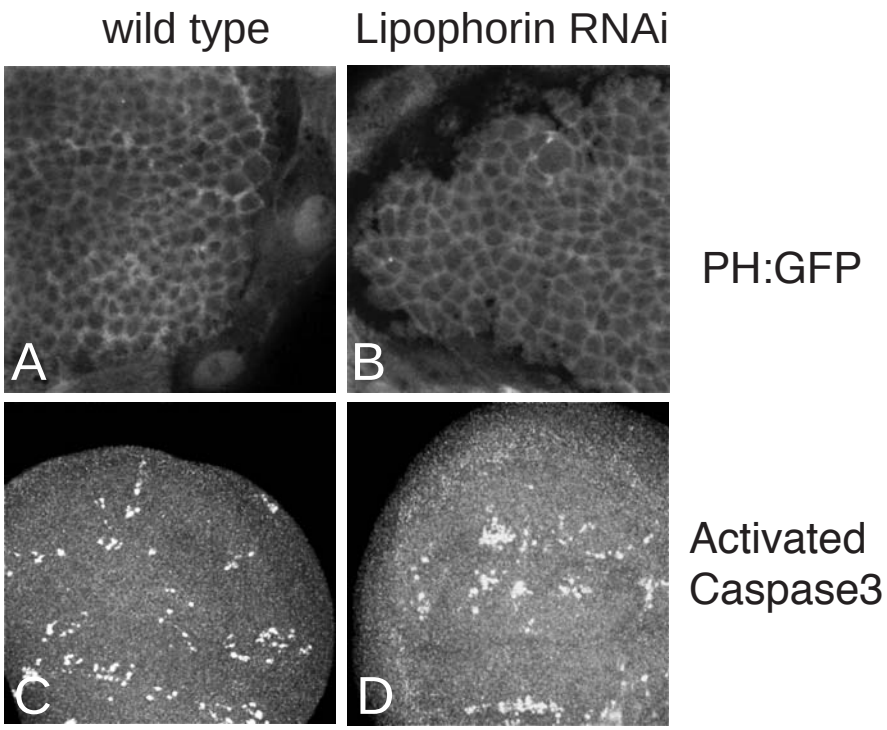


Figure 18: Induction of *lipophorin* dsRNA does not prevent Insulin signaling or elevate the cell death. A,B) confocal images of living imaginal discs from either wild type **(A)** or *hs-flippase*/+; tubP:GFP:PH/+; UAS:*lipophorin* dsRNA/*Tubulin*GAL4 **(B)** larvae that ubiquitously produce a Plekstrin homology domain:GFP fusion protein 6 days after heat shock. **C, D)** confocal images of imaginal discs from either wild type **(C)** or *hs-flippase*/+; UAS:*lipophorin* dsRNA/*Tubulin*:GAL4 **(D)** larvae stained 6 days after heat shock with an antibody to activated Caspase3.

Increased level of apoptosis might be another possible explanation for reduced size of imaginal discs expressing *lipophorin* dsRNA. To test that I compared the levels of activated Caspase3 in these discs (Figure 18D) to that of the wild type discs (Figure 18C). I observed that Caspase3 activation is not altered in Lipophorin RNAi discs, suggesting their small size is not due to the cell death.

Thus, these data show that Lipophorin-mediated transport is necessary for the accumulation of normal levels of stored lipid, and for growth of imaginal disc tissue. The developmental delay caused by reduction of Lipophorin levels further suggests the requirement for Lipophorin in normal larval development.

Trafficking and signaling of lipid-linked morphogens is perturbed by Lipophorin RNAi

We originally proposed that interaction between the lipid moieties of Hedgehog and Wingless and hydrophobic particles might serve to release these proteins from the plasma membrane on a particle and allow them to spread long distances through the developing tissue. To investigate the idea that lipidic particles act as carriers of lipid-linked morphogens, I asked whether the Lipophorin reduction was able to perturb the trafficking or signaling activity of Wingless and Hedgehog.

Wingless function requires Lipophorin

Wingless protein is produced and released from the cells at the dorsoventral boundary of wing imaginal disc epithelia. In the receiving tissue, Wingless is present in a graded distribution of cytoplasmic vesicles. To ask whether Lipophorin RNAi perturbed Winglesss trafficking, I examined its distribution in the wing imaginal discs. I did not observe any changes in the distribution of Wingless positive vesicles in the receiving tissue by conventional immunostaining protocol. In addition, Wingless levels and its distribution in producing cells were not altered, suggesting that in the absence of Lipophorin, Wingless is still released from producing cells as well as endocytosed by adjacent receiving tissue (data not shown).

One possible mechanism for Wingless spread is its diffusion in extracellular space (Strigini, M. *et al.* 2000). We asked therefore, whether extracellular Wingless might be affected in Lipophorin RNAi discs. We observed that extracellular Wingless was less abundant both on apical and basolateral epithelial surfaces of disc cells. Furthermore, extracellular Wingless spread over shorter distances when Lipophorin levels were reduced (Figure 19A-D). Thus, Lipophorin promotes extracellular accumulation of Wingless.

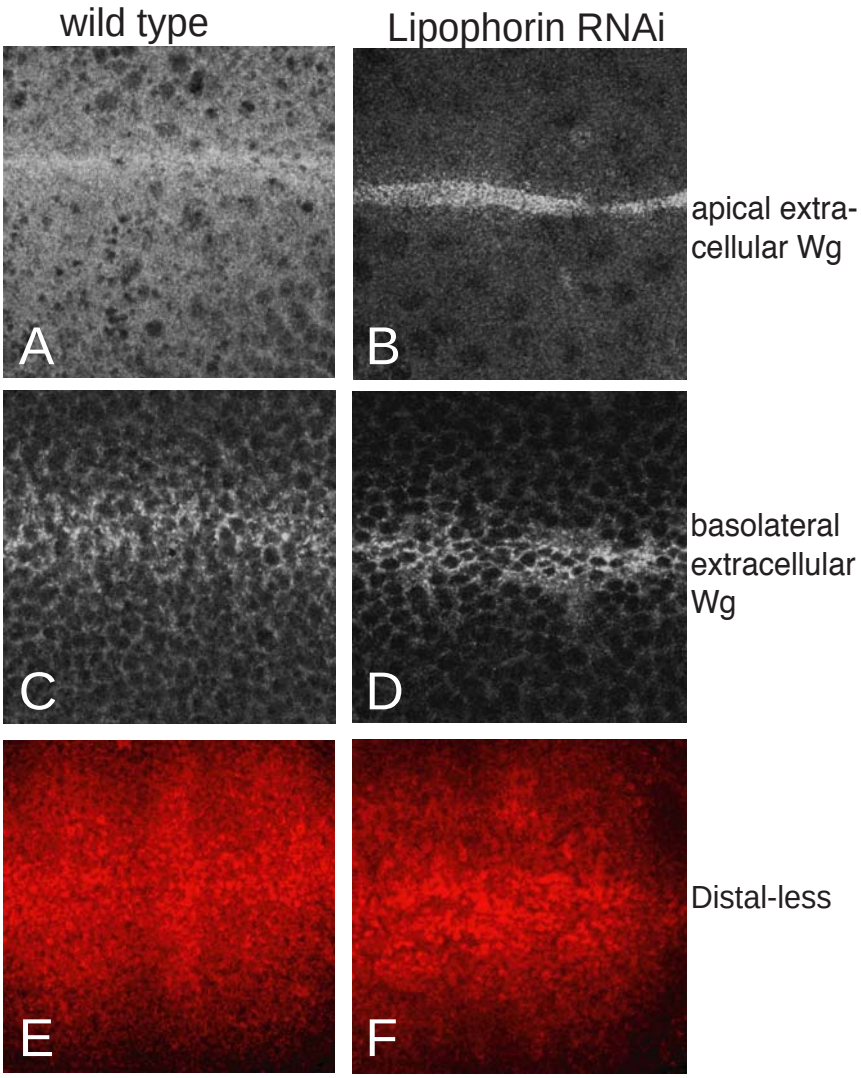


Figure 19: Lipophorin RNAi narrows the range of Wingless signaling. A-D) Apical **(A,B)** and basolateral **(C,D)** sections of wild type **(A,C)** and *hs-flippase*; *Adh*GAL4/+; UAS:*lipophorin* dsRNA/+ **(B,D)** wing discs 5 days after heat shock stained for extracellular Wingless. **E,F)** Distal-less protein accumulation in wild type **(E)** and *hs-flippase*/+; UAS:*lipophorin/Tubulin*GAL4 **(F)** wing imaginal discs 5 days after heat shock. (experiment performed by S. Eaton)

Once released, Wingless activates several target genes at different distances from its site of production. *Senseless* is activated in the tissue immediately adjacent to the Wingless-expressing cells straddling the DV boundary. *Distal-less* expression is induced in gradient-like fashion over a broad domain covering almost the entire wing pouch (Neumann, C.J. *et al.* 1997). To investigate whether Wingless signaling requires Lipophorin, we examined the activation of these two target genes. Expression of a short-

distance target gene *senseless* is unaffected by Lipophorin RNAi (date not shown). On the contrary, the gradient of long-distance target gene *Distal-less* is abnormally narrowed in discs expressing *lipophorin* dsRNA (Figure 19E and F). To compare the range of *Distal-less* activation in wild type and Lipophorin RNAi discs, staining intensities of Distal-less were measured using ImageJ and plotted on the graph shown in Figure 20A; for comparison, individual traces in Lipophorin RNAi discs are shown in Figure 20B.

These data suggest that Lipophorin knock-down specifically perturbs the range over which Wingless can signal and that Lipophorin particles are required for long distance trafficking of Wingless throughout the extracellular space of the wing disc epithelia.

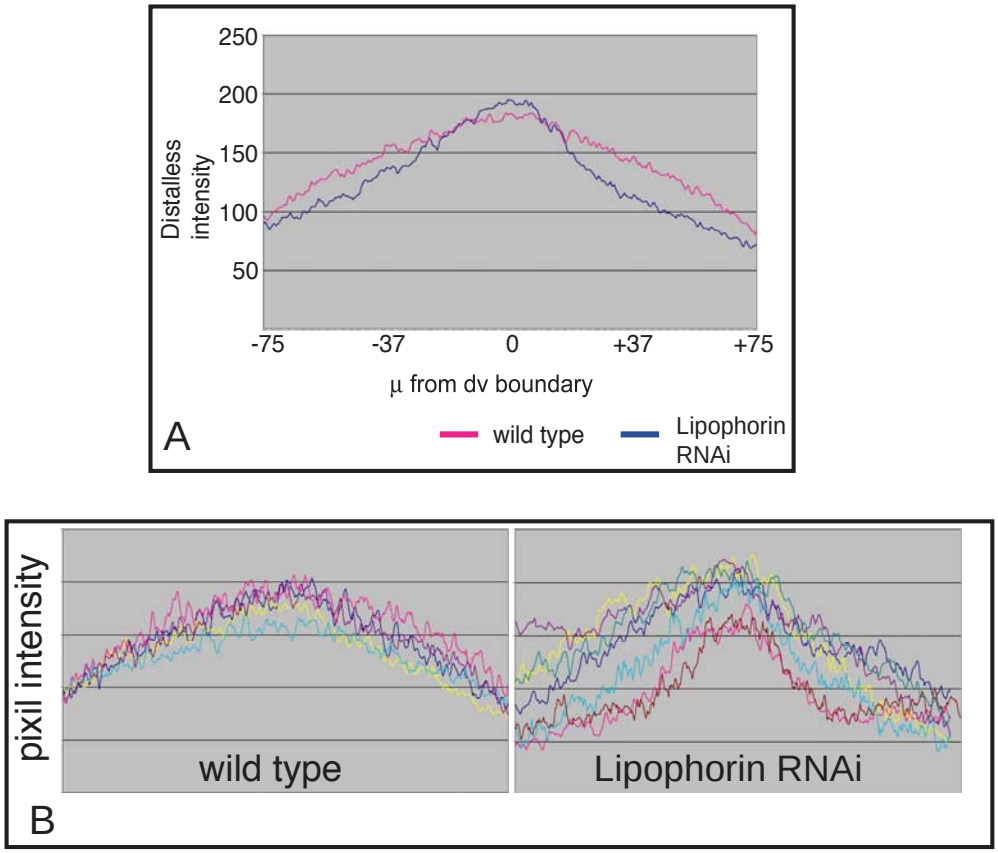


Figure 20: Staining intensities of Distal-less. A) Average Distal-less staining intensity with distance from dorsal-ventral boundary of 5 wild type wing imaginal discs (pink) and 5 *hs-flippase*/+; UAS:*lipophorin* dsRNA/*Tubulin*GAL4 (blue) wing imaginal discs. **B)** Individual traces of Distal-less staining intensity for 5 wild type and 5 *hs-flippase*/+; UAS:*lipophorin* dsRNA/*Tubulin*GAL4 wing imaginal discs. (with S. Eaton)

Hedgehog function requires Lipophorin

In wild type imaginal discs, Hedgehog expressed in the posterior compartment moves across the anterior-posterior (AP) compartment boundary and, as in case of Wingless, activates the transcription of both short and long-distance target genes. Cells immediately adjacent to the AP boundary, which receive the highest levels of Hedgehog, respond by activating the transcription of *patched* (see Figure 23C ad green in B) and *collier* (Figure 21C and green in B). Further away from the source of Hedgehog production, *patched* and *collier* are no longer produced; here, Hedgehog activates the transcription of long-range target gene *decapentaplegic* (Figure 21A and red in B) (Strigini, M. *et al.* 1997; Vervoort, M. *et al.* 1999; Vervoort, M. 2000).

To test whether Lipophorin association was required for Hedgehog function, I examined Hedgehog distribution and signaling in Lipophorin-RNAi larval discs. To compare Hedgehog signaling in wild type (Figure 21A-C) and Lipophorin RNAi (Figure 21D-F) larvae, I monitored the levels of Collier, and a *decapentaplegic* reporter construct (*dppLacZ*) in their imaginal discs. Discs from either wild type or Lipophorin RNAi larvae were dissected and stained in parallel and imaged under identical conditions. These images show that discs from Lipophorin RNAi larvae activate the short-range target gene *collier* at least as efficiently than those of wild type (compare Figure 21C and F). In contrast, the range of activation of the long-range target *dppLacZ* was significantly narrowed in *lipophorin* RNAi discs (compare Figure 21A and D). In wild type discs (Figure 21A), *dppLacZ* was expressed up to 11 cells away from the AP boundary (indicated by the blue line). In contrast, *dppLacZ* production in discs from Lipophorin RNAi larva occurred only within 6 cell diameters from the AP boundary (Figure 21D). Staining intensities from 4 discs of each type were measured using ImageJ, and are depicted graphically in Figure 21G. This data suggests that Lipophorin knock-down decreases the range of Hedgehog signaling.

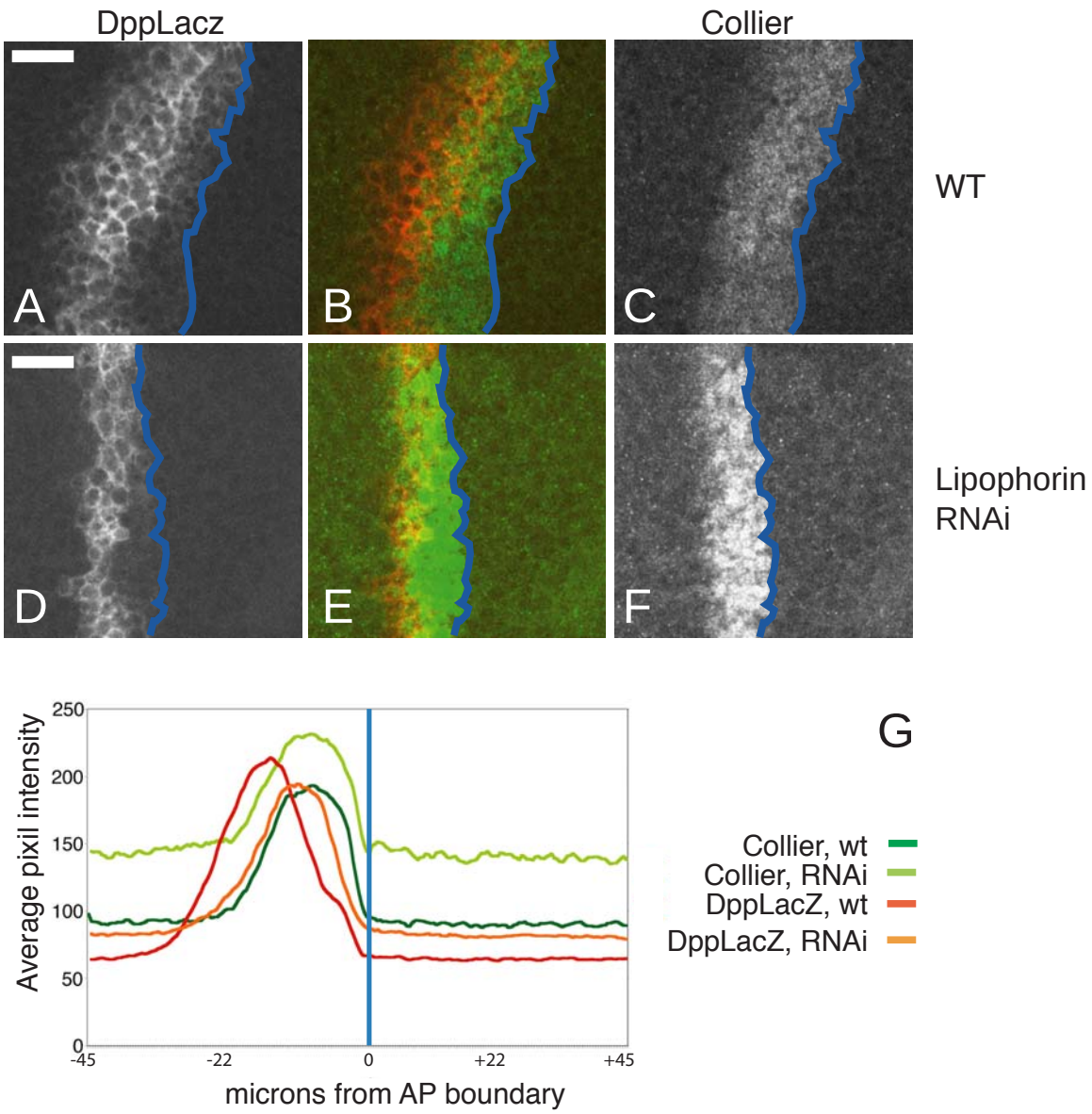


Figure 21: Lipophorin RNAi perturbs Hedgehog signaling. A-C) *dpplacZ*/+ disc 4 days after heat shock stained for β-Gal **(A** and **B** in red**)** and Collier **(B** in green and **C)**. **D-F)** *hs-flippase*/+; *dppllacZ*/+; UAS:*lipophorin* dsRNA/*Tubulin*:GAL4 disc 4 days after heat shock stained for β-Gal **(D** and **E** in red**)** and Collier **(E** in green and **F)**. Blue lines indicate AP compartment boundaries. Scale bars = 10 μ. **G)** Average Collier and DppLacZ (β-Gal) staining intensities for 4 wild type and 4 Lipophorin RNAi discs. Blue lines indicate A/P compartment boundary. Average distance from A/P boundary of peak DppLacZ staining was 16.6 +/- 2.7 μ for wild type and 11.1 +/- 1.5 μ for Lipophorin RNAi.

The activation of Hedgehog target genes is mediated by the transcription factor Cubitus interruptus (Ci). In wild type discs, in the absence of Hedgehog, PKA-dependent proteolytic cleavage generates a truncated form of Ci (Ci-repressor) that blocks the

expression of Hedgehog target genes throughout most of the anterior compartment. Upon Hedgehog stimulation in the region near to AP boundary, the processing of Ci is prevented and that further leads to accumulation of full length form of Ci (Ci-activator) (Figure 22A), which turns on the expression of the target genes like *patched* and *decapentaplegic* (Aza-Blanc, P. *et al.* 1999; Methot, N. *et al.* 2001). To examine whether the reduction in the activation range of *decapentaplegic* in Lipophorin RNAi discs is due to the narrowing of the range over which full length Ci is stabilized, I monitored the distribution of full-length Ci protein in these discs.

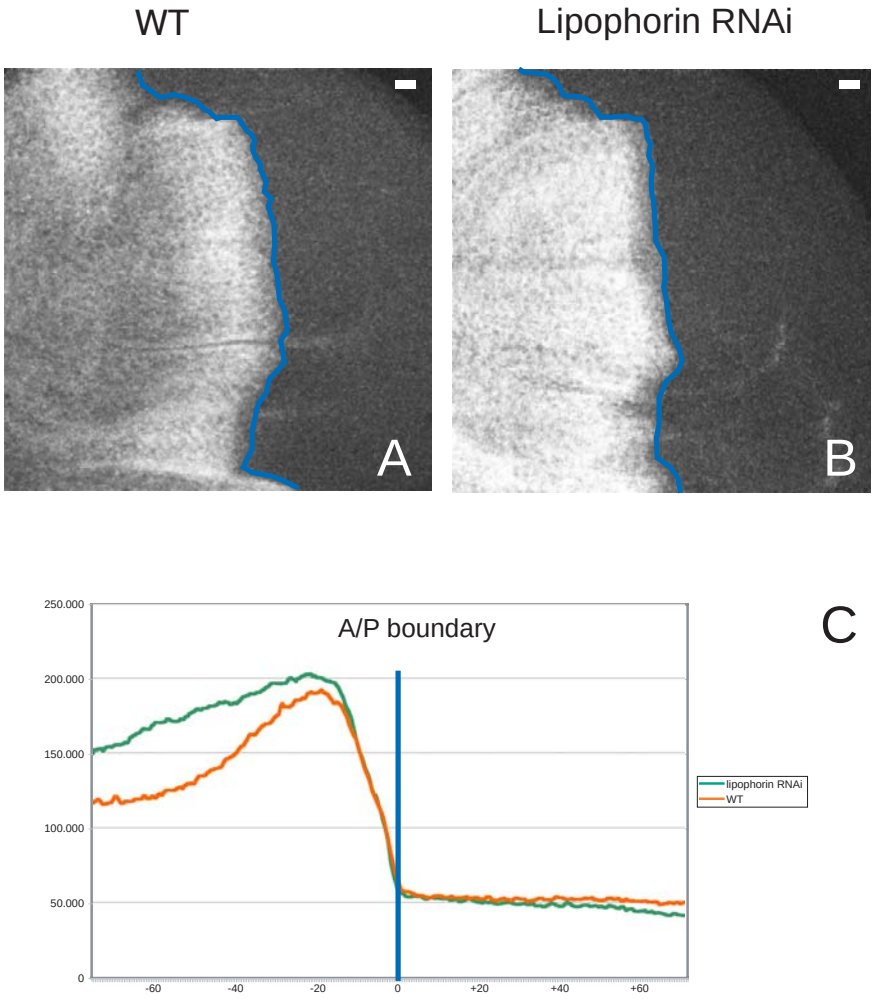


Figure 22: Full-length form of Ci accumulates in Lipophorin RNAi discs. A) Wild type wing imaginal disc or **B)** *hs-flippase*; UAS:*lipophorin* dsRNA/*Tubulin*GAL4 disc stained 4 days after heat shock with antibody against Ci full-length form. Blue lines indicate AP compartment boundaries. Scale bar = 10 μ **C)** Average Ci staining intensities from 3 independent experiments (17 wild type and 19 Lipophorin RNAi discs). Blue line indicates A/P compartment boundary.

Strikingly, distribution of the full-length Ci is dramatically altered in the discs from Lipophorin RNAi larvae. Inspite of the fact that Hedgehog protein does not spread further away (see below), full length Ci protein is not restricted to the AP boundary region. Conversely, it accumulates at high levels throughout the whole anterior compartment (Figure 22B). Staining intensities for wild type and Lipophorin RNAi discs are quantified in Figure 22C. Despite this abnormall accumulation, the full length Ci does not induce the transcription of Hedgehog targets as shown earlier. The stabilization of full length Ci is not sufficient to convert it to a transcriptional activator. Full activation of Ci requires other Hedgehog signaling-dependent post-translational modifications. These data demonstrate that in the absence of Lipophorin, Ci cleavage is prevented. However, the Ci protein is not fully activated and does not translocate to the nucleus, indicating that these steps in the transduction pathway are fully Hedgehog dependent. This finding is consistent with the idea that Lipophorin itself is required to promote the cleavage of full length Ci and its subsequent degradation.

In wild type discs, Hedgehog interacts with its receptor Patched, with which it is endocytosed into the receiving cells. Patched-mediated endocytosis is thought to sequester Hedgehog and limit its spread (Chen, Y. *et al.* 1996; Torroja, C. *et al.* 2004) and Figure 23A-C. To ask whether this narrowed range of signaling results from altered Hedgehog trafficking, I examined the distribution of Hedgehog and its receptor Patched. Released Hedgehog protein that spreads in the anterior compartment, is detected most easily up to 5 rows of cells from the AP compartment boundary (Tabata, T. *et al.* 1994). Although Hedgehog clearly signals over a wider range (see Figure 21A), specific signal is difficult to distinguish from background staining at greater distances. In discs from Lipophorin RNAi larvae, Hedgehog (Figure 23D and red in E) accumulates strongly above wild type levels in the first 5 rows of cells anterior to the AP boundary (indicated by the blue line). I quantified the number of Hedgehog spots in the most apical 10 microns in this area. I counted 380 Hedgehog spots in the wild type disc shown in Figure 23A comparing to 1208 spots of the same region of the Lipophorin RNAi disc shown in Figure 23D: a difference of 310%.

The majority of accumulated Hedgehog in these discs colocalizes with Patched (Figure 23D-E) in large punctate structures. On the other hand, Patched colocalizes more extensively with Hedgehog than it does in wild type tissue. To ask whether the large punctate structures in which Hedgehog and Patched accumulate together are endosomes, I labeled endocytic compartments of Lipophorin RNAi imaginal disc with fluorescent dextran. These structures became labeled within 10 minutes, and are thus early endosomes (Figure 23G-J).

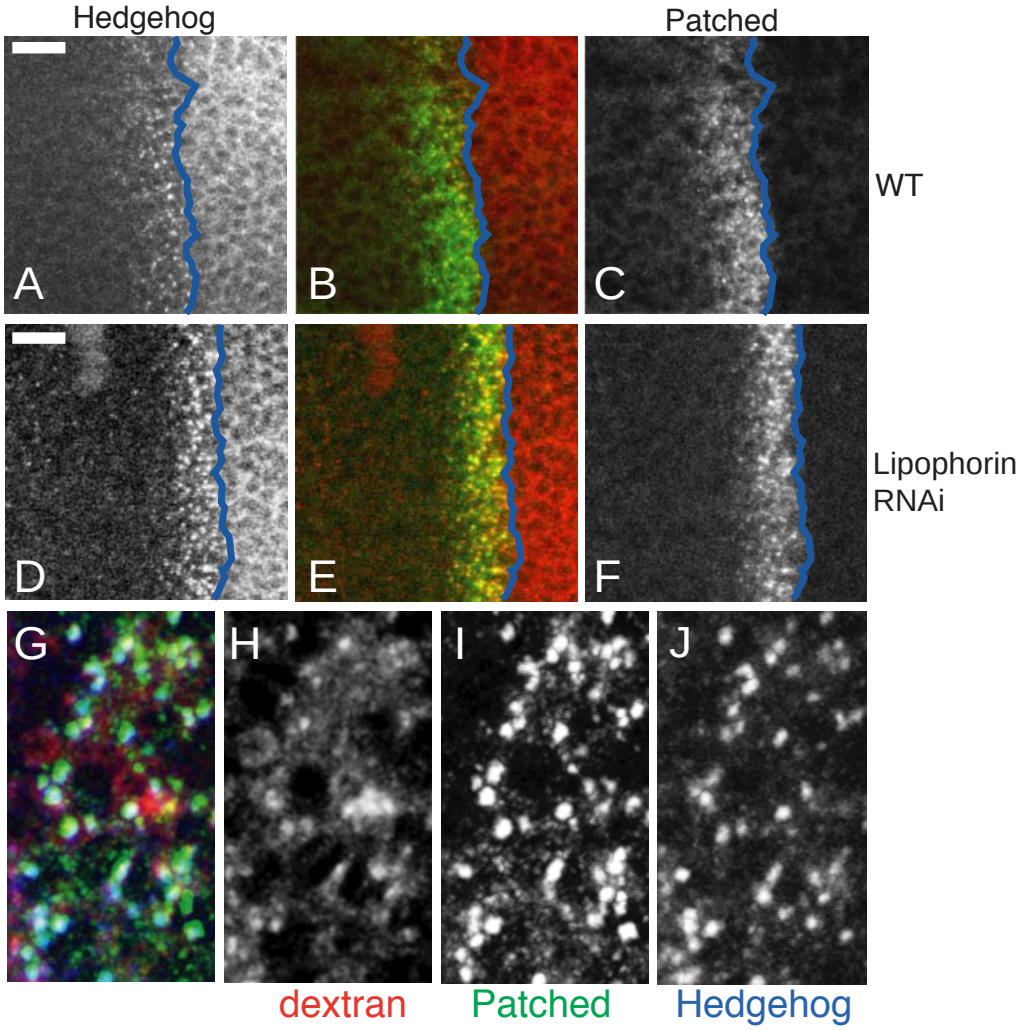


Figure 23: Hedgehog distribution is perturbed in Lipophorin RNAi. A-C) wild-type disc 4 days after heat shock stained for Hedgehog **(A** and **B** in red**)** and Patched **(B** in green and **C)**. **(D-F)** *hs-flippase*/+; UAS:*lipophorin* dsRNA/*Tubulin*GAL4 wing disc 4 days after heat shock stained for Hedgehog **(D** and **E** in red**)** and Patched **(E** in green and **F)**. Scale bars = 10 μ. **G-J)** Hedgehog accumulates with Patched in endosomes in Lipophorin-RNAi larvae. Confocal image of an imaginal disc from a *hs-flippase*/+; UAS:*lipophorin* dsRNA/*Tubulin*GAL4 larva stained with antibodies to Patched **(G** in green and **I)** and Hedgehog **(G** in blue and **J)**. Endosomes were labeled by red dextran uptake **(G** in red and B**)**.

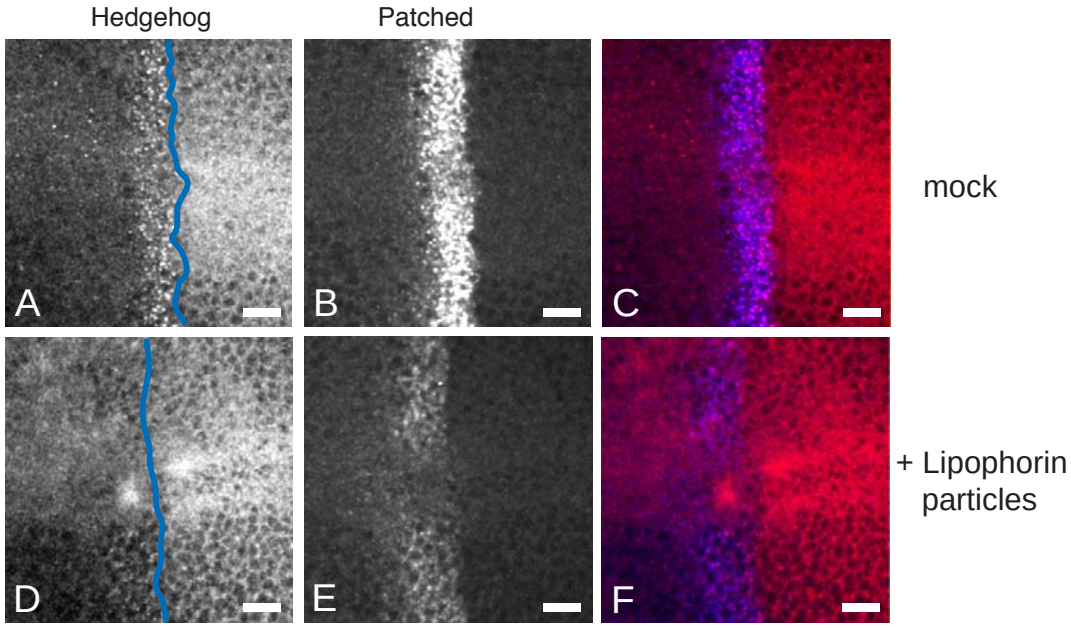
These data indicate that reducing Lipophorin levels either increases the susceptibility of Hedgehog to Patched-mediated endocytosis, or prevents subsequent degradation of the protein.

All together, these data indicate that Hedgehog function requires Lipophorin. Lipophorin particles bind Hedgehog in order to mediate its trafficking and signaling over long distances. In addition, these results suggest that Lipophorin is specifically required in the processing of Ci.

Lipophorin acts directly to control Hedgehog trafficking

It is possible that Lipophorin depletion might affect Hedgehog trafficking indirectly by preventing release of a needed co-factor from another larval tissue. To investigate this hypothesis, I added purified Lipophorin particles to explanted Lipophorin-RNAi discs and examined Hedgehog and Patched distribution. Abnormal Hedgehog and Patched accumulation was strongly reduced after a two-hour incubation of dissected discs with Lipophorin particles (Figure 24; compare mock treated 24A-C with the treated discs 24D-F). To compare the levels of reduction of accumulated Hedgehog, we quantified the number of Hedgehog positive spots in Lipophorin RNAi discs that had been mock treated (Figure 24A and red in C) or incubated with purified Lipophorin particles (Figure 24D and red in F). I focused on the first 2 μm from the apical surface, in the region where the Hedgehog accumulation is the most prominent. The number of Hedgehog spots was reduced by 60% in this apical area of the discs (Figure 24G). Interestingly, the difference between the numbers of Hedgehog spots was not so prominent in more basolateral regions of the disc (data not shown). This suggests that Lipophorin acts directly on Hedgehog accumulated in early apical endosomes. I also noticed that added Lipophorin particles sometimes reduce apical (but not basolateral) Hedgehog staining in producing cells, especially in regions further away from the D/V boundary. We are currently investigating whether it is due to the increased release of Hedgehog by Lipophorin from this region.

I further observed in these experiments that levels of Patched are dramatically reduced upon addition of Lipophorin particles. Measuring the staining intensities of Patched in these discs (Figure 24H) revealed that levels of Patched are reduced by approximately 50%. Because the incubation with Lipophorin particles with the explanted



G

	number of Hh-dots	percentage
mock (n=3)	117 +/- 47 (95, 170, 86)	100
+ Lp-particles (n=5)	52 +/- 12 (51, 48, 61, 34, 65)	43.7
ratio mock/+Lp	2.290	

H

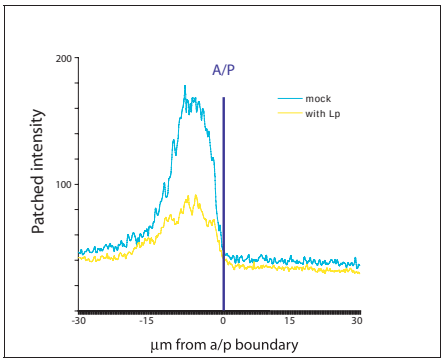


Figure 24: Rescue of Hedgehog accumulation in dissected Lipophorin RNAi discs by in vitro Lipophorin addition. A-C) *hs-flippase*/+; UAS:*lipophorin* dsRNA/*Tubulin*GAL4 wing imaginal disc incubated for 2 hours in Grace's medium. The disc was stained for Hedgehog **(A** and **C** in red**)** and Patched **(B** and **C** in blue**). D-F)** *hs-flippase*/+; UAS:*lipophorin* dsRNA/*Tubulin*GAL4 wing imaginal disc incubated for 2 hours with purified Lipophorin particles. Approximately 1/10 of the estimated concentration in hemolymph was added. The disc was stained for Hedgehog **(D** and **F** in red**)** and Patched **(E** and **F** in blue**).** Scale bars = 10 µ. **G)** Quantification of Hedgehog puncta in mock-treated and Lipophorin-treated discs in the anterior compartment within the apical most 5 µ of discs. The average number of Hedgehog positive spots, standard deviation, and counts in individual discs are shown. **H)** Quantification of Patched staining intensity in 3 mock-treated and 5 Lipophorin particle-treated discs of the most apical 5 µ of each disc. All discs were stained in parallel and imaged at the same day under identical conditions.

discs is time limited, I speculate that this change reflects a direct effect of Lipophorin on Patched degradation rather than an effect caused by changes in signaling.

Thus Lipophorin acts directly in wing imaginal discs to control Hedgehog trafficking, although it is still possible that its effects on signaling are indirect.

Lipid deprivation stalls growth, but does not affect Hedgehog signaling

Drosophila cannot synthesize sterols and relies on uptake from dietary sources. To assess whether reduced uptake of sterols or other lipids might cause the changes we see in Lipophorin knock-down animals, 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 were developmentally delayed; 5-7 days after lipid deprivation, their discs were similar in size to those of early third instar larvae (Figure 25A, B), whereas their yeast-fed siblings had already begun to emerge. Those flies that infrequently eclosed after larval lipid depletion were reduced in size (35-60% of normal body weight) but normally patterned (Figure 25C, D). These data show that lipid depletion stalls growth of imaginal tissues.

We next asked whether Hedgehog trafficking or signaling was affected in discs from lipid-starved larvae. Figure 25H-J shows a disc from a larva deprived of lipid for 6 days, after initially feeding on yeast for 48 hours. Since all yeast-fed controls have pupariated by this time, we compared the distribution of Hedgehog and Patched with younger yeast-fed larval discs of similar size (Figure 25E-G). No obvious changes are apparent. We performed similar experiments to ask whether lipid depletion altered the range of Hedgehog signaling. Figure25K-P shows that distance over which *decapentaplegic* and *collier* expression is activated does not differ between lipid-starved and yeast-fed larval discs.

Thus, lipid starvation does not mimic the effects of Lipophorin knock-down on Hedgehog. These data suggest that lipid deprivation does not inhibit Hedgehog signaling,

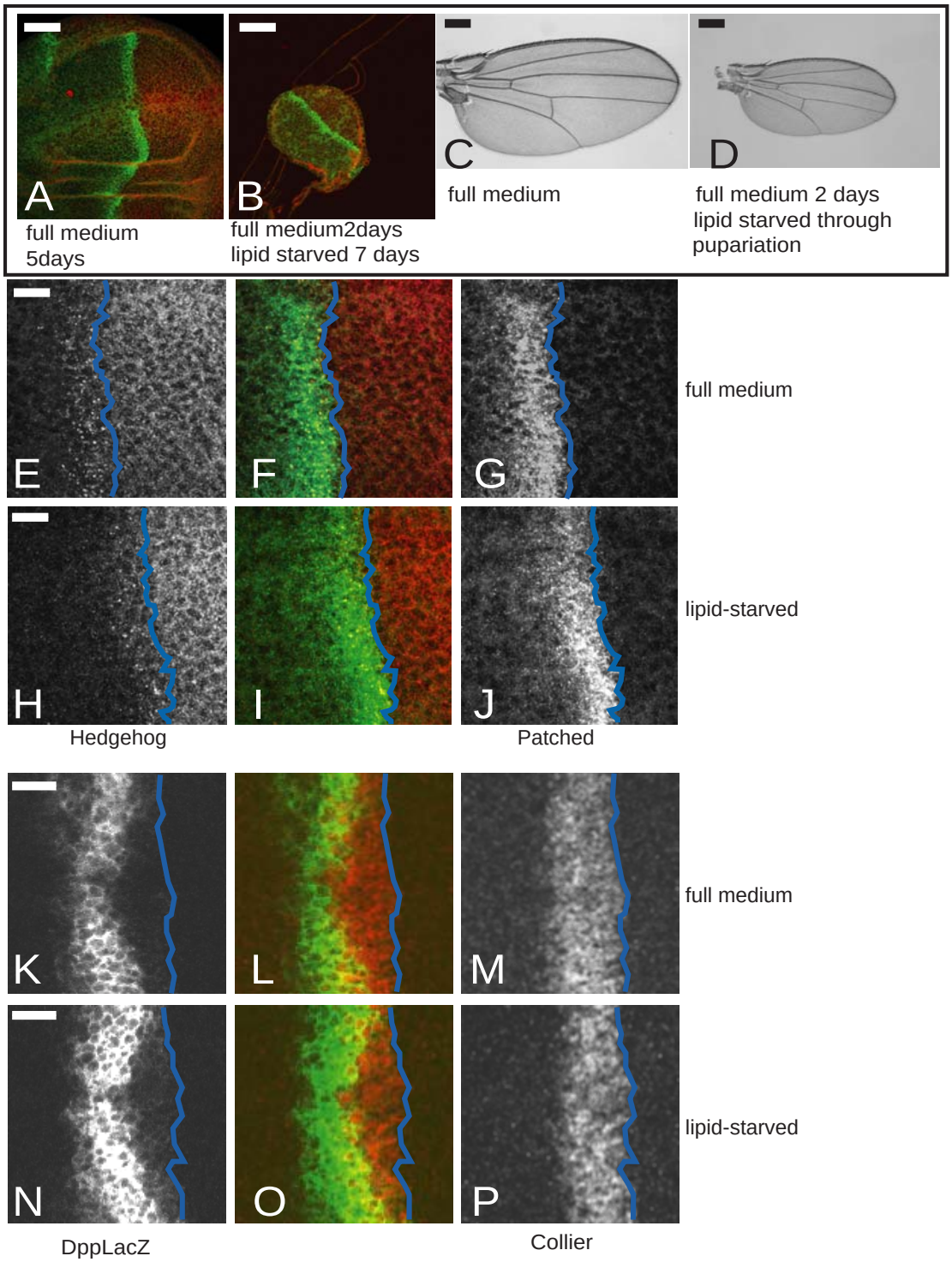


Figure 25: Hedgehog signaling is unaffected by lipid-depletion. A) Wing imaginal disc from a larva fed 5 days on full medium. **B)** Wing imaginal disc from larva fed 2 days on full medium then transferred to lipid-depletion plates for 7 days. **C)** Wing from a fly that fed on full medium throughout larval development. Scale bar = 250 μ. **D)** Wing from a sibling that fed on full medium for 2 days, then on lipid-depletion medium through the rest of larval development. Scale bar = 250 μ. **E-G)** Wing imaginal disc from larva fed on full medium stained for Hedgehog **(E** and **F** in red**)** and Patched **(F** in green and **G)**.

despite the fact that sterols are required for Hedgehog modification and for Smoothened activity. We speculate that the growth arrest that ensues after removal of dietary lipid may prevent membrane sterol concentrations from dropping to levels that would interfere with the Hedgehog pathway. Lipid deprivation stalls growth, but does not affect Hedgehog signaling. Therefore, it seems likely that Lipophorin plays a direct role in supporting Hedgehog movement and signaling, rather than indirectly affecting the pathway via lipid deprivation.

Lipid-linked proteins could be loaded on Lipophorin inside the cells

Our results show that lipid-linked morphogens and gpi-anchored proteins associate with Lipophorin particles *in vivo*. However, the data does not explain how these particular proteins become specifically loaded on such a particle. One possibility is that the lipid-linked proteins are passively extracted from membranes and bind Lipophorin particles without assistance of other interacting partners. The observation that incubation of membranes with purified Lipophorin particles did not result in any additional extraction of Hedgehog (see Figure 14E) supports the idea that association of lipid-linked morphogens with Lipophorin is an active rather than a passive process.

Unlike vertebrate LDL that is taken up by cells via receptor-mediated endocytosis and targeted to the lysosome, a characteristic feature of insect Lipophorin is its ability to transfer lipid cargo without being degraded (e.g. *Locusta migratoria*) (van der Horst, D.J. *et al.* 2002; Van Hoof, D. *et al.* 2002). Hence, another option how specific lipid-linked proteins could bind Lipophorin involves a selective loading of these proteins on a Lipophorin particle within the imaginal discs cells, perhaps in a recycling

Figure 25, continued: Scale bar = 30 μ. **H-J)** Wing imaginal disc from a lipid starved larva stained for Hedgehog **(H** and **I** in red**)** and Patched **(I** in green and **J)**. Scale bar = 30 μ. **K-M)** Wing imaginal disc from *dpplacZ*/+ larva fed on full medium stained for β-Gal **(K** and **L** in green**)** and the transcription factor Collier **(L** in red and **M)**. Scale bar = 10 μ. **N-P)** Wing imaginal disc from a lipid-starved larva harboring the same *dpplacZ* reporter construct stained for β-Gal **(N** and **O** in green**)** and Collier **(O** in red and **P)**. Scale bar = 10 μ. Blue lines indicate the A/P compartment boundary in all panels. (experiment performed by S. Eaton)

compartment. Alternatively, Lipophorin and lipid-linked proteins can become associated even within the biosynthetic pathway. In both cases, *Drosophila* imaginal disc cells would have to possess the machinery in being able to load and recycle Lipophorin particles with new protein and/or lipid composition.

To address this question, we constructed myc and GFP tagged versions of ApoLII (Figure 26A). First, we examined whether the tagged constructs are functional and can be associated with Lipophorin particles *in vivo*. We expressed ApoLII:myc in the fat body, the site of endogenous Lipophorin production, using an *Adh*GAL4 driver and prepared isopycnic density gradients from whole larvae. We observed that the majority of ApoLII:myc migrated to the top low density fraction of the gradient (Figure 26B, top panel). In addition, ApoLII:myc co-immunoprecipitates with ApoLII (see Figure 14F, fourth panel) and thus is incorporated into the Lipophorin particles. To ask whether tagged ApoLII could be transported from the fat body to imaginal discs, I examined living wing imaginal discs from larvae in which ApoLII:GFP had been expressed specifically in the fat body (Figure 26C). Live confocal imaging of FM4-64-stained discs revealed that the fusion protein was present in endosomes of disc epithelium (Figure 26 D). These data suggest that the tagged ApoLII fusion construct is functional and behaves like endogenous Lipophorin.

To investigate whether imaginal disc cells might be capable of assembling Lipophorin particle from exogenously provided Lipophorin, I expressed ApoLII:myc exclusively in the wing imaginal discs using an *en*GAL4 driver. I prepared the isopycnic density gradient from these dissected discs. Strikingly, approximately half of the tagged protein migrates to the low density fraction, suggesting that it becomes associated with the Lipophorin particles (Figure 26B, lower panel). These data indicate that *Drosophila* imaginal disc cells are able to specifically incorporate ApoLII:myc into the Lipoprotein particles.

Lipophorin particles normally contain both ApoLI and ApoLII. Since ApoLII:myc was incorporated into low density particles by imaginal disc cells, I wondered whether these particles might also contain endogenous ApoLI. To address this question, I examined the distribution of ApoLI in imaginal discs expressing ApoLII:myc

under the control of *patched*GAL4 (Figure 26E). Strikingly, the same cells that over-expressed ApoLII:myc also over-accumulated the exogenous, fat body-derived ApoLI (Figure 26F). Furthermore, ApoLI was also found in large punctate structures in

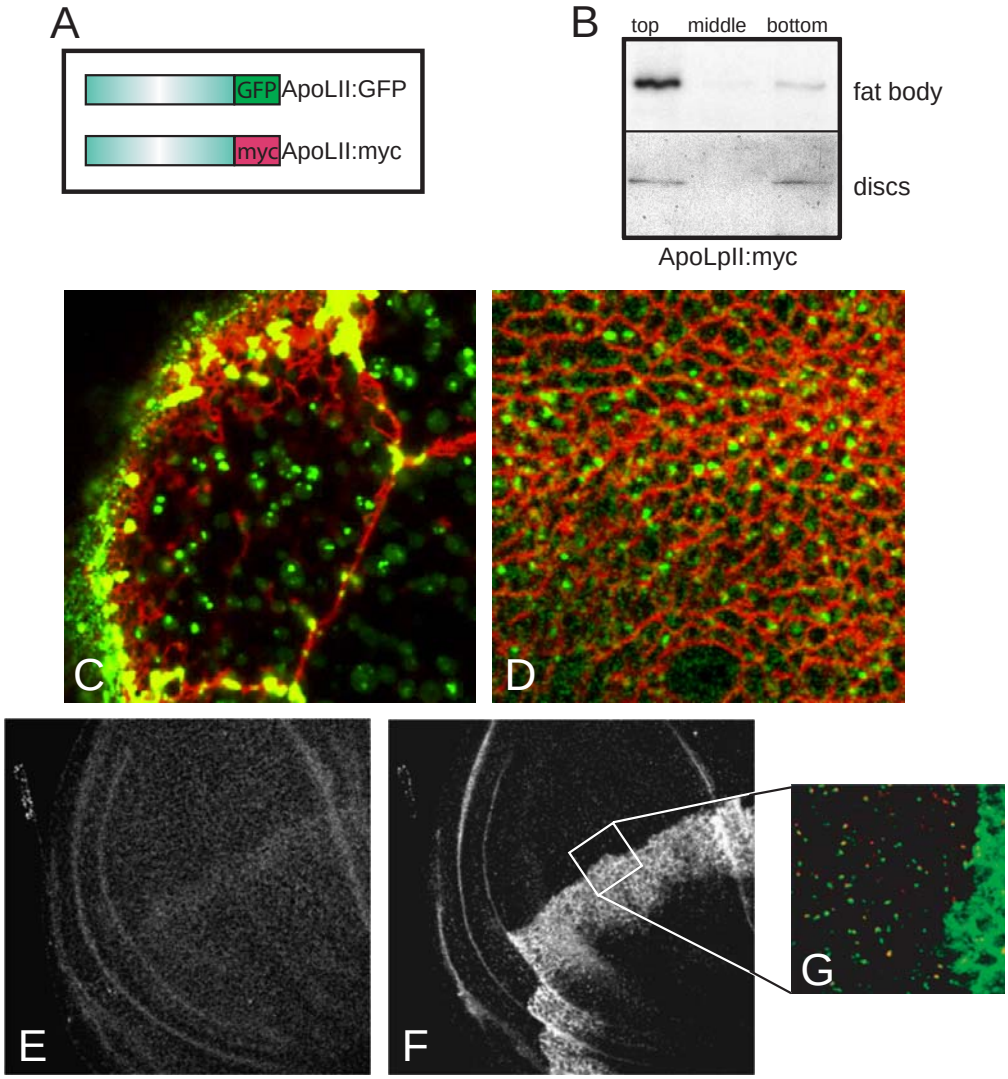


Figure 26: Imaginal disc cells possess machinery for assembly of Lipophorin particles: A) Schematic picture of tagged fusion constructs of ApoLII. **B)** top panel: Western blot of fractions from isopycnic density gradient prepared from whole larvae expressing ApoLII:myc in the fat body using *Adh*GAL4 driver and probed against anti-myc antibody (together with H. Sprong). Lower panel: Western blot of fractions from isopycnic density gradient prepared from dissected discs expressing ApoLII:myc in the imaginal discs using *en*GAL4 driver and probed against anti-myc antibody. **C,D)** Fat body of larva expressing ApoLII:GFP using *Adh*GAL4 driver **(C)** and wing imaginal disc from the same larva **(D)**. ApoLII:GFP is transported from fat body and endocytosed by imaginal disc cells. Membranes and endosomes are stained red with FM4-64. **E,F)** Wing imaginal disc expressing ApoLII:myc using *ptc*GAL4 driver stained with anti-ApoLI antibody **(E)** and anti-myc antibody **(F)**. **(G)** Inset of (E,F), ApoLI is in red and ApoLII:myc in green.

surrounding tissue that colocalized extensively with ApoLII:myc (Figure 26G). These results indicate that expressing high levels of ApoLII:myc in the disc cells recruits internalized ApoLI, and suggests that both proteins are re-assembled into new Lipophorin particles.

The assembly of lipoproteins is dependent on the endoplasmic reticulum protein, microsomal triglyceride transfer protein (MTP) The role of MTP is specifically required in biosynthesis of lipoproteins, particularly in their lipidation (Hussain, M.M. *et al*. 2003). To examine whether imaginal discs transcribe *mtp*, I performed in situ hybridization and observed that imaginal discs express mRNA encoding MTP (data not shown). Thus, imaginal disc cells, which do not synthesize Lipophorin protein *de novo*, nevertheless possess the machinery to assemble Lipophorin particles. Intriguingly, after 20 minutes incubation of living imaginal discs with the Lipophorin-Alexa488 particles, I observed the labeling of internal structures that resembled endoplasmic reticulum (data not shown). This fact makes it possible to speculate that lipid-linked proteins might be able to associate with Lipophorin in the biosynthetic pathway.

Taken together, these data indicate that the fat body-derived Lipophorin can be internalized by imaginal disc cells and incorporated into new particles. This further raises the possibility that imaginal disc cells can selectively load and unload specific proteins on Lipophorin during this process. This suggest that lipid-linked morphogens might be loaded onto Lipophorin particles during re-assembly and recycling of Lipophorin within the cells of the wing disc epithelium.

Lipophorin particles can carry glypicans Dally and Dally-like

A body of evidence indicates that the transport of the morphogens through the developing tissue is a highly controlled process. It is intriguing to speculate that Lipophorin particles, in addition to signaling molecules, associate with proteins that regulate particle trafficking or its interaction with the binding partners. The fact that many gpi-anchored proteins associate with Lipophorin and that vertebrate lipidic particles

interact with glypican molecules (Mahley, R.W. *et al.* 1999) (Camejo, G. *et al.* 1998) prompted us to test the idea whether two *Drosophila* glypicans, Dally and Dally-like, also bind Lipophorin particles.

Dally and Dally-like are present in the imaginal discs in multiple forms

To study the function of these proteins in developing wing epithelia, fusion proteins Dally:GFP and Dally-like:GFP were constructed in our lab; GFP was inserted at the N termini of the proteins (A. Mahmoud). Confocal imaging revealed that they are associated with the membranes of producing cells as predicted from their structure. To ask whether they could be released from the producing cells, their distribution in the wing disc tissue was monitored. Surprisingly, we observed that only Dally, but not Dally-like, was found in non-expressing tissue (Ch. Eugster, V. Greco, S. Eaton, unpublished). To ask which forms of Dally might account for this distribution, I decided to take advantage of biochemical assays.

To determine whether the observed sizes of Dally:GFP and Dally-like:GFP fusion proteins are consistent with those predicted, I first tested their mobility on SDS-PAGE. Both proteins migrate with a broad band of higher molecular weights than predicted. This is consistent with the possibility that the core proteins are modified by heparan sulfate glycosoaminoglycan side chains. Incubation of imaginal discs expressing Dally:GFP with Heparinase I, resulted in narrowing of an observed band (data not shown) that indicates that indeed a core protein is modified with HS side chains.

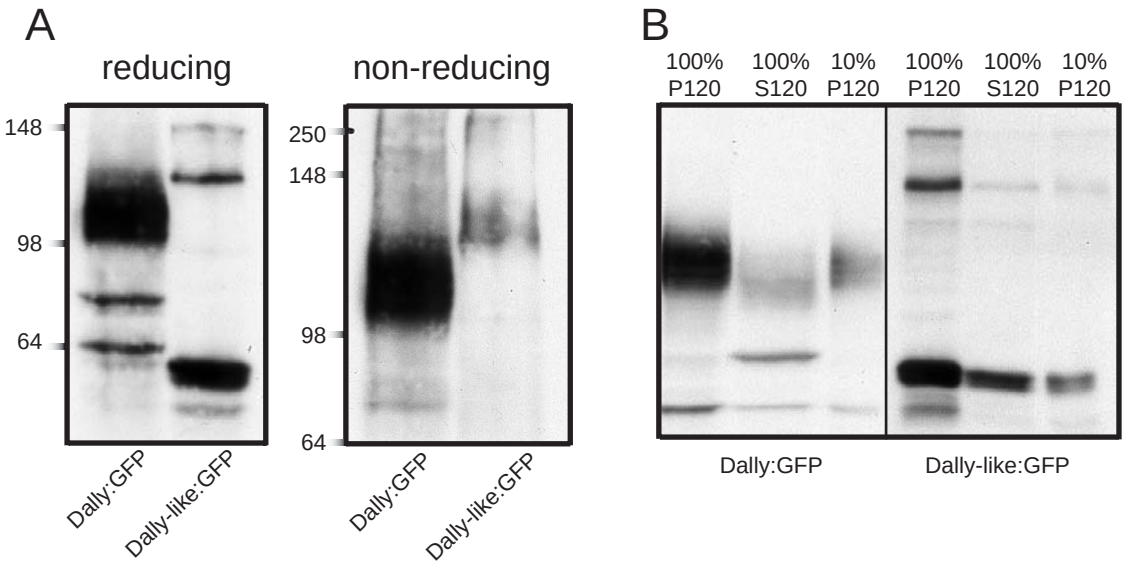
In addition to a broad band of full-length size of Dally:GFP, I detected two additional bands, ~75 kDa and ~64 kDa, under reducing conditions of SDS-PAGE (Figure 27A, left panel). To ask whether these bands might be the result of a cleavage event that generates fragments of the proteins that than remain associated via disulfide bonds, I ran SDS-PAGE under non-reducing conditions (Figure 27A, right panel). I observed that the 75 kDa band is still present, however the 64 kDa band is no longer apparent. I named these three observed isoforms of Dally:GFP: a full-length size –Dally-FL (~98 kDa band), a cysteine-rich fragment that is released from the rest of the protein –

Dally-CR (~75 kDa band) and a cleaved form that remains associated with the rest of the protein via disulfide bonds, a full-length processed form – Dally-FLP (~64 kDa band) (Figure 27D).

I performed the same experiments with larvae expressing Dally-like:GFP. Under reducing conditions, I observed two bands: a full-length size and additional band that migrates at ~60 kDa. Under non-reducing conditions, this ~60 kDa band is no longer apparent. I named these observed isoforms of Dally-like:GFP as follows: a full-length size – Dally-like-FL (~120 kDa band) and a cleaved form that remains associated with the rest of the protein via disulfide bonds, a full-length processed form – Dally-like-FLP (~60 kDa band) (Figure 27D).

To address the possibility that insertion of GFP to the proteins causes artifactual cleavage, we inserted GFP at the C terminus of Dally. Under both, reducing and non-reducing conditions I observed the fragments of the predicted sizes (data not shown). I conclude that the insertion of the GFP tag does not cause additional processing of either protein.

Thus, this data suggests that Dally and Dally like are present in wing imaginal discs in multiple forms. Dally protein exists in at least three different forms; as a Dally-FL, as a Dally-CR and as a Dally-FLP (Figure 27D). Dally-like is likely to be present in at least two forms, as Dally-like-FL and as a Dally-like-FLP (Figure 27D). This data further suggests that Dally is proteolytically processed to a lesser extent than Dally-like, as a majority of the protein exists in the imaginal discs in its full-length size form. Most of the Dally-like protein is proteolytically cleaved to fragments that remain associated via disulfide bonds.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148
98
64
Dally:GFP
Dally-like:GFP
Dally:GFP
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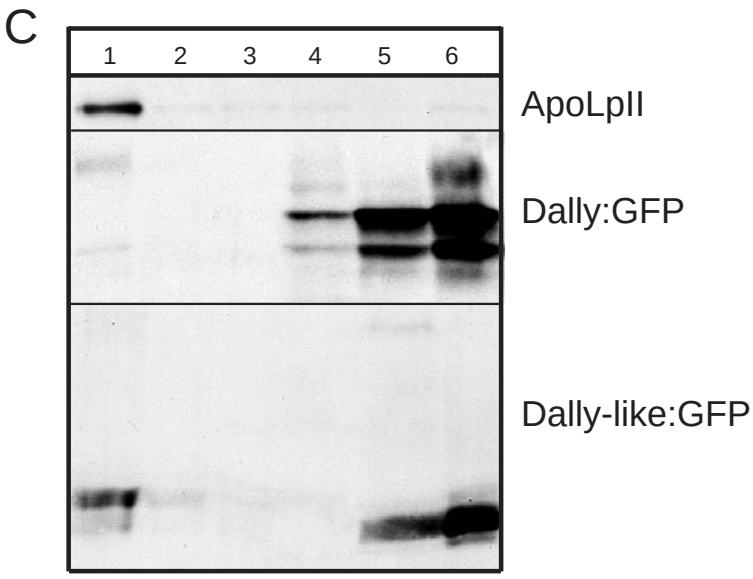
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ApoLpII
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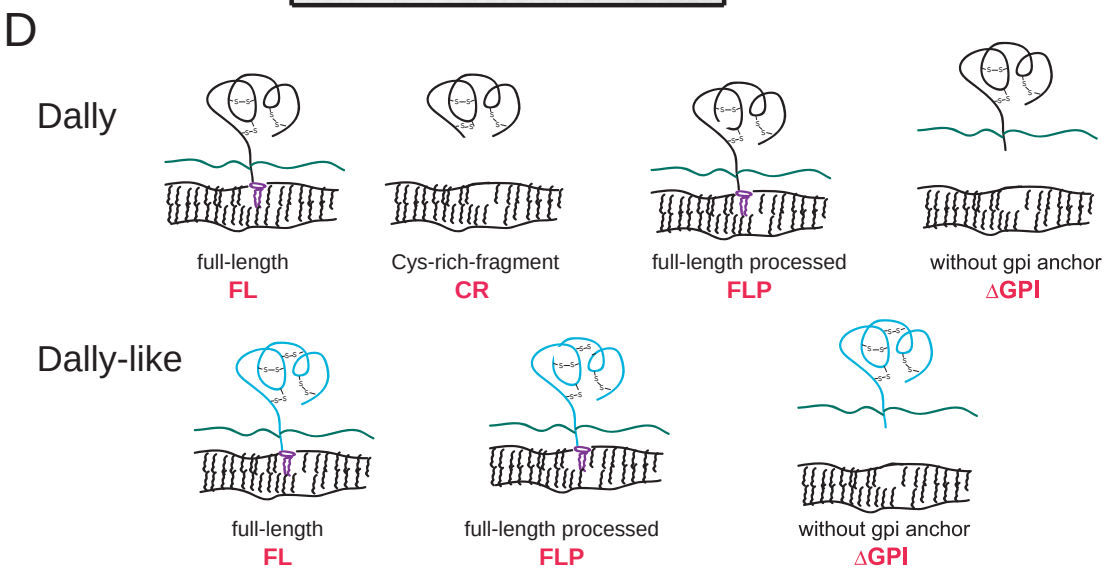
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FLP
without gpi anchor
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Dally and Dally-like associate with Lipophorin particles

Our imaging data suggested that Dally but not Dally-like is released from the expressing cells. However, we could not exclude the possibility that Dally-like also behaves similarly. To determine which of these isoforms of Dally and Dally-like are released from cells, I monitored their sedimentation properties in biochemical assays.

First, I examined behaviour of Dally and Dally-like in fractionation assay. I observed that the majority of Dally and Dally-like is present in the pellet (P120). However, a significant fraction of Dally fails to pellet at 120 000 *g* (Figure 27B, left). Strikingly, despite the fact that we do not observe Dally-like in any non-expressing tissue by confocal imaging, approximately 20% of Dally-like is also found in S120 (Figure 27B, right). Thus, a fraction of both, Dally and Dally-like is released from the cells.

To test whether the released forms of Dally and Dally-like associate with Lipophorin, I prepared isopycnic density gradients from dissected imaginal discs expressing Dally:GFP or Dally-like:GFP under the control of an *en*GAL4 driver. The fraction of the Dally-FL and the Dally-FLP co-migrate with Lipophorin in the isopycnic gradients. The data indicate that these forms are loaded onto Lipophorin particle via their gpi anchor. The Dally-CR and the fraction of the Dally-FLP migrate to the higher density fractions of the isopycnic gradient and behave like soluble proteins, suggesting that they might have lost their lipid anchorage. Interestingly, the fact that full-length size of Dally also migrates to the higher density fractions indicates that some Dally-FL is also released from cells without binding to Lipophorin (Figure 27C). Because other gpi-anchored proteins do not bind Lipophorin when they are cleaved off their lipid anchorage, I named these isoforms of Dally-FL and Dally-FLP that do not associate with Lipophorin as Dally-ΔGPI forms.

Figure 27: Dally and Dally-like are present in imaginal discs in multiple forms. A) Western blots from dissected discs expressing Dally:GFP or Dally-like:GFP using *en*GAL4 driver run under reducing (left) or non-reducing (right) conditions. **B)** Post nuclear supernatants from dissected imaginal discs expressing Dally:GFP or Dally-like:GFP using *en*GAL4 driver were centrifuged at 120,000*g* for three hours. Western blots of supernatants and the indicated proportion of pellets were probed with anti-GFP antibody. **C)** Western blots of fractions from density gradients prepared from dissected discs expressing Dally:GFP or Dally-like:GFP using *en*GAL4 driver and probed with anti-ApoLII or anti-GFP antibody (together with Ch. Eugster). **D)** Schematic drawing of different forms of either Dally or Dally-like that are present in imaginal disc tissue (core protein-black, Dally or blue, Dally-like; gpi-anchor-violet; HS side chains-green).

Similarly, the Dally-like-FLP co-migrates with Lipophorin to the low density fraction of isopycnic gradient. However, we never observed the Dally-like-FL in this fraction. A fraction of both, the Dally-like-FL and the Dally-like-FLP migrate to the higher density fraction of the isopycnic gradient, indicating they are also released from the cells in non-Lipophorin associated form (Figure 27C); I named them as Dally-like-ΔGPI forms.

Thus, I demonstrate that both Dally and Dally-like exist in the wing imaginal disc tissue in multiple forms (Figure 27D). They can be present as proteolytically cleaved fragments or as cleaved fragments that remain associated via disulfide bridges. Furthermore, while some forms associate with Lipophorin particles, presumably via a gpi anchor, other forms do not bind Lipophorin, suggesting that they have been cleaved off their lipid moiety. The results further indicate that beside the plasma membrane forms of Dally and Dally-like, several other forms are present in the developing wing epithelium such as the Lipophorin associated forms that can be transported across the tissue or as the soluble forms that can exert its function on broad field of cells. It is intriguing to speculate that apart from an autonomous role of Dally and Dally-like at the plasma membrane, they might be responsible for other non-autonomous effects. Such a high degree of capacity of these proteins puts another level of complexity of their role in morphogen trafficking.

3. DISCUSSION

Here, we establish the principle that lipid-linked proteins of the exoplasmic face of the membrane bilayer associate with Lipoprotein particles. These include a wide variety of gpi-linked proteins with diverse functions, as well as the lipid-linked morphogens Wingless and Hedgehog. Lipid moieties tether Wingless and Hedgehog to the membranes. However, these molecules are able to travel through the developing epithelia to induce target gene expression many cell diameters from their source of production (Bradley, R.S. *et al.* 1990; Papkoff, J. *et al.* 1990; Porter, J.A. *et al.* 1996; Reichsman, F. *et al.* 1996; Pepinsky, R.B. *et al.* 1998). Gpi-anchored proteins are also released and transported between cells with their gpi anchor intact (Ilangumaran, S. *et al.* 1996). However, the mechanisms allowing long-range dispersal of lipid-linked proteins is not understood. The finding that these proteins exist in both membrane-tethered and Lipophorin-associated forms suggests that their reversible binding to Lipophorin particles is a plausible mechanism for intercellular transfer. The fact that lowering Lipophorin levels in *Drosophila* larvae resulted in impaired Wingless and Hedgehog signaling further supports this idea.

Chapter 3.1. What are argosomes?

We initially proposed that lipid-linked morphogens travel through the epithelium on carriers named argosomes. We hypothesize that argosomes were exosome-like vesicles with an intact membrane bilayer and that lipid-linked morphogens must assemble on these particles in order to be secreted by producing cells. Because specific proteins can be sorted and transported on the exosomes between cells, we speculated that exosomes could facilitate the spread of membrane bound morphogens. Here, we established that *Drosophila* wing imaginal disc cells are capable of producing exosomes. Next, we used biochemical assays to show that lipid-linked proteins do not behave like

the exosomal marker CD63:GFP. Furthermore, we do not observe any significant colocalization between CD63:GFP-labeled exosomes and lipid-linked morphogens. Thus, we conclude that it is unlikely for the lipid-linked morphogens Wingless and Hedgehog to associate with the exosomes. However, the fact that the cells of the wing epithelium produce exosomes makes it intriguing to speculate that other membrane-bound ligands such as Boss or Notch (Cagan, R.L. *et al.* 1992; Klueg, K.M. *et al.* 1999; Parks, A.L. *et al.* 2000) are internalized on exosomes by the receiving cells.

What are argosomes? The model presented in this thesis differs significantly from our concept of argosome production. We first used the biochemical assay to show that lipid-linked proteins are present in two forms; membrane-tethered form and released form that retains its lipid moiety. The latter group associates with the *Drosophila* lipoprotein, Lipophorin, and we refer further to that form as Lipophorin-associated form. Lipophorin is produced by the fat body cells and is transported through the hemolymph to the peripheral tissues, including the wing imaginal discs. Hence, argosomes are not produced by wing imaginal disc cells. Instead, argosomes are exogenously derived Lipophorin particles that associate with lipid-linked proteins in the wing epithelium where they facilitate their transport across long distances.

Thus, in addition to their well-established role in lipid transport, we assign a new function to the Lipophorin particles that is to transfer lipid-linked proteins, including signaling and regulatory molecules, throughout the developing organism.

Chapter 3.2. Lipophorin promotes long-range movement of Wingless and Hedgehog

We used several biochemical assays to characterize the nature of argosomes. We showed that argosomes are fat body derived Lipophorin particles and that lipid-linked proteins associate with them in wing imaginal disc tissue.

Using RNA interference, we established a functional link between lipid-linked morphogens and Lipophorin particles. By lowering the Lipophorin levels, Hedgehog and Wingless distribution was significantly altered. Hedgehog accumulated to an abnormally

high level in cells near the source of its production, suggesting that Hedgehog was not capable of spreading across longer distances. In addition, the range over which Hedgehog normally moves is restricted by Patched-mediated endocytosis. In the wing imaginal discs from Lipophorin RNAi larvae, accumulated Hedgehog co-localized with Patched in endosomes that indicates that it was more efficiently sequestered by Patched. Our data showed that extracellular Wingless distribution in Lipophorin RNAi discs was also strongly impaired. Wingless was confined to the producing cells straddling the D/V boundary and did not disperse further away.

In addition to restricted long-range movement of both lipid-linked morphogens, we showed that both Hedgehog and Wingless long-range signaling is inhibited. However, the short-range target genes are expressed normally and seemed to be unaffected.

Thus, these data indicate that Lipophorin promotes long-range dispersal of Hedgehog and Wingless. The fact that only long-distance but not short-distance movement and signaling is perturbed, raises the possibility that lipid-linked morphogens might spread by two independent processes; one controlling the movement over short distances, the other one over long distances. The mechanism supporting the spread across long distances is Lipophorin dependent.

Chapter 3.3. How do lipid-linked morphogens associate with Lipophorin particles?

Here, we introduce the novel mechanism of Lipophorin dependent dispersal of lipid-linked morphogens. However, we do not show how these morphogens become associated with Lipophorin particles. There exist at least two possibilities how morphogens might be loaded onto Lipophorin particles.

Extraction and re-insertion

The data that Hedgehog and Wingless distribution in the receiving tissue is altered in Lipophorin RNAi discs is consistent with the idea that Lipophorin is continuously required for cell-to-cell transfer of lipid-linked morphogens. In this model, lipid-linked morphogens are passively transferred from the plasma membrane of one cell to that of adjacent one by subsequent steps of extraction and re-insertion without aid of any receptor or any regulatory protein. Association of lipid-linked morphogens with Lipophorin would be in this case reversible.

Lowering Lipophorin levels resulted in massive Hedgehog accumulation. The model of extraction and re-insertion predicts that at the low levels of Lipophorin, Hedgehog should spend longer time in the plasma membrane before becoming associated with Lipophorin. This should slow its rate of transfer and increase the probability of being endocytosed by Patched 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.

The fact that Wingless travels shorter distances in Lipophorin RNAi discs than in wild type ones, also favours the idea that Lipophorin is required for the cell-to-cell transfer. In this case, lowering the Lipophorin levels would increase the time that Wingless spends on the membrane and would be subsequently lead to the higher rate of Wingless endocytosis. The fact that we do not detect any change in the intracellular distribution of Wingless positive endosomes in the receiving cell suggests very efficient degradation of Wingless in lysosomes. All these predictions are consistent with our observations.

In line with these predictions, several lipoproteins, so called exchangeable lipoproteins, can associate with the lipidic particles only temporarily. This dissociation and re-association can occur in the plasma membrane or extracellular space (Shelness, G.S. *et al.* 2001). Thus, one possible model is that reversible loading of Hedgehog and Wingless onto Lipophorin particles facilitates their transfer from the plasma membrane one cell to that of the next one. Moreover, the lipid modifications of the proteins

themselves allow their transfer from one membrane to another. It would be intriguing to investigate whether Lipophorin particles can alter the half-life that lipid-modified morphogens spend associated with the donor membranes and thus mediate the release of lipid-linked morphogens from the producing cells.

Recycling or re-assembly

Another possible mechanism for the binding of lipid-linked morphogens to Lipophorin is that they are selectively loaded on a Lipophorin particle intracellularly. In this model, Lipophorin particle would be endocytosed by receptor-mediated endocytosis and targeted to the recycling endosomes. Here, it could associate with lipid-linked morphogens and then be recycled and exocytosed back to the extracellular space. One line of evidence supporting this idea, is that insect Lipophorin acts as a reusable shuttle, meaning that is endocytosed and recycled rather than degraded upon uptake (van der Horst, D.J. *et al.* 2002; Van Hoof, D. *et al.* 2002). Furthermore, our data suggest that Wingless and Hedgehog can be endocytosed either alone, or in the context of a Lipophorin particle.

Additionally, Lipophorin particles are endocytosed and can be re-assembled by the wing imaginal disc cells. We showed that ectopically expressed tagged ApoLII can be incorporated into the Lipophorin particles produced by imaginal disc cells. The observation that wing imaginal discs do express mRNA encoding Microsomal Triglyceride Transfer Protein, a lipoprotein assembly enzyme, leads us to speculate whether the association of lipid-linked morphogens with Lipophorin might even occur in the biosynthetic pathway. Interestingly, upon uptake, Lipophorin-Alexa488 particles localize to internal structures that are very reminiscent of endoplasmic reticulum. Although, we do not have a direct evidence for this model, it is tempting to hypothesize that Lipophorin coming from the fat body enters the endoplasmic reticulum of wing imaginal disc cells, in order to be re-lipidated and loaded with lipid-linked proteins into a new particle.

Whether, these models are feasible with the long-distance dispersal of lipid-linked proteins, will be the subject of further studies.

Chapter 3.4. Lipophorin-associated morphogens endocytosis and LRP receptors

The uptake of vertebrate LDL particles was extensively studied in the context of LDL receptor-mediated endocytosis. Intriguingly, the LDL receptor related proteins (LRP) Arrow and Megalin, have been implicated in Wingless and Hedgehog signaling pathways. Analyses of Arrow function have shown that it acts as a co-receptor for Wingless ligand and its activity is required for receiving cells to transduce the Wingless signal (Tamai, K. *et al.* 2000; Wehrli, M. *et al.* 2000). Another member of LRP receptor family, Megalin, has been implicated in Hedgehog endocytosis. It has been shown that Sonic hedgehog binds to Megalin with high affinity and that Megalin efficiently endocytoses Sonic hedgehog ligand (McCarthy, R.A. *et al.* 2002). Despite their function is essential for proper Wingless and Hedgehog signaling, their exact role in regulating these pathways is poorly understood.

Our finding that Wingless and Hedgehog associate with Lipophorin particles is consistent with the idea that LRP receptors might be implicated in endocytosis of lipid-linked morphogens in the context of a Lipophorin particle. On the other hand, it is also possible that these receptors might be involved in recycling Lipophorin-associated form of Wingless and Hedgehog.

Lipophorin particles are endocytosed throughout the whole wing pouch and Arrow and Megalin are not the only LDL receptor related proteins encoded in the *Drosophila* genome (Culi, J. *et al.* 2003). It would be interesting to further investigate the role of these proteins in the embryonic patterning and development.

Chapter 3.5. Lipophorin can interact with HSPGs during its spread

Many questions remain as to how the spread and the cell interactions of the Lipophorin particles are regulated. Clearly, heparan sulfate proteoglycans are essential for the movement of Hedgehog and Wingless into receiving tissue (The, I. *et al.* 1999) (Takei, Y. *et al.* 2004) (Han, C. *et al.* 2004). Despite the central importance of HSPGs to Wingless and Hedgehog signaling, the cell biological mechanisms they control are not completely understood.

Interestingly, heparan sulfate binds to vertebrate lipoprotein particles. It has been reported that HSPGs play a critical role in enhanced LDL uptake, not only in the initial sequestration but also as an integral component of HSPG-LRP pathway (Mahley, R.W. *et al.* 1999) (Camejo, G. *et al.* 1998). Furthermore, heparin antagonizes Megalin-dependent endocytosis of Sonic hedgehog, indicating that HSPGs might indeed act as co-receptors of LRPs (McCarthy, R.A. *et al.* 2002). In addition, HSPGs appear to function alone as receptors for specific isoforms of apolipoprotein E (Mahley, R.W. *et al.* 1999). Intriguingly, it has been shown that heparinase treatment of imaginal discs results in the depletion of Wingless within receiving cells as well as from producing cells (Greco, V. *et al.* 2001). Thus, it is possible to speculate that HSPGs regulate morphogen movement or sequestration via binding to Lipophorin. Alternatively, HSPGs might function differently in the binding Lipophorin-associated form of morphogen in the producing cells comparing to the receiving cells.

Conversely, we find many HSPGs, Dally and Dally-like on Lipophorin particles themselves. Thus, Lipophorin particles might act as carriers not only of signaling molecules but also regulatory proteins. These associated proteins may have the potential to modulate the cellular affinities or trafficking properties of Lipophorin particles and the morphogens they carry.

Chapter 3.6. Cholesterol and Hedgehog transport: a possible link

Cholesterol is crucial for proper Hedgehog signaling and has the potential to modulate the activity of the Hedgehog pathway at many different points (Porter, J.A. *et al.* 1996; Mann, R.K. *et al.* 2000; Jeong, J. *et al.* 2002; Cooper, M.K. *et al.* 2003). The modification of Hedgehog with a cholesterol moiety is required for its spatial distribution as well as for its signaling properties (Porter, J.A. *et al.* 1996; Lewis, P.M. *et al.* 2001).

Spatial distribution of Hedgehog and cholesterol

One way that cholesterol modulates the spatial distribution of Hedgehog is by acting as a targeting signal to localize Hedgehog to lipid rafts. Lipid rafts are thought to assemble and concentrate the ligands on the membranes and thus facilitate their multimerization before the release from the membranes (Zeng, X. *et al.* 2001). Only the cholesterol unmodified form of Hedgehog can propagate through the cells missing HSPGs (Bellaiche, Y. *et al.* 1998; The, I. *et al.* 1999). Thus, cholesterol plays a role in the extracellular trafficking of cholesterol-modified Hedgehog and in its distribution to the receiving cells.

In addition, the cholesterol-modified form of Hedgehog present in producing cells is thought to interact with Dispatched, a transmembrane protein with a sterol-sensing domain. Dispatched is specifically required for releasing the lipid-linked Hedgehog (Burke, R. *et al.* 1999). Our data showed that lowering Lipophorin levels impair Hedgehog trafficking over long distances. However, Hedgehog release from producing cells is not affected in Lipophorin RNAi discs. Thus it is possible to speculate that Dispatched might function independently of Lipophorin and releases Hedgehog that spread does not require Lipophorin association.

Cholesterol and Patched-Smoothened activity

Patched (Ptc), another transmembrane protein with a sterol-sensing domain, plays a role in the receiving cells in Hedgehog sequestration, and thus limits its mobility (Nakano, Y. *et al.* 1989; Tabata, T. *et al.* 1994; Torroja, C. *et al.* 2004). Patched acts as a Hedgehog receptor and functionally antagonizes the activity of Smoothened (Smo), another component of the Hedgehog receptor and transduction complex. In the absence of Patched, Smoothened constitutively activates the Hedgehog pathway. Sequestration of Hedgehog by Patched is thought to release Smo from the Ptc-Smo complex and thus induce the Hedgehog transduction pathway (Chen, Y. *et al.* 1996; van den Heuvel, M. *et al.* 1996; Chen, Y. *et al.* 1998). However, the mechanism as to how does Ptc release Smo have never been described.

Interestingly, the activity of Smoothened is particularly sensitive to cholesterol depletion. Inhibition of cholesterol biosynthesis downregulates Smoothened function in the Hedgehog-receiving cells (Cooper, M.K. *et al.* 2003). In addition, it has been reported, that Patched can act as a pump for the small inhibitory molecules, similar to plant-derived teratogen cyclopamine, that specifically restrain Smoothened function (Taipale, J. *et al.* 2000). In contrast, the idea that Patched is capable of pumping small molecules raises the possibility that such a molecule could in fact be promoting Smoothened function instead of inhibiting it. Could cholesterol be this molecule? In such a scenario, in the absence of Hedgehog, Ptc inhibits Smo by depleting cholesterol in the vicinity of Smo. Upon binding of Hedgehog that is associated with a Lipophorin particle carrying cholesterol, Ptc pumping activity is either restricted or the local levels of cholesterol increase such that the basal activity of Ptc cannot inhibit the Smo for a certain period of time.

Transport of cholesterol and Hedgehog signaling

Perturbations of cholesterol homeostasis phenocopy Hedgehog signaling defects. Patients with Smith-Lemli-Opitz Syndrome lack the activity of 7-dehydrocholesterol reductase and as a consequence have abnormally low levels of serum cholesterol (Salen, G. *et al.* 1996). A low percentage of these patients displays holoprosencephaly, a phenotype typical for Hedgehog loss of function (Chiang, C. *et al.* 1996). A similar developmental defect was observed in mouse mutant embryos lacking apolipoprotein B (Farese, R.V., Jr. *et al.* 1995), in which transport of cholesterol is impaired. However, this latter observation is also consistent with our finding that lipoproteins associate with Hedgehog and other lipid-modified proteins.

Thus, it is possible that changes in the level of cellular cholesterol play a functional role in regulating the activity of Hedgehog pathway. Here we show that Hedgehog interacts with a particle that delivers sterol to the cells. We speculate that internalization of Hedgehog is linked to the sterol uptake via Hedgehog interaction with Lipophorin. Thus, Hedgehog on Lipophorin particle might help to regulate the amount of sterols and other lipids that particle delivers to the cells or might effect the intracellular lipid trafficking. On the other hand, interaction of Hedgehog and Lipophorin together with other proteins like HSPGs might regulate the level of Hedgehog available to the developing tissues.

Lipid delivery, growth and Hedgehog

A principal role of lipoprotein particles is to transport lipids, or in other words nutrition, to the peripheral tissues. We observed that perturbing the lipid homeostasis by reducing the Lipophorin levels lead to the enormous accumulation of neutral lipids in the gut and to the lowering of the amount of lipid droplets, the lipid storage organelles, in the fat body and imaginal discs of Lipophorin RNAi larvae. This resulted in the reduction of growth rate and the narrowing of the range over which could lipid-linked morphogens

Wingless and Hedgehog signal. Interestingly, growing the animals in the absence of lipids, stalls larval growth, but never causes a reduction in Hedgehog signaling.

Morphogens do not only act as signaling molecules that organize the patterning of the tissue, but they also control the rate of proliferation and growth of the organism. It is not very well understood how the shape and size of the developing organ is controlled. Our observation that lipid-linked morphogens are associated with the Lipophorin particles allows us to speculate that one way how these morphogens can achieve the fine regulation of growth and proliferation is to control the particular amount of lipids that Lipophorin particle brings to the cell. Thus, this fact allows us to link nutrition, growth and signaling during development.

4. MATERIALS AND METHODS

Fly stocks

The wild type Oregon K flies, *ap*Gal4, *ptc*Gal4, *en*Gal4, *Tub*Gal4, *hs*-flippase fly stocks are available from the Bloomington stock center. The *wg*Gal4 driver line was a gift from Steve Cohen, *Adh*Gal4 (Britton, J.S. *et al.* 2002), *C765*Gal4 (Gomez-Skarmeta, J.L. *et al.* 1996), Transgenic lines: UAS:GFPgpi (Greco, V. *et al.* 2001), UAS:secreted GFP (Entchev, E.V. *et al.* 2000), UAS:cytoplasmic GFP (Bloomington), UAS:Wingless:GFP was a gift from Jean-Paul Vincent, UAS:CD8:GFP (Lee, T. *et al.* 1999), UAS:PH:GFP (Britton, J.S. *et al.* 2002), UAS:Hh:HA, UAS:ApoLII:myc, UAS:ApoLII:GFP (Sprong, H.), *lipophorin* dsRNA constructs: HlipA4B in pUhr and FLA1 in pFRIPE (Marois, E.) UAS:CD63:GFP, UAS:Dally:GFP, UAS:Dally-like:GFP, tubP:Rab5:CFP, were constructed in the lab of Suzane Eaton.

Western blot of larval, discs or fat body extracts

For larval extracts, larvae were collected in an eppendorf tube. A drop of ice-cold PBS containing protease inhibitors (ROCHE Complete (Roche) or CLAP: Chymostatin, Leupeptin Hemisulfate salt, Antipain Hydrochlorid, Pepstatin A (25 mg) (Sigma-Aldrich) dissolved in 2,5 ml of DMSO) was added and larvae were homogenized using biovortexer and tight fitting pestle. The volume was usually increased to 80 μl; when not indicated, reducing Laemmli sample buffer was added to the sample. For imaginal discs or fat body extracts, the third instar larvae were dissected in ice-cold PBS plus protease inhibitors. In most cases the imaginal discs from 10 larvae were collected in eppendorf tubes on ice; to compare amount of ApoLI and II in fat body to the discs, fat bodies and discs from 4 larvae were dissected. Samples were then boiled in the Laemmli sample buffer and analysed by SDS-PAGE and Western blotting. Primary antibodies were used

as follows: anti-ApoLI, 1:1000; anti-ApoLII, 1:1000; anti-GFP (Santa Cruz), 1:750. Secondary HRP-conjugated antibodies (Jackson) were usually used in the dilution 1:5000.

Fractionation

5 ml of larvae from wild type or transgenic lines expressing different fusion constructs under the control of GAL4:UAS system were homogenized in a tight fitting douncer using loose and tight pestle, with 5 ml of TNE (100 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.2 mM EGTA) plus protease inhibitors on ice at 4°C. Homogenate was centrifuged for 10 minutes at 1000 *g* yielding pellet and post-nuclear supernatant. The post-nuclear 1000 *g* supernatant was centrifuged for 3 hours at 33,600 rpm (120,000 *g*) at 4°C in a SW40Ti rotor (Beckman) generating a pellet (P120) and supernatant (S120). The dissected imaginal discs from 100 larvae were homogenized and centrifuged similarly using TLA-55 rotor (Beckman) at 50,000 rpm. For analyzing P120/S120, P120 was re-dissolved in TNE to the equal volume of S120; the samples were CH_3Cl/MeOH precipitated and either 10% or 100% of the sample was loaded on SDS-PAGE and further analyzed by Western blotting.

For isopycnic density centrifugation, the S120 was mixed with the solution of 0.5 g/ml KBr (Merck) in dH_2O to the final concentration of 0.35 g/ml and centrifuged for 48 hrs at 40,000 rpm or 50,000 rpm (285,000 *g*) at 10°C in the SW40Ti rotor or TLS-55 rotor (Beckman) in Ultra-Clear tubes $^{9}/_{16}$ x 3 ^{¾} in. or $^{7}/_{16}$ x 1 $^{3}/_{8}$ in. (Beckman), respectively. Equal fractions were carefully taken from gradients and CH_3Cl/MeOH precipitated. Samples were analyzed on SDS-PAGE followed by Western blotting.

Primary antibodies were used in following dilutions: anti-GFP (Santa Cruz), 1:750; anti-myc (Santa Cruz) 1:1000; anti-HA (Santa Cruz), 1:1000; anti-ApoLII 1:1000; anti-Wg (Strigini, M. *et al.* 2000), 1:200; anti-Hh 1:500; anti-Fasciclin I (Hortsch, M. *et al.* 1990), 1:50; anti-Connectin (Nose, A. *et al.* 1992), anti-Klingon (Butler, S.J. *et al.* 1997) and

anti-Acetylcholineasterase (Incardona, J.P. *et al.* 1996). Secondary HRP-conjugated antibodies (Jackson) were used in dilution 1:5000.

Immunoprecipitation

Protein A-sephacryl CL4B beads (Amersham) were washed 5 times with 10 volumes of PBS, 0.5% BSA. Beads were pelleted in between the washes at max 2000 *g* for $\pm$ 5 min. Beads were then incubated with antibodies overnight at 4 ^{o}C on head-over-head rotating wheel; usually 0.5 ml of antibody per 1ml beads was used. After overnight incubation, beads were washed 5 times with 10 volumes of PBS, 0.5% BSA. S120 was prepared as described above. To pre-clear S120, supernatant was incubated with the washed beads for at least 3 hours at 4 ^{o}C on a rotating wheel. Beads were pelleted and supernatant was incubated with antibody-coupled beads for 3 hours to overnight at 4 ^{o}C on the rotating wheel. Beads were pelleted and supernatant was saved for further analysis. Beads were washed with 10 volumes of PBS, 0.5% BSA, then PBS, and eluted using Laemmli sample buffer or RIPA buffer (150 mM NaCl, 2 mM EDTA, 100 mM Tris-Cl pH 8,3, 0.5% w/v Nonidet –P40, 0.5% w/v sodiumdeoxycholate and 0.1% w/v SDS). With Laemmli sample buffer, beads were boiled under reducing conditions for 10 min at 90 ^{o}C. Using RIPA buffer, beads were shaked for 10 min at 4 ^{o}C then pelleted and supernatant avoided of any beads was carefully transferred to a new eppendorf tube. Samples were then analysed on SDS-PAGE followed by Western blotting. Primary and secondary antibodies were used as indicated above.

Antisera

(Sprong, H. and Thiele, Ch.)

Hedgehog: Rabbits were immunized with synthetic peptide LEGVIRRDSPKFKDL (Hedgehog amino acids 123-138), conjugated to keyhole limpet hemocyanin

(Eurogentec, Seraing, Belgium). Antibody was affinity-purified on peptide-conjugated affigel-15 columns (Biorad, Hercules, CA).

Apolipophorin I and II: DNA encoding amino acids 195-509 or 891-1070 (parts of ApoLII and ApoLI, respectively) was amplified from incomplete cDNA clone GH18004 (Resgen) and cloned into pQE30. His-tagged fusion proteins (Qiagen, Valencia, CA) were used to immunize rats or rabbits.

Expression constructs

CD63:EGFP: CD63:EGFP encoding sequence with incorporated NotI sites at 5' and 3', respectively, was amplified using PCR from pEGFP-C1-bos (gift from Gillian Griffiths) and cloned into NotI site of pUAST expression vector (Brand, A.H. *et al*. 1993).

ApoLII:myc (Sprong, H.): ApoLII was amplified and ligated into pUAST between the EcoRI and XhoI sites. A myc9-epitope or GFP was inserted in frame just before the stop-codon.

RNA interference

(RNAi system was cloned and developed by Eric Marois.)

RNA-interference was induced by expressing inverted repeats derived from two different regions of the pro-apolipophorin cDNA, one 3' of the open reading frame (607 bp ending 47 bp from stop codon), and one corresponding to the first 500 coding base pairs starting at ATG. The first fragment was amplified and inserted into pENTR2B (Invitrogen, Leek, The Netherlands). Using the Gateway system, it was then inserted 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. Heat shock-mediated excision of the cassette allows GAL4-driven expression of a direct inverted repeat.

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

Flies containing Lipophorin RNAi constructs were crossed with others harbouring heat shock-inducible Flippase and one of several GAL4 drivers. After 5 days at 25°C, larvae were heat shocked for 90 minutes at 37°C to remove the HcRed cassette. 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; here approximately 50% of the cells excise the HcRed cassette even when maintained at 18 °C, possibly due to the higher basal activity of the heat shock promoter in this tissue (see results).

Lipophorin dsRNA was expressed under the control of *Tubulin*GAL4 (ubiquitous), *Adh*GAL4 (fat body and part of the gut) or *C765*GAL4 (disc-specific). Expression with *Adh*GAL4 or *Tubulin*GAL4 was semi-lethal and produced identical larval phenotypes. Ran was usually induced using *Tubulin*GAL4 rather than *Adh*GAL4 because the viability of larvae in these crosses was better. No phenotype was ever observed when *lipophorin* dsRNA was expressed in imaginal discs.

Immunohistochemistry

Imaginal discs were dissected in PBS and collected in eppendorf tubes on ice. Afterwards they were fixed in 4% paraformaldehyde in PBS for 20 min and then permeabilized with 0.05 % Triton X-100 in PBS (PBT) twice for 10 min. The imaginal discs were then blocked three times for 15 min in PBT + 1mg/ml BSA + 250 mM NaCl, then incubated overnight at 4 ^{o}C with primary antibody in PBT + 1mg/ml BSA. Then they were washed twice for 20 min with PBT + 1mg/ml BSA and twice for 20 min with the blocking solution PBT + 1mg/ml BSA + 4% normal goat serum (NGS). The imaginal discs were then incubated for at least 2 hours in a dilution of the secondary antibody. The antibody was removed by washing three times for 15 min with PBT and three times for 15 min in PBS. Last, the imaginal discs were mounted with Prolong Anti Fade (Molecular Probes).

Primary antibodies were diluted as follows: anti-Wg (Strigini, M. *et al.* 2000), 1:200; anti-Hh (Taylor, A.M. *et al.* 1993), 1:500; 1:100; anti-Ptc (Capdevila, J. *et al.* 1994), 1:50; anti-βGal (Promega Z378A), 1:100; anti-Col (Vervoort, M. *et al.* 1999), 1:200, anti-Ci 2A1 (The, I. *et al.* 1999), 1:10. Secondary antibodies: Alexa488-, Cy3- and Cy5-conjugates (Molecular Probes) were used in dilution 1:1000. Extracellular staining was done as described by Strigini and Cohen (Strigini, M. *et al.* 2000); anti-Wg antibody was used in the dilution 6:200. To compare wild type and Lipophorin RNAi animals, tissues were stained in parallel and imaged the same day under identical conditions with either LSM Zeiss 510 or Leica TCS SP2 confocal microscope.

Image Analysis

Hedgehog-positive spots in wild type and Lipophorin-RNAi discs were quantified in 10 confocal sections 1μ apart. Signal threshold was adjusted to 130 and images were despeckled using ImageJ. Grids were overlaid on the processed image and spots were counted manually. Similarly, the Hedgehog-positive spots were counted in the experiment where fluorescently labeled Lipophorin particles were added to the Lipophorin-RNAi discs. Here, just first two apical sections were included into the quantification.

We used ImageJ to quantify the Hedgehog signaling range in 5 projected apical sections of Col and Dpp stained discs. For each image, we determined pixel intensity along 10 lines centered at the AP boundary using Plot Profile and averaged them to obtain a plot for each disc. Average plots from 4 discs of each type were generated using Microsoft Excel. Distalless range in Figure 20, Ci range in Figure 22 and Patched staining in Figure 24 were quantified similarly.

Live imaging

Wing imaginal discs were dissected from third instar larvae in Grace's insect medium (Sigma) at room temperature. Live imaginal discs were then transferred onto glass slides into drops of medium delimited by a chamber of double-sided adhesive tape (Greco, V. *et al.* 2001). Imaginal discs were always oriented with the apical surface facing the cover slip. For the live imaging, the membranes were usually stained with a vital dye FM4-64 (Molecular Probes) diluted in Grace's 1:1000 from 16 mM stock solution in DMSO. Imaginal discs were imaged using either Leica TCS SP2 or Zeiss LSM 510 confocal microscope.

Red Dextran assay

Third instar larvae were dissected in Grace's insect medium (Sigma). To stain endocytic compartments, discs are then transferred to a drop of Red Dextran solution; 10,000 MW lysine fixable Red Dextran is diluted 1:10 in Grace's medium. For staining the early endosomes, discs are incubated in Red dextran for 10 min at room temperature. To stop the fluid phase uptake, discs are washed with ice-cold medium or PBS at least three times. The disc were fixed and stained as described in Immunohistochemistry.

LysoTracker assay

Third instar larvae were dissected in Grace's insect medium. To stain acidic endocytic compartments, discs are then transferred to a drop of 50 nM LysoTracker solution, which was diluted in Grace's medium from 1mM stock solution. For staining acidic endosomes, discs are incubated with LysoTracker for 30 min at 25 ^{o}C. The discs are then washed with medium at least three times and transferred to a chamber for live image acquisition.

Nile Red assay

(the assay was conducted by Suzanne Eaton)

Third instar larvae were dissected in Grace's insect medium. To stain intracellular lipid droplets, the discs are transferred to a drop of Nile Red solution, which was diluted 1:100 in Grace's medium from 100 μg/ml stock solution in acetone (Greenspan, P. *et al.* 1985). The Discs are incubated with the dye for 10 min at room temperature and then washed with medium at least three times and transferred to a chamber for live image acquisition.

Lipid Starvation

(the assay was conducted by Suzanne Eaton)

Eggs were collected on apple juice/agar plates + yeast for 24 hours, 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 seconds with 50% Na hypocholorite, and rinsed with sterile H_20. 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 0.3g chloroform extracted yeast autolysate (for lipid starvation), or 0.3g yeast (for lipid-fed controls).

Labelling Lipophorin with Alexa488

Lipophorin particles were fluorescently labeled with Alexa Fluor 488 (Molecular Probes) according to 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, pH7.4, 100 mM NaCl, 10% sucrose.

Incubation of dissected discs with Lipophorin particles

For experiments shown in Figure 15 and Figure 24, the imaginal discs were incubated at 29ºC with 50 g/ml Lipophorin particles for 20 minutes and 2 hrs, respectively. Based on 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 hemolymph. After incubation, the disc are washed three times in PBS, fixed and stained as described in Imunohistochemistry.

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