Release and Transport of the secreted signal encoded by *wingless*

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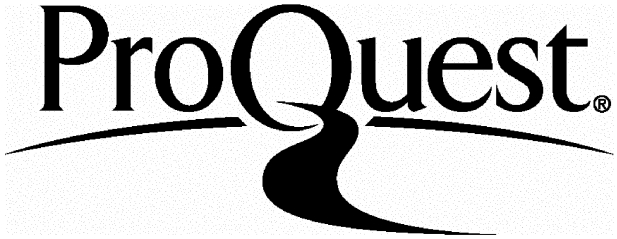

Abstract

One of the questions of outstanding interest is how a secreted protein such as Wingless is able to travel a distance in order to pattern the tissue away from its source.

The Wingless protein of *Drosophila* (Wg) is a member of the Wnt family of secreted glycoproteins. In the embryonic epidermis it is expressed in one row of cells per segment and acts asymmetrically from the source, while in imaginal discs the spread of Wingless is symmetrical and over a greater distance. The mechanism of Wingless transport is still poorly understood although it has been postulated that Heparan Sulfate Proteoglycans could be involved. HSPGs are abundant at the cell surface and consist of a protein core to which sulfated sugar chains are attached. It has been suggested that Wingless binds to these sugar chains, on secreting or receiving cells. I found that Wingless is mostly retained at the cell surface of secreting cells while very little is observable at the surface of receiving cells. HSPGs are responsible for this retention since when no functional proteoglycans are present, Wingless no longer accumulates at the surface of secreting cells. Interestingly, much Wingless is also lost from the secretory pathway in the absence of mature proteoglycans, suggesting a role for the HSPGs in secreting cells.

Although much Wingless remains associated with expressing cells, the signal must be released since it acts at a distance. The mechanism and form by which Wingless is released from secreting cells is not known so I set out to investigate this process and also to ask how it is influenced by HSPGs. Using insect cell lines, I found Wingless to be released to the supernatant of expressing cells in an insoluble and a soluble form. Preliminary characterization of the insoluble form suggests that is membrane bound.

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I dedicate this thesis to my parents for their support and friendship.

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Abbreviations

μg	micro gram
μM	micro Molar
β-gal	β galactosidase
APT-1	Acyl-protein thioesterase-1
Asn	Asparagine
BMP	Bone Morphogenetic Protein
Bp	base-pair
cDNA	complementary deoxyribonucleic acid
DAB	3,3' diaminobenzidine
DNA	Deoxyribonucleic acid
DOC	Sodium Deoxycholate
Ds	double stranded
DTT	DL- dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
ER	Endoplasmic reticulum
EtOH	ethanol
FGF	Fibroblast growth factor
Xg	Relative centrifugal field
g/ml	grams per mililitre
GAG	glycosaminoglycan
GFP	Green fluorescent protein
GlcA	Glucoronic acid
GlcNAc	N-Acetylglucosamine
GPI	Glycosyl phosphatidyl inositol
GTP	Guanidine triphosphate
HA	haemaglutinin
Hr	hour(s)
HRP	Horseradish peroxidase
Hs	Heat-shock
HS	Heparan Sulfate
HSPG	Heparan Sulfate Proteoglycan

Kb	Kilo base-pairs
kDa	Kilo Dalton
min	minute(s)
mRNA	messenger ribonucleic acid
MW	Molecular Weight
NDST	N-deacetylase/ N sulphotransferase
NP-40	Nonidet P-40
OD	Optical density
OPD	o-phenylenediamine
PAT	Palmitoyl acyltransferase
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PCR	Polymerase Chain Reaction
PG	Proteoglycan
PI-PLC	Phosphatidyl inositol- phospholipase C
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RNAi	RNA interference
Rpm	revolutions per minute
RT	Reverse Transcription
S	supernatant
S2	Schneider-2
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
secr	secreted
SFM	Serum free medium
Ss	single stranded
TCA	Tricloroacetic acid
TGF-β	Transforming Growth Factor β
TM	transmembrane
UDP	Uridine diphosphoglucose
v/v	volume/volume ratio
w/v	weight/volume ratio
WT	Wild-type

Chapter 1- Introduction

CHAPTER 1- INTRODUCTION

1.1 *wingless* and the Wnt family of genes

The Wnt family of genes encode for secreted glycoproteins that are involved in many processes during embryogenesis (reviewed by Wodarz and Nusse, 1998). Wnt signalling is also clearly implicated in cancer (reviewed by Taipale and Beachy, 2001).

The *Drosophila wingless (wg)* gene is the best-understood gene of this family and one of the first to be characterised. A mutation in *wingless* (wg^1) was first reported in 1973 (Sharma, 1973) and adult flies carrying this viable allele showed loss of the wing and duplication of the notum, giving the gene its name (Sharma and Chopra, 1976). Later, its function in segmentation was identified in a genetic screen for zygotic lethal mutations that affect larval cuticle pattern (Nusslein-Volhard and Wieschaus, 1980). The embryos bearing the *wg* mutation showed segmentation defects and an abnormal cuticle pattern, characteristic of segment-polarity mutations. *wg* was subsequently shown to be required for the maintenance of *engrailed* (*en*) expression, another segment-polarity gene (reviewed by DiNardo et al., 1994). Later in development, *wg* has a major role in the patterning and growth of larval imaginal discs.

All members of the Wnt family of genes show a high degree of homology and since *wg* was first identified nearly 30 years ago, nearly 100 Wnt genes have been found in species ranging from the nematode, C. elegans, to human (reviewed in Cadigan and Nusse, 1997; Wodarz and Nusse, 1998)

In *Drosophila* six other Wnt genes have been identified, although their function is still poorly understood. These are: DWnt2, DWnt3/5, DWnt4, DWnt6, DWnt8 and DWnt10 and of these only DWnt2, DWnt3/5 and DWnt 4 have been studied. DWnt2 seems to be involved in trachea development together with *wg* (Llimargas and Lawrence, 2001), in patterning of the male reproductive tract (Kozopas et al., 1998)

and in muscle patterning (Kozopas and Nusse, 2002). DWnt3/5 has been shown to have a role in axon guidance (Yoshikawa et al., 2003) and DWnt4 in cell motility (Cohen et al., 2002).

1.2 Wingless/Wnt Signalling Pathway

The Wnt proteins in vertebrates can act through three signalling pathways: the so-called "canonical" pathway, the planar polarity pathway and the Wnt/Ca^{2+} pathway. In *Drosophila*, Wg activates the canonical signalling pathway and it is not yet known if a *Drosophila* Wnt activates the planar polarity pathway. The Wnt/Ca^{2+} pathway has not been described in *Drosophila* (reviewed by Strutt, 2003).

1.2.1 The canonical signalling pathway

The Wg/Wnt pathway downstream of the receptor has been extensively studied. Briefly, binding of Wg to the receptors, the seven-pass transmembrane proteins Fz or Dfz2, and to Arrow, a single-pass transmembrane LDL-receptor related protein (LRP) homologous to LRP5 and LRP6 in vertebrates, at the cell surface leads to the accumulation and activation of Armadillo (Arm), a homologue of the vertebrate β-catenin. This requires the activity of *dishevelled* (*dsh)* and is negatively regulated by the kinase Shaggy/Zw3 (GSK3β). Accumulated Armadillo forms a complex with dTCF which translocates to the nucleus leading to the transcriptional activation of target genes (reviewed by Cadigan and Nusse, 1997) (Wehrli et al., 2000) (see Figure 1.1).

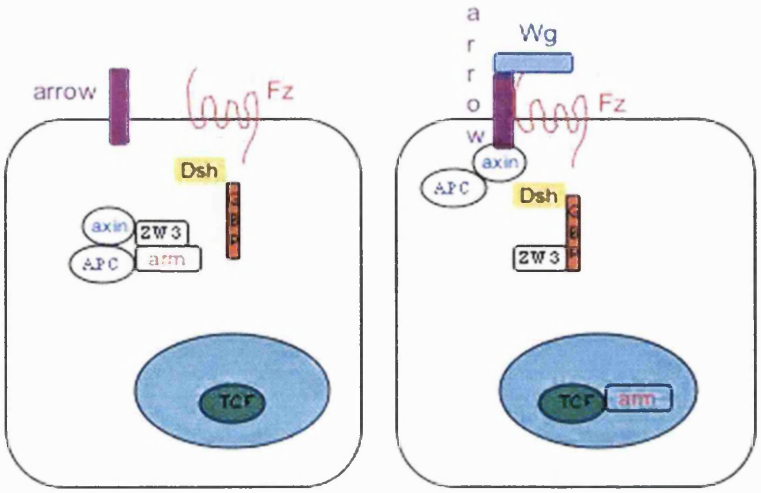


Figure 1.1 Simplified model of the wg signalling pathway.

In the absence of Wg/Wnt, Armadillo is bound to a complex with several proteins, such as Axin, APC and ZW3 that targets it to degradation. Upon Wg/Wnt binding to the receptors Fz and LRP/Arrow, Dsh binds to the ZW3 complex blocking Arm degradation and allowing it to accumulate in the cytoplasm. Accumulated Arm translocates to the nucleus, binds a complex that includes TCF and activates the target genes (diagram based on the two-state model in http://www.stanford.edu/~rnusse/wntwindow.html).

In vertebrates there are several molecules that act as inhibitors of signalling such as WIF (Hsieh et al., 1999), Frp (Rattner et al., 1997), Cerberus (Piccolo et al., 1999), Dkk (Glinka et al., 1998) and WISE (Itasaki et al., 2003). The first three seem to bind directly to Wnts, while the last two interact with LRP/Arrow. These molecules have not yet been identified in Drosophila, although the fly has a gene, *notum/wingful*, that codes for an inhibitor of *wg* activity that acts extracellulary (Gerlitz and Basler, 2002; Giraldez et al., 2002). This gene will be further described later.

1.2.2 The planar polarity pathway

Some proteins that are components of the canonical signalling pathway are also used in an alternative *arm*-independent pathway that specifies planar cell polarity (reviewed by Strutt, 2003). In vertebrates this pathway is activated by a Wnt ligand

(reviewed in Tada et al., 2002), although no Dwnt has been shown to be involved in the PCP pathway in *Drosophila*.

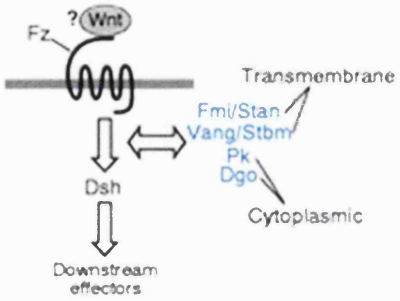


Figure 1.2 Planar polarity pathway

The downstream effectors include the small GTPases Rho and Cdc42 and the Jun N-terminal kinase (JNK). The proteins shown in blue are though to modulate the activity of the pathway by forming a multiprotein complex together with Fz and Dsh (adapted from Strutt, 2003)

Dsh is used for both canonical and planar polarity pathways so the discrimination between the two sets of downstream components is an important issue. It has been suggested that different domains of the Dsh protein have specific roles in the two pathways. The DEP domain is essential for planar polarity signalling and the DIX domain for the canonical signalling pathway (Axelrod et al., 1998; Boutros et al., 1998; Penton et al., 2002; Rothbacher et al., 2000).

1.3 Canonical wingless signalling and patterning of the embryonic epidermis

The ventral embryonic epidermis is a monolayer of cells decorated by a segmentally repeated pattern. Each segment is about 11 cells wide, of which 6 produce denticles, cuticular protrusions secreted by each cell, while the remaining cells produce naked cuticle (reviewed in DiNardo et al., 1994).

Patterning of the embryonic epidermis involves several steps. The first steps of embryonic segmentation involve the subdivision of the anterior-posterior axis through

a cascade of gap, pair-rule and segment-polarity genes (reviewed in DiNardo et al., 1994; Sanson, 2001; Wolpert et al., 1998). The pair-rule genes initiate, within each parasegment, the expression of key segment-polarity genes: *wg* in one row of cells and *hh/en* (Hedgehog, Hh, is secreted by the en expressing cells) in the adjacent posterior row. The segment-polarity genes are expressed in 14 stripes, each corresponding to a parasegment (Figure 1.3).

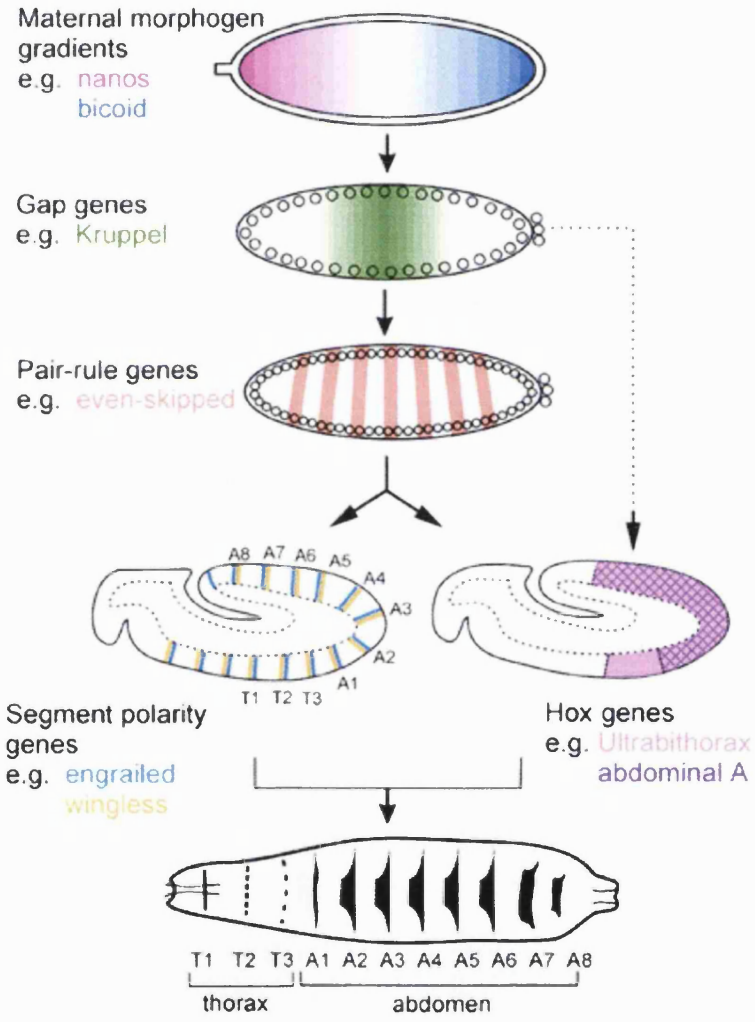


Figure 1.3 Patterning of the *Drosophila* embryo

A cascade of maternal and zygotic genes is activated in the syncitial embryo and divides the ectoderm in smaller domains. After the activation of the pair-rule genes the embryo cellularizes and gastrulates. This set of genes will in turn activate the expression of the Hox genes and the segment polarity genes

and together these act in the differentiation of each segment of the future larva. A1 to A8 represent the abdominal segments 1 to 8 and T1, T2 and T3 represent the three thoracic segments (Sanson, 2001).

To date all analysed segment-polarity genes seem to be positive or negative regulators of either the wg or hh pathways (reviewed by Perrimon, 1994).

After activation of wg, hh and en, subsequent patterning events can be divided in two phases: the stabilization and the fate specification phase. Initially at stage 9-10, in the stabilisation phase, wg is required for the expression of hh/en and reciprocally hh is required to maintain wg expression (DiNardo et al., 1988; Hidalgo and Ingham, 1990; Martizez Arias et al., 1988). These interactions stabilize and maintain the parasegmental boundary, which is crucial for the correct patterning of the larval cuticle.

Later, at the end of stage 10, en expression becomes independent of wg expression and the distribution of the Wg protein, which was symmetrical, becomes now asymmetrical (Sanson et al., 1999). It has recently been shown that the asymmetry of Wg is partly due to the endocytosis and degradation of the protein posteriorly to the source (Dubois et al., 2001) (Figure 1.4).

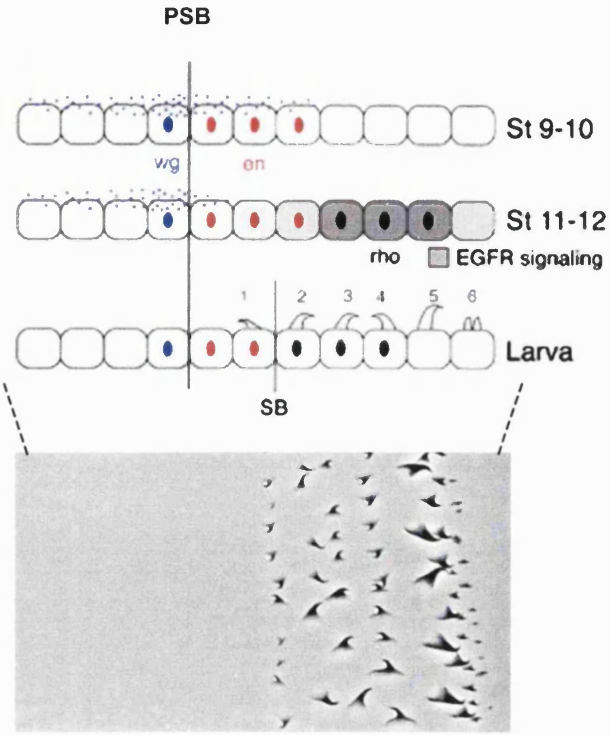


Figure 1.4 Specification of naked cuticle by wg

Wg is expressed in one row of cells per segment and the protein is initially distributed symmetrically from the source. At late stage 10 Wg spreads asymmetrically 3 cells anteriorly to the source and 1 cell posteriorly. In this domain it specifies naked cuticle, as shown. PSB and SB represent, respectively, the parasegmental and segmental boundaries (adapted from Dubois et al., 2001)

This asymmetry in the range of Wg corresponds to an asymmetry in wg signalling activity. This is manifested, for example, in the asymmetric specification of naked cuticle. *wg* specifies naked cuticle over a range of 3 to 4 cells at the anterior and only over 1 cell posteriorly (Alexandre et al., 1999; Sanson et al., 1999).

Due to its easy read out of wg signalling, the ventral embryonic epidermis provides a good system to study short-range transport.

1.4 Role of *wingless* in the wing imaginal disc

Imaginal discs are larval structures that give rise to adult appendages. The discs develop according to the segment they originate from. For instance the leg, wing and haltere discs arise, respectively from the thoracic segments 1, 2 and 3 (T1, T2 and

T3) (Wolpert et al., 1998). The third instar wing imaginal discs have been extensively studied due to their large size and accessibility. The wing imaginal disc is a single layer columnar epithelium in which the dorso-ventral and anterior-posterior axes are defined as the disc grows during larval development. Engrailed is expressed in the posterior compartment and defines the anterior-posterior axis and Apterous, a LIM-homeodomain protein, is expressed in the dorsal compartment, defining the dorso-ventral axis (Blair, 1993; Diaz-Benjumea and Cohen, 1993; Lawrence and Struhl, 1996; Williams et al., 1993) (Figure 1.5). The division of the anterior-posterior axis is inherited from the embryonic epidermis (Cohen et al., 1993; Cohen et al., 1991; Morata and Lawrence, 1975), while the dorso-ventral axis is established in the second larval instar (Blair, 1993; Diaz-Benjumea and Cohen, 1993), (reviewed by Lawrence and Struhl, 1996).

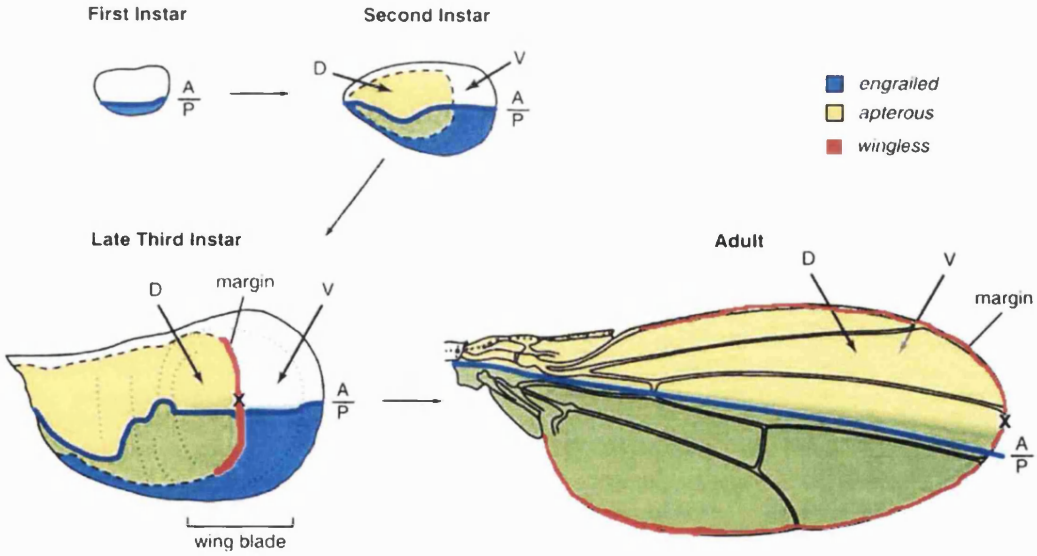


Figure 1.5 Development of the wing imaginal disc.

At first instar the anterior-posterior axis is already formed, identified by *engrailed* expression, in blue. In the mid second instar the disc subdivides into the dorsal and ventral compartments, identified by the *apterous* expression in the dorsal compartment, in yellow. In the third instar, *wingless*, in red, restricts its expression pattern to the dorso-ventral boundary and it is responsible for wing margin formation.

wingless expression in earlier stages is not represented. Only the wing pouch will contribute to the wing blade. The centre of the *wingless* expression domain, represented by a cross, will give rise to the more distal tip of the wing blade (adapted from Blair, 1995).

Early during imaginal disc development, at early second instar, *wg* is required to specify the wing primordium. If *wg* is removed the tissue will develop as body wall and no wing will be formed and, conversely, *wg* ectopic expression will lead to an ectopic wing (Ng et al., 1996; Williams et al., 1993).

In the third instar wing imaginal disc, wg is expressed in a stripe of approximately 3 cells wide at the dorsoventral boundary and its expression is induced by the ligand Serrate through the Notch receptor (Diaz-Benjumea and Cohen, 1995) (Figure 1.5). However, wg is first expressed in the second instar. In mid second instar, wg is expressed strongly in the ventral compartment and less dorsally, starting to be restricted to the dorso-ventral boundary by late second instar (Ng et al., 1996; Williams et al., 1993). The range of Wg is symmetrical and extends 10 to 15 cell diameters to each side of the source.

wg expressed at the dorso-ventral boundary induces neighbouring cells to later differentiate into bristles. High wingless activity is also necessary for the formation of the wing margin (Couso et al., 1994; Phillips and Whittle, 1993). Indeed, wg mutant clones that straddle the dorso-ventral boundary show tissue loss, notching, and the absence of margin bristles. Conversely, excess wingless causes tissue overgrowth (Diaz-Benjumea 95)

Interestingly, the patterning events in the disc occur during extensive growth of the tissue. wg, together with dpp (a member of the TGF-β family of genes), had long been thought to be responsible for proliferation of the wing disc, since a reduction or loss of wg or dpp activity compromises cell proliferation and leads to reduced wing mass (reviewed by Serrano and O'Farrell, 1997). Recent data, however, argue that wg does not directly induce cell growth but instead promotes cell survival during the

rapid growth phase of the immature discs, while patterning is taking place. Later in larval development, when wing patterning is nearly complete, wg slows down growth (Johnston and Sanders, 2003) .

1.5 Wingless as a morphogen

Wingless in the wing imaginal disc acts at a longer range than in the embryo, reaching 10 to 15 cells away from the source. This was shown by studying the expression of two putative target genes, *distal-less (dll)* and *vestigial (vg)*. Using a temperature-sensitive mutation of *wg* it was demonstrated that dll expression is lost 2 days after a shift to the non permissive temperature and vg expression is also drastically reduced (Zecca et al., 1996). Some cells still express the gene, but these cells have been shown to be directly responsive to the Notch signalling, via Delta and Serrate (Kim et al., 1996) Conversely, ectopic activation of wg leads to up regulation of dll and vg (Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1997; Zecca et al., 1996).

As mentioned above, wingless is also required for bristle specification in the wing margin. These bristles arise from neuroblasts that segregate to both sides of the wg expressing cells, and can be visualised by expression of a reporter gene insertion in the *neuralized (neu)* gene (Couso et al., 1994; Zecca et al., 1996).

Thus, wg regulates the expression of dll, vg and bristle specification at different distances within the tissue. The question of whether the secreted Wingless acts directly, through a gradient of the protein, or indirectly, through a relay mechanism in these cells, was first addressed by Zecca and co-workers (1996). The authors constructed a membrane-tethered form of Wingless and a constitutively active form of Armadillo and monitored wingless activation using *distalless*-lacZ, *vestigial* and *neuralized*-lacZ. If Wingless functions through a relay mechanism, by misexpressing the membrane-tethered Wingless and the constitutively active Armadillo in clones in the wing imaginal disc, it would be expected the same non-autonomous effect at the

distance on the target genes as when the wild type wingless is expressed. However, these forms of Wingless and Arm are not able to induce a wg response outside of the clone, indicating that Wingless acts directly on distant cells. Interestingly, the authors note that the membrane-tethered Wingless is still able to act on cells immediately adjacent to the clone, while the constitutively active form of Armadillo is not able to do so. Zecca and co-workers also show, using *wg* overexpression clones, that different responses are elicited at different distances from the Wingless source. *vestigial* is activated at a greater distance than *dll*-lacZ and both further than *neuralized*-lacZ.

These observations were later confirmed by Neumman and Cohen (1997), in a set of experiments that used a temperature sensitive mutation of *wingless* to reduce Wg activity to intermediate and low levels, at temperatures of 22° C and 24° C respectively. At permissive temperature the three target genes are activated. When the discs are shifted to 22° C, *neuralized*-lacZ expression is lost and dll expression is reduced, while vg expression is not affected. At 24° C, dll expression is completely lost and vg expression is reduced.

Wingless also has a direct and long-range action in the leg and eye imaginal discs. (Zecca et al., 1996)

Proteins, such as Wingless, that are secreted in a localised source, signal directly to distant cells and induce dose-dependent responses are called morphogens. The experiments described above led to the definition of Wingless as a morphogen. Although this is widely accepted, it has been argued that Wingless, and the Wnt family of proteins in general, do not comply with all the pre-requisites to be a "classical morphogen" (Martinez Arias, 2003). The author argues that the previously mentioned experiments that led to the definition of Wg as a morphogen do not take into consideration important parameters such as the growth of the wing and the order of the onset of gene expression, especially that of *vestigial* and *distal-less* expression. The author considers the role of Wg to be of maintenance of gene

expression instead of induction of gene expression and proposes the revision of the morphogen concept to this family of molecules.

1.6 Mechanisms of Wingless transport

The mechanism by which secreted signals move across tissues has been considerably debated in the last years. Two simple, and contrasting, ideas were put forward: either the signal diffuses freely in the extracellular space or an active mechanism takes place in which the ligand is endocytosed by the adjacent cell and recycled back to be endocytosed by the then next cell, in a process that came to be known as transcytosis (Figure 1.6).

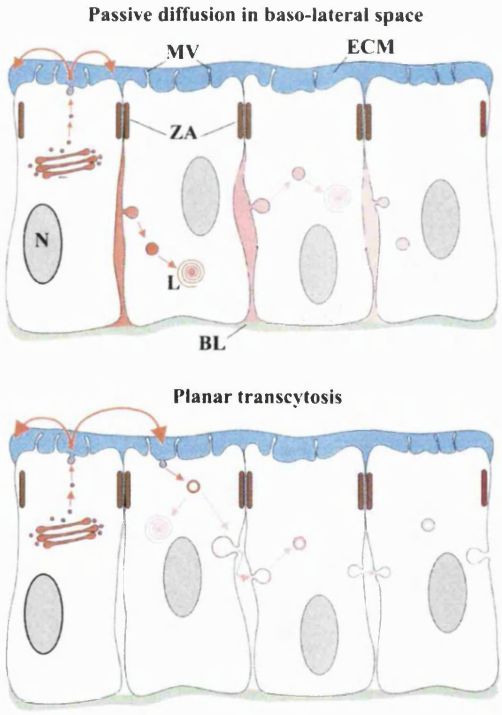


Figure 1.6 Main models of Wg transport

The diagram represents the two models in a wing imaginal disc epithelia, in which there are microvili (MV), zonula adherens (ZA) and basal lamina (BL). L represents a lysosome. In the diffusion model, the ligand is secreted apically and diffuses basally, forming a gradient shown in tones of red. After signalling, the degradation of the ligand is done by targeting to lysosomes.

In the planar transcytosis model, the ligand is transported by consecutive cycles of endocytosis and recycling to the membrane. The gradient forms by degradation of some ligand during the process (adapted from Vincent and Dubois, 2002).

Which model applies to Wg has been difficult to assess because of the difficulty in observing the Wg gradient and devising experiments that are specific to transport and do not affect other processes such as Wg production and degradation. In the last couple of years, however, new tools or techniques have been developed that allow the observation of the extracellular gradient of Wg in the wing (Strigini and Cohen, 2000) and also of one other well studied morphogen, Dpp. (Entchev et al., 2000; Srinivasan et al., 2002; Strigini and Cohen, 2000; Teleman and Cohen, 2000).

It has been argued that if Wg is transported by diffusion it cannot do so by free diffusion, since tissues such as the leg or the wing disc are extremely convoluted and the signal would be lost. In order to diffuse within the plane of the epithelium the protein would have to remain associated with the cell surface (Strigini and Cohen, 2000; Vincent and Dubois, 2002). Such association could be mediated by glycosaminoglycan chains of cell surface proteoglycans. I will return to this issue later.

Wg could also be constrained to the plane of the epithelium if it spreads by planar transcytosis, involving repeated cycles of endocytosis and exocytosis. In an attempt to address if Wg is transported through a process that involves clathrin-mediated endocytosis, several groups used a temperature-sensitive mutation in the dynamin homologue in the fly, *shibire (shi)*. If Wg transport requires endocytosis, it should be blocked in tissues where the endocytic machinery is blocked. Experiments in the embryo showed that in *shibire* mutants the range of Wg is reduced. The restriction of the range of Wg leads to a concomitant restricted domain of action to one or two cells, instead of 4 cells (Bejsovec and Wieschaus, 1995; Moline et al., 1999). These results suggest that Wg transport might require endocytosis.

In contrast, in wing imaginal discs, the Wg gradient does not seem to be affected when endocytosis is blocked (Strigini and Cohen, 2000). In an analysis of *shibire* mutant clones in the wing disc, Wg is observed far from the source and is able to travel through the mutant clone.

In this study, the authors developed a technique to detect only the extracellular Wg gradient and showed that in wild-type discs Wg is detected in a region that roughly corresponds to its domain of action. Using this technique they found that extracellular Wg is no longer produced at the surface of *shi* mutant expressing cells leading the authors to propose that *shibire* has an effect in secretion of Wg and that by shifting the larvae to the permissive temperature the gradient is established *de novo* in 1 hr. The authors propose that Wingless is transported by diffusion in the wing imaginal disc.

This view is supported by theoretical studies that analyse the experimental data (Lander et al., 2002). According to these authors, another explanation for the requirement of endocytosis is that it is necessary for the down regulation of the receptors at the surface. A block in endocytosis would lead to accumulation of the receptors and trapping of the ligand near the source. The authors also argue that the rate of gradient formation (proposed by Strigini and Cohen, 2000) would be incompatible with transcytosis.

Despite the above work, a variant of transcytosis has received renewed attention (Greco et al., 2001). The authors observed GPI-GFP (Glycosyl phosphatidyl inositol anchor fused to green fluorescent protein) vesicles that travel long distances in the wing imaginal disc and colocalise with endossomal markers in receiving cells. Importantly, Wg colocalizes with these suggesting that they could play a role in Wg transport. The exovesicles are constituted mainly of plasma membrane and were named argosomes. The authors propose two alternative models for the spread of the vesicles: either the vesicles are formed as exovesicles from multivesicular bodies

and are endocytosed and exocytosed by the next cell (model 1, Figure 1.7) or the exovesicles are formed by trans-endocytosis (model 2, Figure 1.7)

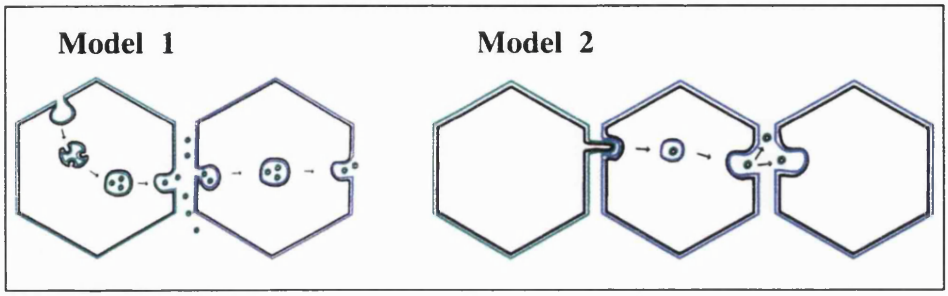


Figure 1.7 Argosome formation in wing imaginal discs

In model 1, argosomes are formed from multivesicular bodies, and released as exovesicles (exosomes) to the next cell. In model 2 argosomes are formed by trans-endocytosis. The expressing cells are represented in green and the receiving cells in blue (Greco et al., 2001)

If these vesicles are to be used as carriers of Wg, the rate of production of the argosomes should be similar to the rate of gradient formation seen by Strigini and Cohen (2000). The authors found that the distribution of new argosomes that spread to 15 cells away is complete 3 hrs after photobleaching, which could be consistent with a mechanism of Wg dispersal in the disc. However, this mechanism would require endocytosis of the ligand and Strigini and Cohen (2000) showed that Wg transport does not require clathrin-mediated endocytosis, leaving open the question of whether Wg in fact uses this mechanism, if it uses more than one mechanism or if the argosomes are carriers for other morphogens.

Importantly, the planar transcytosis model would imply that the receptors are required for transport. It has not yet been possible to address this because cells lacking Fz and Dfz2 do not survive in wing discs. Thus, the mechanism of Wg transport remains to be elucidated.

Although work in the imaginal disc has clearly shown that Wg does spread along an epithelium, there is at least one situation where this is dispensable. In the fly embryo,

experiments with a membrane tethered form of Wg have shown that release of Wg from expressing cells is not required for normal range of action (over 3-4 cell diameters). This is because Wg can also be carried by the daughters of expressing cells as they spread (Pfeiffer et al., 2000). Such a form of transport may be particularly suited to situations when transport occurs over a short range.

While with Wg the weight of evidence does not favour transcytosis, the reverse is true for Dpp, another morphogen (reviewed by Entchev and Gonzalez-Gaitan, 2002). Therefore, one cannot assume that all morphogens are transported by the same mechanism or that each morphogen only uses one type of mechanism.

1.7 Regulation of Wingless distribution

Several molecules have been shown to modulate the Wg gradient and here I will focus on the heparan sulfate proteoglycans.

1.7.1 Regulation of Wg distribution by heparan sulfate proteoglycans

It has been suggested that Wg interacts with heparan sulfate proteoglycans at the cell surface. This is based on experimental evidence both in tissue culture and genetic studies.

Heparan sulfate proteoglycans are a class of proteins composed of a protein core, which contain at least one heparan sulfate chain covalently bound to it. These are widely distributed in animal tissues and Glycosyl phosphatidyl inositol (GPI)-anchored proteoglycans are called glypicans, while proteoglycans carrying a transmembrane domain are called syndecans (Yanagishita and Hascall, 1992). Most of the heparan sulphate found at the cell surface is in either of these forms (Baeg & Perrimon 2000, Bernfield et al, 1999).

The first evidence for the involvement of heparan sulfate proteoglycans in wingless signalling came from cell culture studies. Reichsman et al (1996) showed that

Wingless binds heparin and heparan sulfate in S2 cells and that when these molecules are depleted from cells, by heparinase treatment or by inhibiting sulfation of proteoglycans with a drug, wingless signalling is impaired, using an Arm accumulation assay. Moreover, when heparin or heparan sulfate is added in small quantities to these cells bound Wg is released from the cell surface. The authors also showed that Wg binds heparin with high affinity and specificity.

In vivo evidence came from the identification in three genetic screens of a gene encoding a homologue of vertebrate UDP-glucose dehydrogenase, *sugarless* (*sgl*) (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997). This enzyme is essential for the biosynthesis of heparan sulfate, which is added to the protein core. Embryos mutant for this gene have a cuticle phenotype reminiscent of *wg* mutants. In a *sugarless* mutant, wg signalling is impaired in the embryo, e.g. *en* transcripts fade after stage 10.

Interestingly, when wg is overexpressed in a *sgl* embryo, in a domain that comprises roughly the wg domain of action (using *prd*-gal4), wg can partially restore naked cuticle suggesting that wg signalling can be activated in the absence of *sgl*, at least in an overexpression situation (Hacker et al., 1997).

Heparinase injections in WT embryos mimic the *sgl* phenotype and, consistently, heparan sulfate injected in a *sgl* embryo rescues the phenotype. Also, *sgl* embryos lack heparan sulfate chains as shown by probing with an antibody against these in Western Blot (Binari et al., 1997; Haerry et al., 1997). These experiments confirm that in a *sgl* mutant, the heparan sulfate sugar chains are not present in the HSPGs and that these are important for *wg* function.

Like *sgl*, *sulfateless* (*sfl*) also encodes an enzyme involved in proteoglycan processing and also affects *wg* signalling. The gene encodes a N-deacetylase/N-sulphotransferase (NDST), an enzyme required for the specific modification of heparan sulfate (Lin and Perrimon, 1999). As shown in Figure 1.8, it acts

downstream of UDP-glucose dehydrogenase encoded by *sgl* (Lander and Selleck, 2000).

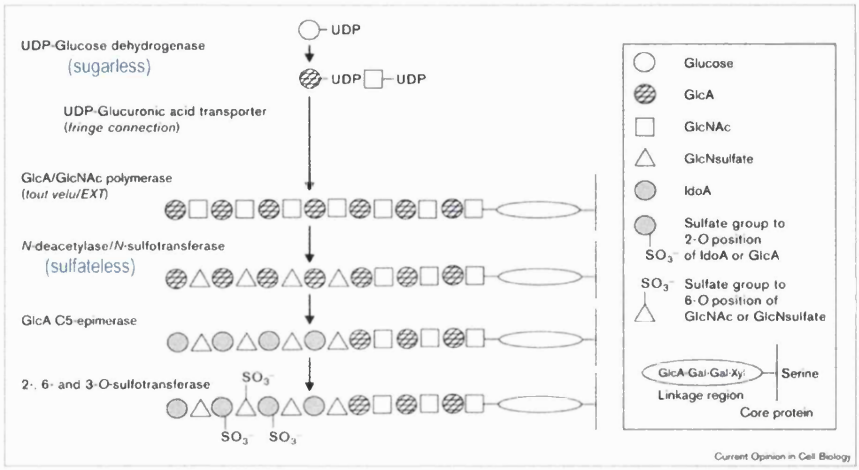


Figure 1.8 Structure of the heparan sulfate chains

The HS chains are linked to serine residues of the core protein. HS is synthesised by the addition of alternating GlcA and GlcNAc residues. The chain is then enzymatically modified by N-deacetylation/N-sulfation, epimerization and O-sulfation (adapted from Baeg and Perrimon, 2000)

sfl mutant embryos also have a cuticle phenotype that is reminiscent of *wg* mutants and are impaired for wg signalling (Lin and Perrimon, 1999).

The *wg* mutant cuticle phenotype is identical to the *hh* mutant phenotype and during embryonic stages a feedback loop makes the expression of wg and hh mutually dependent. Therefore the observed cuticle phenotypes of *sgl* and *sfl* are equally consistent with an effect on hh. However more detailed analysis of embryos, e.g. of the stomatogastric nervous system, which requires wg activity but not hh, has confirmed that *sgl* and *sfl* specifically affect wg signalling (Hacker et al., 1997; Lin and Perrimon, 1999).

The observation that two genes coding for glypicans (*dally* and *dally-like*) might be implicated on wg signalling (Baeg et al., 2001; Lin and Perrimon, 1999; Tsuda et al., 1999) led to increased interest in this class of molecules. *division abnormally delayed* (*dally*) was first identified in a screen for cell division defects in the

developing central nervous system of *Drosophila* (Nakato et al., 1995). More recently *dally-like* (*dlp*) was also identified (Khare and Baumgartner, 2000). These are the only known genes to encode glypicans in *Drosophila*. Glypicans are GPI-anchored cell surface proteoglycans and share a characteristic pattern of 14 conserved cysteines that form a globular domain, glycosaminoglycan attachment sites and a GPI anchor at the C-terminus (Bernfield et al., 1999; Fransson, 2003) (Figure 1.9). These proteins are always substituted with heparan sulfate but removal of the central domains leads to substitution with chondroitin sulfate (Fransson, 2003)

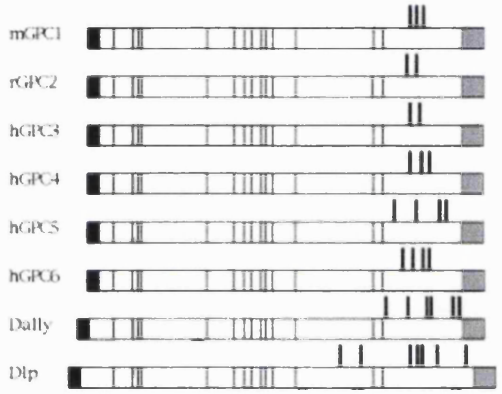


Figure 1.9 Schematic representation of some glypican core proteins, including Dally and Dlp

The black boxes at the N-termini correspond to signal sequences, the grey lines within the white rectangles represent conserved cysteines, the vertical black lines outside of the rectangles represent the potential GAG binding sites and the grey boxes at the C-termini correspond to the GPI anchor (adapted from Khare and Baumgartner, 2000).

dally starts to be expressed early in the embryo, at stage 5 in which it is expressed uniformly in the blastoderm, at stage 8/9 it has been reported to be expressed in stripes, 3 to 4 cells anterior to the wg expressing cells (Lin and Perrimon, 1999), while another group reports it to be expressed in the en cells, adjacent and posterior to the wg expressing cells (Tsuda et al., 1999). *dlp* is also expressed uniformly after stage 5 and at stage 10 shows a striped pattern overlapping the wg domain, being

expressed in the wg expressing cells and 2 to 3 cells anteriorly (Khare and Baumgartner, 2000).

Phenotypic analysis of loss of function conditions is confusing. The only alleles of *dally* available are weak and, undoubtedly, these have a weak, poorly penetrant phenotype that could be reminiscent of *wg* mutants (Tsuda et al, 1999; Lin & Perrimon, 1999). To further block activity, RNA interference (RNAi) was used and published results are not consistent. Two groups found that embryos in which *dally* or *dlp* dsRNA is injected exhibit a cuticle phenotype similar to a *wg* mutant phenotype, presenting ectopic denticles and polarity defects (Baeg et al., 2001; Lin and Perrimon, 1999; Tsuda et al., 1999). When a combination of both dsRNA from *dally* and *dlp* are injected, the embryos have a stronger cuticle phenotype: all embryos are smaller and all naked cuticle is transformed into denticles (Baeg et al., 2001), which may imply partial redundancy. As mentioned before, these cuticle phenotypes could be due to an effect on hh signalling resulting in the same segment-polarity phenotype. This hypothesis was discarded based on genetic interactions between *dally* and components of the wg pathway (Lin and Perrimon, 1999; Tsuda et al., 1999).

However, a recent study has cast doubt on this interpretation and provide evidence that *dally* loss-of-function has no effect on wg signalling (Desbordes and Sanson, 2003). These authors also show that *dlp* is required for hh signalling in the embryonic epidermis, which is consistent with what has been demonstrated in a tissue culture system (Lum et al., 2003).

These studies leave open the question of what is the exact role of *dally* and *dlp* on wg signalling and if indeed these have an effect, at least in the embryo.

Overexpression data is no less confusing, overexpression of *dally* in the embryonic epidermis with a heat-shock transgene, *hs-dally*, leads to an expansion of naked cuticle and loss of denticles, as expected from an increase in *wg* signalling. *hs-dally*

can also partially rescue a weak *wg* mutant (Tsuda et al, 1999). *dlp* and *dally* require *sfl* for sugar modification and both interact genetically with *sgl*. Dally has also been shown to be modified by Sgl (Lin & Perrimon, 1999; Tsuda et al, 1999; Baeg et al, 2001).

Flies in which *dlp* has been overexpressed in the wing margin, show margin defects and loss of sensory bristles, as if *wg* activity were decreased. This phenotype has been suggested to be due to accumulation of extracellular Wg, limiting protein diffusion. This is consistent with the finding that extracellular Wg is decreased in *sfl* mutant clones (Baeg et al, 2001).

Two main models have been suggested for the role of the HSPGs in Wg distribution and signalling. The HSPGs could act as co-receptors in a complex with Dfz2. Alternatively, the HSPGs could capture Wg limiting its free diffusion, increasing its local concentration and the probability of interaction with high-affinity signalling receptors (Baeg et al., 2001; Lin and Perrimon, 1999; Tsuda et al., 1999). Existing evidence, namely the observation that Wg can signal in the absence of HSPGs, does not support the first model. The second model could explain the effect of *dlp* on wg signalling, at least based on overexpression data, but it is not clear if it would apply to all HSPGs.

1.7.2 Regulation of Wg distribution by a modifier of HSPGs

Two groups have recently identified a gene, *notum* or *wingful (wf)*, that encodes for a secreted extracellular protein that acts negatively on wg pathway and is activated by Wg (Gerlitz and Basler, 2002; Giraldez et al., 2002). In the wing, *notum* is expressed at the dorsoventral boundary, as is *wg*. *wf/notum* overexpression leads to *wg* loss of function phenotypes and reversely *wf/notum* loss of function leads to higher wg activity, consistent with its role as an inhibitor. The encoded Wf/Notum protein is structurally homologous to a subfamily of hydrolases and is related to plant pectin acetylesterases. Based on this homology, this gene has been proposed to act by

inhibiting the activity of the proteoglycans *dally* and *dally-like*, by deacetylating its glycosaminoglycan chains both in the secretory pathway and at the cell surface, by competition with another enzyme that substitutes the acetyl for sulfate, NDST (which is encoded by *sulfateless*) (Giraldez et al., 2002). According to these authors, reduced *notum* activity leads to higher binding of Wg to the HSPGs and consequent stabilisation, increasing the domains of the target genes.

1.7.3 Regulation of Wg distribution by signalling receptors

In wing imaginal discs, Dfz2, a key signalling receptor of Wg, is expressed in a complementary pattern to that of Wg. It has been shown that Wg represses Dfz2 expression and that when Dfz2 is overexpressed in the wing disc, Wg accumulates at the cell surface (Cadigan et al., 1998). The authors argue that the repression of Dfz2 by wg is essential to shape the Wg gradient since the overexpression of Dfz2 leads to an expansion of the domains of the low and intermediate Wg target genes.

1.8 Properties of the Heparan Sulfate Proteoglycans

Proteoglycans are abundant cell surface and extracellular matrix molecules constituted by long and unbranched glycosaminoglycan (GAG's) chains covalently attached to a protein core. In addition to the GAG chains most core proteins also contain N and/or O- linked sugars (Kjellen and Lindahl, 1991). GAGs can be galactosaminoglycans or glucosaminoglycans. The glycosaminoglycan chains attach to serine residues in the protein core, although there is not a consensus sequence for GAG attachment (Kjellen & Lindahl, 1991). The GAG chains have a high negative charge as a result of the presence of acidic sugar residues and/or modification by sulfate groups. The HS chains are GAG chains composed of repeating disaccharide units of uronic acid linked to glucosamine (Bernfield et al., 1999; Prydz and Dalen, 2000). The HS chains are synthesised and modified in the lumen of the Golgi apparatus. After synthesis the HSPGs are secreted to the cell surface or ECM. The

pathway taken from the Golgi to the cell surface is largely unknown (Prydz and Dalen, 2000).

The membrane of epithelial cells, such as cells from the fly embryonic epidermis or wing imaginal disc, are separated in two different domains: the apical and basolateral surfaces. Due to this polarization, proteins need to be sorted to the correct surface domain. Proteoglycans have been shown to localise both to apical and basolateral domains and although the GPI linkage and the presence of N-glycans are thought to favour apical sorting, many proteoglycans are found basolaterally. The HS chains have been proposed to counteract the apical sorting signal, since a form of glypican that lacks the HS attachment sites shifts its localisation from basolateral to apical (reviewed by Fransson, 2003; Prydz and Dalen, 2000).

The mechanism used for sorting is also largely unknown, although it has been proposed that GPI-anchored proteins, such as Dally and Dally-like, associate with membrane domains called rafts which are found mostly at the apical surface of epithelial cells (Simons and Ikonen, 1997).

1.9 Wingless synthesis and processing

The Wingless protein, and its mammalian homologue Wnt-1, contains a hydrophobic signal sequence at the N terminus followed by a consensus signal peptidase cleavage site and a pattern of 23 cysteine residues, which is conserved in all Wnt proteins (Wodarz and Nusse, 1998). Although Wingless and Wnt-1 are very conserved at the sequence level, there is a major difference in size. Wingless is 98 amino acids longer that its mammalian homologue, due mainly to an 85 amino acids hydrophilic region that is not present in Wnt-1 (Rijsewijk et al., 1987).

1.9.1 Post-translational modifications

1.9.1.1 N-Glycosylation

In order for Wingless to be optimally secreted it needs the activity of the segment polarity gene *porcupine* (*porc*). In embryos that lack *porcupine's* maternal and zygotic contribution, Wingless is retained inside producing cells and the embryos develop a cuticle phenotype that is very similar to that of a *wg* mutant. *porc* also impairs wg function in other tissues such as wing imaginal discs (Kadowaki et al., 1996). The distribution of the Wingless protein in a porc mutant is similar to that of a temperature sensitive *wg* allele (*wg* ^{IL114}) that carries a substitution of one of the cysteines (Cys 104) to a serine. In vitro studies using Schneider-2 (S2) cells show that in *wg* ^{IL114} mutant Wg is not secreted, being seemingly retained in intracellular vesicles (van den Heuvel et al., 1993). Another wg mutant (*wg*^{IN67}), in which a glycine (Gly 221) is substituted by aspartic acid, shows the same phenotype in transfected cells (van den Heuvel et al., 1993).

The role of *porcupine* in Wingless processing was further elucidated by (Kadowaki et al., 1996) and (Tanaka et al., 2002). *porcupine* encodes a multipass transmembrane protein that is localised to the ER and is required for N-glycosylation of Wg (Kadowaki et al., 1996).

In S2 cells, Wg is expressed in three forms of similar molecular size, as detected by Western Blotting (forms I, II and III). However only form I (the lowest MW species) is present when the cells are treated with tunicamycin, an inhibitor of N-glycosylation, indicating that the other two forms are glycosylated (Tanaka et al., 2002). In Wild-Type *Drosophila* embryos and imaginal discs, most Wg is detected as form III, although some form II is also present. Forms II, III were shown to contain, respectively, one and two N-glycans (Tanaka et al., 2002).

When *porcupine*, alone or together with *wg*, is overexpressed in these cells, Wg is mainly detected as the more glycosylated form (III). The same result is seen in imaginal discs in which *porc* is overexpressed. On the other hand, in imaginal discs

lacking *porcupine* only form II is detected. These observations indicate that *porcupine* activity is required for efficient N-glycosylation (Tanaka et al., 2002).

To test whether Wg processing occurs inside the ER or in translocational intermediates through the ER membrane, Tanaka and co-workers treated wg expressing cells with exogenous proteases and found forms II and III, and form I to a lesser extent, to be resistant to the treatment, suggesting that the glycosylation takes place inside the ER.

Since Wingless can be modified with two N-glycans, one would expect two N-glycosylation sites to be accessible in the wg protein. Tanaka et al. showed that these two sites are Asn^{103} and Asn^{414} and that binding of Porcupine to an N-terminal 24 amino acid domain (residues 83–106) is essential for the glycosylation at Asn^{103}.

The role of *porc* on Wg processing seems to be quite specific, since *porc* does not have an effect on dpp or hh signalling in midgut development and Hh secretion is not affected when *porcupine* is overexpressed in Kc cells, as was shown to happen for Wingless (Kadowaki et al., 1996).

It should also be mentioned that the N-glycosylation sites are highly conserved in other Wnt family members (Tanaka et al., 2002). Homologues of *porc* were identified in mouse (M*porc*) and in Xenopus (X*porc*) and M*porc* can substitute for the fly gene (Tanaka et al., 2000). Moreover, the identification in C. elegans of a gene with 29% identity to *porc*, Mom-1, that is required for the processing of Mom-2 (a protein with 34% identity to Wg and 35% identity to human Wnt2) (Rocheleau et al., 1997; Thorpe et al., 1997) suggest that the porc gene family is evolutionary conserved and is generally involved in processing of the Wnt family of proteins.

1.9.1.2 Palmitoylation

Wnt proteins have been shown recently to be palmitoylated near the N-terminus (Willert et al., 2003). Palmitoylation is a common pos-translational modification that

consists of the covalent attachment of fatty acids, such as palmitate, stearate, oleate and arachidonate. The attachment occurs by thioester linkage to cysteine residues. The modification increases protein hydrophobicity, facilitates protein interactions with lipid bilayers and has a role in protein sorting and consequently its function, (reviewed by Bijlmakers and Marsh, 2003; el-Husseini Ael and Bredt, 2002; Resh, 1999).

Wnts are modified by the addition of a palmitate, a 16 carbon saturated fatty acid. Using liquid chromatography tandem mass spectrometry Willert and co-workers (2003) found the palmitoylation to occur on a conserved cysteine, C77 in Wnt3a and C51 in Drosophila Wnt-8.

The authors showed that the palmitoylation renders secreted Wnt3a hydrophobic by using a Tx-114 phase separation assay. Most Wnt3a partitions to the detergent phase. When the C77 is mutated to alanine the protein partitions to the water phase, consistent with a loss of hydrophobicity. Moreover, treating purified Wnt3a with an enzyme that removes the palmitate, APT-1, also shifts the protein to the water phase and importantly also blocks its signalling activity in L cells, seen by its inability to stabilise β-catenin. However, in another cell line, 293, transfection of the mutated Wnt3a does not totally block signalling, suggesting that at high levels this mutated, non hydrophobic, form is able to signal.

It is interesting to note that when palmitoylation is the only lipid modification on a protein, it often occurs on multiple adjacent cysteines (el-Husseini Ael and Bredt, 2002) and yet only one cysteine was found to be modified in Wnt3a, leaving open the question as to whether the Wnt proteins carry other modifications besides palmitoylation and N-glycosylation.

The features required for palmitoylation are poorly understood. Preferred sites seem to be cysteines that are close to membrane–interacting domains (TM domains or membrane associated domains in non-integral membrane proteins), possibly to be

close to the enzyme PAT, palmitoyl acyltransferase, found to be membrane associated in yeast. These recently identified enzymes, Erf2p-Erf4p complex and Akr1p, had been shown to localise, respectively, to the ER and Golgi (Lobo et al., 2002; Roth et al., 2002).

The lack of a consensus sequence and the observation that several amino acids influence palmitoylation makes it more likely that common structural features are the key factors that specify palmitoylation (Bijlmakers and Marsh, 2003).

Also, the site of palmitoylation has proved difficult to find. In many situations proteins pass through cycles of palmitoylation and re-palmitoylation, so they can be acylated in more than one cellular location.

Myristoylated and palmitoylated peripheral membrane proteins, such as the members of the Src family of nonreceptor tyrosine kinases Lck and Fyn are palmitoylated at different locations in the exocytic route. For instance, the cytosolic protein Lck, expressed in T-lymphocytes, interacts with the TCR co-receptors CD4 and CD8 but to do so it needs to be attached to the membrane and palmitoylated. And as this interaction occurs early after Lck synthesis the palmitoylation occurs early in the exocytic pathway and the protein travels as a membrane bound form through the exocytic route to the plasma membrane (Bijlmakers and Marsh, 1999). In contrast, Fyn is palmitoylated only at the plasma membrane (van't Hof and Resh, 1997). Other proteins are modified in intermediate locations.

The main factors that are likely to determine where palmitoylation occurs are the subcellular distribution and substrate specificity of different palmitoyl acyltransferases (PATs) and the interactions that influence targeting to PATs (Bijlmakers and Marsh, 2003). In spite of their importance and the effort taken to purify active enzymes that mediate palmitoylation, the majority of these are still unknown.

Interestingly, Porcupine, which is required for Wg N-glycosylation, may actually be mediating the palmitoylation. Porcupine is a member of a superfamily of membrane-

associated acyltransferases that transfer organic acids, typically fatty acids, onto hydroxyl groups of membrane-associated proteins (Hofmann, 2000). The substrate of acylation can be either a lipid or a carbohydrate. It has been suggested that Porcupine anchors Wg to the ER membrane through acylation, facilitating N-glycosylation (Tanaka et al., 2002). So if membrane association is required for efficient glycosylation and secretion one would expect that a non-palmitoylated protein is not membrane associated and therefore not properly glycosylated and secreted. Indeed, in embryos mutant for an allele of wg in which the conserved cysteine is substituted for a tyrosine (wg ^{S21}) (supposedly lacking palmitoylation) Wingless does not seem to be secreted. However, the lack of palmitoylation seems to render Wnt-3a less active and hydrophilic but it is still secreted (Willert et al., 2003). It is important to point out that many cysteine mutations cause general misfolding of a protein, which in turn causes secretion impairment. Free cysteines residues have free sulphydril groups that will have the tendency to make disulphide bonds with other cysteines, so it is understandable that mutations in cysteines lead to misfolded proteins. Due to its high content in cysteines, Wnt proteins are prone to misfolding and retention in the cell (Nusse, 2003).
Further investigation is needed to fully understand the role of Porcupine in Wg processing.

The figure below shows a stretch of the amino acid sequence of Wingless with the reported sites of palmitoylation and glycosylation and an alignment with other members of the Wnt family indicating the conserved amino acids.

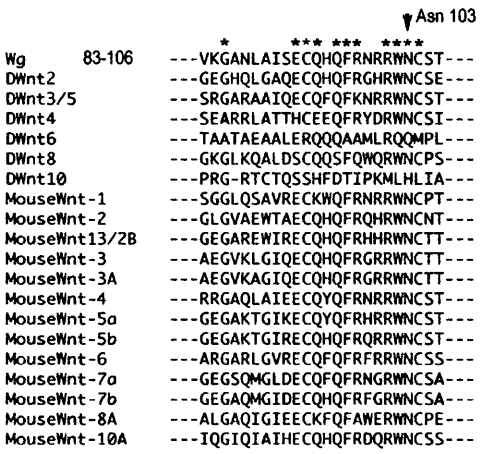


Figure 1.10 Alignment of an N-terminal stretch of the amino acid sequence of Wg with those of other members of the Wnt family. The asterisks indicate the conserved amino acids. The Asn 103 site of glycosylation and the cysteine where palmitoylation is thought to occur in Wg are shown in red and blue, respectively. (adapted from Tanaka et al, 2002 and Willert et al, 2003).

The palmitoylation of a secreted protein is highly unusual and, as I will discuss below, intriguingly, the other known secreted and palmitoylated protein is Hedgehog (and its vertebrate counterpart, Sonic hedgehog).

1.9.1.1.1 Hedgehog Palmitoylation

Hedgehog proteins are synthesized as 45Kd precursor proteins that then undergo autocatalytic cleavage to give rise to a 22 Kd signalling N-terminal domain (hh-N). While cleavage takes place a cholesterol moiety is attached to the C-terminus (Porter et al., 1995). In addition cleaved Sonic Hedgehog (Shh) is acylated at the N terminus. Palmitate was found to be the fatty acid covalently attached to the cysteine (Cys 24). Absence of this modification leads to a 30-fold decrease in activity. In a binding assay for patched-1, one of the Shh receptors, both palmitoylated and unpalmitoylated forms showed similar binding capability (Pepinsky et al., 1998), suggesting that the modification is not necessary for the binding event *per se*.

Dual lipid modification leads to a much higher level of membrane association (Pepinsky et al., 1998). This property, if biologically significant, could be relevant in

tissues in which long or short range signalling properties are required. Indeed, this would be consistent with the observation that acylation is required for Hh function at short range in the *Drosophila* wing (see below) but not for the long-range action of Hh in the vertebrate limb (Lee et al., 2001).

In *Drosophila*, four independent groups identified a putative palmitoyl acyltransferase, named *skinny hedgehog* (*ski*) (Chamoun et al., 2001) *sightless* (*sit*) (Lee and Treisman, 2001) *central missing* (Amanai and Jiang, 2001) and *rasp* (Micchelli et al., 2002), required for Hedgehog palmitoylation. Mosaic analysis in imaginal discs showed that *ski* is required in hh expressing cells suggesting a role for *ski* either in the maturation or secretion of Hh. Secretion of Hh is not affected, since no protein accumulates in the producing cells and the expression of *hh* is also unaffected (Chamoun et al., 2001).

Ski is as a highly hydrophobic protein with similarities to members of the superfamily of membrane bound acyltransferases (Chamoun et al., 2001; Lee and Treisman, 2001). It is interesting to note that Porcupine, which is required for correct wingless processing is also a member of this family of proteins. Since *ski* encodes a putative acyltransferase it is likely that Hedgehog is acylated. Indeed, Chamoun and co-workers (2001) found that Hh is amino-terminally palmitoylated.

1.10 Wg release

Wingless was first shown to be released in an active soluble form in an elegant set of experiments in tissue culture cells (van Leeuwen et al., 1994). The authors used S2 cells expressing wg under the heat-shock promoter (S2hsWG) as the producing cells and non-transfected clone-8 cells as the receiving cells. By co-culturing the two cell lines the authors were able to show an increase in wg signalling in the receiving cells, indicating that Wg produced in one cell line was able to activate signalling in the other cell line. To assess if the ECM and supernatant from producing cells had

signalling activity, the authors collected either ECM or conditioned supernatant, cleared at 100 000g for 1 hr, from S2hsWg cells and applied it to the receiving cells. In both cases, signalling is activated, as seen by Arm accumulation. These observations demonstrate that both Wg bound to the ECM and secreted soluble Wg are biologically active. It is interesting to note that the amount of soluble Wg is very small, approximately 10x less than the protein detected in the ECM. This quantity is, nevertheless, sufficient to activate signalling at similar levels than what is obtained when the two cell lines are co-cultivated or when Wg bound to the ECM is applied (van Leeuwen et al., 1994).

The release of Wg and its extracellular localisation was further addressed in another important study in tissue culture cells (Reichsman et al., 1996). The authors report that most secreted Wingless is bound to the cell surface and to the ECM (approximately 83%, of which 33% is bound to ECM and 50% to the cell surface) while less than 20 % is detected in medium cleared at 20 000g.

Addition of heparan sulfated glycosaminoglycans releases the most glycosylated form of Wingless from the cell surface and ECM and Wingless binds directly to heparin-agarose beads, leading the authors to suggest that Wingless is bound to heparan sulfated proteins at the cell surface. Because of this association, active Wnt proteins had been found difficult to obtain from the supernatant of expressing cells. Besides Wg, the only Wnt proteins that had been recovered from the medium are Dwnt-3 (Drosophila Wnt-3), Wnt-5a and Wnt-1, the vertebrate homolog of Wg (Rijsewijk et al., 1987). Dwnt-3 has been shown to be secreted at low levels to the medium in both soluble and insoluble forms and is detected at high levels in the ECM, when expressed in clone-8 cells, a cell line derived from wing imaginal discs (Fradkin et al., 1995). Active Wnt-5a was recovered from the medium when expressed in 293 cells (Austin et al., 1997). Early attempts to recover Wnt-1 from the medium were unsuccessful. The protein was shown to be released only upon addition of suramin or heparin to the cells, which are thought to release proteins from

the ECM and cell surface. The secretion is inefficient, as only 10% of the total Wnt-1 is released (Bradley and Brown, 1990; Papkoff and Schryver, 1990). The inefficient secretion, usually observed on Wnt proteins, has been reported to be due to misfolding of the proteins in the ER upon overexpression (Burrus and McMahon, 1995). The detection of Wnt-1 in the medium was achieved when the protein was expressed in a mammary epithelial cell line (C57MG) (Bradley and Brown, 1995). The authors show that approximately 50% of Wnt-1 is released to the medium in a soluble form and that it is biologically active, as seen in a transformation assay.

The difficulty in the purification and isolation of Wnt proteins has been due to its strong binding to the ECM, to protein misfolding when overexpressed and its insolubility. The purification and isolation of several active Wnt proteins was finally accomplished recently (Willert et al., 2003) and allowed the authors to show that the Wnt proteins are insoluble and lipid-modified.

It appears that Wg, and the Wnt proteins in general, binds tightly to the membrane, it is lipid modified and that only a small amount is released as a soluble form. From these observations an important question arises: how is a lipid-modified, membrane bound protein released from the cell? There are two main possibilities, either some other molecule detaches it from the membrane releasing it to the medium, in a way similar to what has been suggested for Hh (Burke et al., 1999), or the protein is released in membranar structures.

1.10.1 Possible mechanisms of release

The active hedgehog protein, Hh-Np is palmitoylated and cholesterol modified so, as with Wingless, the problem arises of how is it released from expressing cells? Some inroad was made with the identification of *dispatched* (*disp*), a gene that encodes a protein required for Hh-Np release from the membrane (Burke et al., 1999). Dispatched is a 12-pass transmembrane domain protein that contains a SSD domain

(sterol sensing domain). Genetic studies demonstrated that it is required in the Hh-producing cells for the release of the lipid-modified Hedgehog. However, the exact mechanism by which it acts is unknown. The authors favour the view that it is either acting by displacing the cholesterol tether through direct binding of Hh-Np to Disp with the help of a co-factor, or by having mainly a regulatory action through its SSD domain (Burke et al., 1999).

It has been shown that Shh-Np is also released as a soluble form (s- ShhNp). This form is not membrane associated and is active (Zeng et al., 2001). The authors demonstrate that this soluble ShhNp forms a multimeric complex and propose that it diffuses by having the lipid attachments sequestered inside the multimer.

A Disp protein required for ShhNp release was also identified in mammals (Caspary et al., 2002; Kawakami et al., 2002) and Zeng and co-workers (2001) postulate that mDisp would be involved in releasing soluble ShhNp from the membrane.

Dispatched is specific for Hh-Np release and it remains to be seen whether Wingless also requires a dedicated protein for its release from the membrane.

Alternatively, Wingless could be released still attached to membranes, through the formation of exovesicles. The possibility that morphogen release and transport could be mediated by exovesicles has recently been suggested by Greco and colleagues (2001). These authors show that membrane fragments containing GPI-GFP are released from expressing cells and travel in wing imaginal discs. Because of their putative role in Wg transport these exovesicles were named argosomes.

Wingless colocalises with argosomes, at least in part. This association could be mediated by proteoglycans since Wg is lost from argosomes and elsewhere after treatment with heparinase I, an enzyme that removes heparan sulfated sugars. Since heparinase treatment does not affect argosome formation, Greco and co-workers postulate that argosomes are produced independently of Wingless.

Nevertheless, the authors propose that Wingless might use these vesicles as means of release and transport.

Although a novel idea as means of morphogen release, membrane vesiculation is a common feature of exosome formation in cell types such as B -lymphocytes, dendritic cells and platelets. Exosomes (40-90 nm vesicles) are formed by the fusion of multivesicular late endosomes/lysosomes with the plasma membrane (Denzer et al., 2000).

Blood platelets have been shown to release exosomes upon activation, either by agonists such as thrombin or by surface adhesion. It is not yet clear what is their exact function but it has been suggested that the exosomes are released at sites of vascular injury (Heijnen et al., 1999). Antigen presenting cells, such as B-lymphocytes and dendritic cells are thought to secrete exosomes containing MHC class molecules that stimulate T cell proliferation (Raposo et al., 1996).

1.10.2 Direction of Release

Wingless is localised apically in the embryo and it has been demonstrated that this distribution is determined by the localisation of the mRNA (Simmonds et al., 2001).

These authors show a correlation between the distribution of transcripts and protein. Wg expressed from an apically localised mRNA is localised apically, while protein expressed from a uniformly distributed mRNA is more diffused, extending laterally. Moreover, apical localisation of transcripts, and hence the apical distribution of the protein, is required for wg full signalling activity.

Although Wingless is localised apically in the embryo and indeed apical secretion of GFP-Wg has been observed (Pfeiffer, S. pers comm.), Wingless is also detected in deposits between the cells, basolaterally, by electron microscopy (van den Heuvel et al., 1989). In another study Wingless is seen in a similar location between the wg and en expressing cells in what seems like small vesicles, and at low level apically not associated with the membrane (Gonzalez et al., 1991).

In the wing imaginal disc, extracellular Wingless has been shown to be localised basolaterally on receiving cells (Strigini and Cohen, 2000), although is not yet clear if it is secreted apically or basally.

1.11 Aims and outline of the thesis

In broad terms, transport of Wg involves 3 steps: release from the membrane, movement to the receiving cell and integration in the receiving cell to signal. By knowing how each of these processes occurs, we will have more information on how the whole process works. This thesis tries to give some contribution to the understanding of the first two steps by addressing three main questions:

1. What is the general effect of changing the distribution of heparan sulfate proteoglycans on the distribution of Wingless?
2. How is Wingless released from the producing cell?
3. How do the heparan sulfate proteoglycans affect this release?

I address the first question in Chapter 3, using the Drosophila embryo and wing imaginal disc as experimental systems.

The second question is addressed in Chapter 4, making use of insect cell lines as a simpler system to study and ultimately purify released Wingless.

I addressed the third question in Chapter 5, making use of the assays in Schneider cells developed in Chapter 4.

Chapter 2 – Materials and Methods

CHAPTER 2 - MATERIALS AND METHODS

2.1 *in vivo*

2.1.1 Drosophila stocks

The following stocks were used: *armadillo*-GAL4; *engrailed*-GAL4; *patched*-GAL4; *prd*-GAL4/TM3; UASlacZ/TM3; *wingless*-GAL4/cyo; *ftz*-GAL4; UAS-lacZ/Cyo; *engrailed*-GAL4 UAS-nuclacZ; *dpp*-GAL4 UAS-GFP (gift from S. Cohen); *w*, UAS-wingless-GFP (produced by C. Alexandre); UAS-HA-wingless/+; *prd*-GAL4, FRT*sgl*/TM3 (gift from A. Manoukian); w, UAS-*dally*/Cyo (gift from S. Selleck); UAS-*dally-like* (gift from S. Baumgartner); UAS-dally-like-HA (gift from S. Cohen); hsflp, FRTovoD2A/TM3 TM6 (gift from G. Struhl); UAS-dally-FLAG (produced by C.Alexandre); *decapentaplegic*-GAL4/TM6 ; *apterous*- GAL4/Cyo; *apterous*-GAL4 *wingless*-GAL4 /Cyo (produced by JP Vincent); UAS-*dally-like*-HA *distaless*-lacZ (produced by JP Vincent); UAS-*dally*-FLAG *distaless*-LacZ (produced by JP Vincent); w, FRT*sgl* (P1731) and yw.

2.1.2 Fly crosses and stock maintenance

Unless stated otherwise, all stocks were maintained at 18° C and the crosses carried out at 25° C in plastic vials containing standard organic media supplemented with dry yeast. Approximately 10 to 15 females and 5 to 10 males were used in most crosses. If necessary, the required progeny of a cross was identified by selection against dominant markers on balancer chromosomes.

2.1.3 Embryo manipulation

For embryo collection, dechorionation and fixation the procedure was done as described (Stern and Sucena, 2000), unless otherwise stated, with the following differences. The eggs were allowed to develop for 6 hrs, until stage 11 and fixation was done in glass vials.

2.1.4 *in situ* hybridisation

RNA double in situ hybridisation was done by fixing and hybridising the embryos with digoxigenin and fluorescein labelled single stranded RNA probe as described (Jowett, 1997), except that no proteinase K treatment was performed.

2.1.5 Immunofluorescence in Embryos

Staging of embryos was done according to (Campos-Ortega, 1985).

Total antibody staining was done according to standard procedures (Vincent and O'Farrell, 1992).

Extracellular Wg antibody staining was developed by adapting a method devised for detection of extracellular protein in wing imaginal discs (Strigini and Cohen, 2000). Embryos in stages 10 and 11 were dechorionated and devitellinized by hand in a petri dish in iced PBS. These were incubated in primary antibody (mouse anti-wg/ mouse anti-HA, rabbit anti-βgal) for 1 hour in ice, washed 2 times in iced PBS and fixed in PBS/Formaldehyde 4% for 20 minutes. After fixation embryos were washed in PBS several times and incubated in primary antibody for the antigen to be detected intracellulary (rabbit anti-en) overnight. After this step the procedure is like a standard antibody staining as above, with the exception that visualisation and photographing is done immediately after staining.

2.1.6 Immunofluorescence in Wing Imaginal Discs

Overnight collections of embryos were made and allowed to age for five to six days within the same vial. Wandering third instar larvae were collected from the side of the vials.

Larvae were dissected in solid glass dishes with forceps and stained for total protein as described (White, 1998), with the following differences. Dissected larval heads and associated imaginal discs were kept on ice until fixation, in solid watch glasses. These were fixed in 3.8% formaldehyde (from a 38% stock solution, Merck) for 20

minutes, at room temperature. The heads were then rinsed three times in PBS, washed for 20 minutes in PBS and then washed for other 20 minutes in PBS/Triton 0.1% (PTX), on a rotating platform at room temperature.

Blocking and antibody incubation was done as described. The wing imaginal discs were dissected from the larval heads and mounted individually in Vectashield with or without DAPI (Vector Laboratories).

Immunofluorescent imaginal discs were analysed initially on a Leica FLUO-TM dissection microscope. Where appropriate genotypes were selected. Approximately for each experiment, 15 to 20 imaginal discs were examined and 5 were selected for confocal imaging. Samples were imaged on a Leica TCS SP laser scanning confocal microscope with Leica TCS NT software or on a Biorad radiance 2100 confocal microscope with radiance 2100 software. The images were processed using NIH image and Adobe Photoshop or just Adobe Photoshop.

2.1.7 Antibodies

The primary antibodies used for immunofluorescence in embryos and discs were the following: mouse anti-Wingless (4D4, a gift from S. Cohen, EMBL) diluted 100 fold, rat anti-HA (affinity purified, from Roche) diluted 500 fold, rabbit anti-βgal (Capell), diluted 12000 fold, mouse anti-FLAG (M2, from Roche), diluted 1000 fold and rabbit anti-Engrailed (a gift from C.H. Girdham and P. O'Farrell, UCSF) diluted 100 fold.

The secondary antibodies used were the following: anti mouse, anti-rabbit and anti-rat Alexa ™ fluorescence conjugates, Alexa ™ 488 and Alexa ™ 592 from Molecular Probes, diluted 300 fold, and anti- rabbit Cy5 (Jackson ImmunoResearch Laboratories), diluted 200 fold.

2.1.8 Cuticle preparations

Embryos were collected for 15 hours at 18° C in a grape juice plate. These were aligned in a fresh grape fruit plate, counted and allowed to age until cuticle formation, approximately 24 hours post-laying.

The periviteline membrane was removed and the embryos were mounted in Hoyer's medium and photographed on a Zeiss Axioplan 2 microscope coupled to a Leica DC500 camera with a Leica Firecam 1.1.1 software or with a film camera with 64T Ektachrome film (Kodak).

2.1.9 Wing preparations

The wings of adult flies of the correct genotype were stored in ethanol in airtight tubes. The wings were dissected and mounted individually in Euparal (Agar Scientific) and baked overnight at 65° C. Wings were photographed on a Zeiss Axioplan 2 microscope coupled to a Leica DC500 camera.

2.1.10 Misexpression experiments

To misexpress *dally* and *dally-like* in a spatial and temporal manner I used the Gal 4 system, as described (Brand and Perrimon, 1993). *dally* was misexpressed by crossing UAS-*dally* flies to the following drivers: *prd* gal4, *ftz* gal4, *arm* gal4, *ptc* gal4, *en* gal4, *wg* gal4, ap gal4, wg,ap gal4 and *dpp* gal4. *dly* was misexpressed in the *wg* and *en* and *dpp* domains by crossing UAS-dly flies to *wg* gal4, *en* gal4 and *dpp*-gal4 flies, respectively.

2.1.11 Production of *sgl* germline clones

sugarless germline clones were produced as described (Chou and Perrimon, 1996), crossing hsflp/*prd*-GAL4, FRTsgl/FRTovoD2A female flies to UAS HA-Wingless/+; FRT*sugarless*/+ males.

2.2 Cell culture

2.2.1 Culture conditions

Five Drosophila Schneider Line 2 (S2) cell lines were used. The Wg-expressing, S2hsWg(+), cell line contains the *wg* cDNA under the control of a heat shock promoter while the S2hsWg(-) cell line has the cDNA in the antisense orientation (Reichsman et al., 1996). The S2HRP-Wg cell line contains the HRP-*wg* cDNA under the control of the pMK33 promoter and the S2WgGFP cell line contains the *wg*-GFP cDNA under the control of the same promoter. The S2 cell line was also used as a control. The S2HRP-Wg and S2WgGFP stable lines were developed by E. Piddini. The heat shock cell lines referred above were a gift from N. Itasaki and all cell lines were cultured as described (Reichsman et al., 1996).

2.2.2 Immunofluorescence of cultured cells

Cells were allowed to grow on round coverslips. At the time of staining, the medium was removed and cells were fixed for 15 min in 4% paraformaldehyde/PBS. Cells were then washed, quenched for 10 min in 50mM Amonium Chloride/PBS and incubated for 10 min in 0.1% Triton/PBS. This step was skipped for cell surface stainings. Cells were then washed 3x in PBS and incubated 1hr in 1%BSA/PBS. Incubation with primary antibodies was done at room temperature for 1 hr, by placing the coverslips in parafilm and adding a volume of 200 μl on top of the coverslip. The coverslips were then washed carefully in PBS 5 x 5 minutes and incubation in secondary antibody was done for 1 hour. The coverslips were washed, mounted in Mowiol ® 4-88 (calbiochem) and photographed in a Biorad radiance 2100 confocal microscope with a radiance 2100 software. All samples were photographed at 60x magnification with a 2x zoom, unless otherwise stated.

The antibodies used were as follows: mouse anti-Wg (4D4) diluted 100 fold, mouse anti-HA (BABCO) diluted 1000 fold, rabbit anti-FLAG (ab 6711, from abcam) diluted

1000 fol, rat anti-HA (affinity purified, Roche) diluted 2000 fold, rabbit anti-HA (santa cruz) diluted 400 fold and mouse anti-FLAG (M2, Roche), diluted 1000 fold. The following fluorescence conjugates were used: anti-mouse and anti-rabbit Alexa ™ 488 and Alexa ™ 592 and anti-rat Alexa ™ 594 from Molecular Probes, diluted 300 fold, and anti- rabbit Cy5 (Jackson ImmunoResearch Laboratories), diluted 200 fold.

2.2.3 Electron Microscopy of released material

Cells transiently transfected with HRP-Wg, secreted-HRP or mock transfected were allowed to grow and express for 2 days in serum free medium (Gibco). After this period the culture medium was spinned once at 1000 rpm for 3 min, collected and used for immunomagnetic isolation using Dynabeads® M-500 subcellular (Dynal Biotech), following the direct isolation procedure, according to the manufacture's protocol. The beads were coated with the secondary linker antibody (affinity purified goat anti-mouse IgG, Jackson Immunoresearch Laboratories) at a concentration 10 μg/10^7 Dynabeads®. I then used anti-Wingless (4D4) at a concentration of 4 μg to 10^7 of Dynabeads®.

After binding of Wingless to the beads, these were washed, and fixed in 2.5% glutaraldehyde/PBS for 20 min at 4° C. The beads were washed 3x 5 min in PBS/BSA 0.1% and stained for HRP using the DAB substrate in the following buffer: 1000 μl PBS/BSA 0.1%, 25 μl DAB (from a 1g/ml stock, kept at – 20° C) and 6 μl H_2O_2 to start the reaction. The reaction was allowed to proceed for 30 min at room temperature and the beads rinsed 4x in PBS and processed for electron microscopy. For this the beads were mixed with 2% agarose (50 μl) and centrifuged once at 6000 g to make a pellet. This was kept in ice for 20 min, washed, cut to a smaller size to include just the sample and transferred to a clean eppendorf tube. The pellet was post-fixed in 2.5 % glutaraldehyde/PBS for 1 hr and then again in 1% osmium tetroxide (OsO_4)/ 0.6% Potassium Ferricyanide ($K_3Cn(Fe)_6$) in PBS for 1 hr and

rinsed 3 x in PBS. The pellets were then dehydrated through a graded ethanol series (30%, 50%, 75%, 90% and 95%) and incubated in propylene oxide for 1 hr before Epon (Agar Scientific) embedding. Dehydration at 100% ethanol was omitted because it adversely affects the HRP signal. Sectioning and mounting of the samples was done by Elizabeth Hirst (Electron Microscopy lab, NIMR) as follows. 50nm ultra thin sections were cut and mounted on pioloform coated slot grids and stained for 5 minutes with standard Reynold's lead citrate. The samples were analysed and photographed in a Jeol 1200 EX electron microscope.

2.2.4 Western Blotting

Western Blotting was done as described (Sambrook, 1989). The samples were run on reducing 10% gels and Immobilon™-P membranes and the following antibodies were used: mouse anti-Wingless (4D4, from Developmental Studies Hybridoma Bank) diluted 500 fold, mouse anti-GFP (Living Colors® monoclonal antibody from BD Biosciences) diluted 1000 fold, mouse anti-FLAG (M2, from Sigma) diluted 2500 fold. The blots were probed with an HRP-conjugate rabbit anti-mouse antibody (from Sigma) diluted 15000 fold and the detection was done using the ECL or ECL plus detection systems (Amersham) and developed using BioMax MR films (Kodak). In all blots equal loading was checked by Ponceau (Serva) staining or using Sypro Ruby protein stain (Molecular Probes).

2.2.5 Stripping and Reprobing of membranes

When a membrane needed to be reprobed, stripping was performed by using Re-Blot Plus Strong solution (Chemicon Intl). The membranes were washed once in TBST, incubated in the stripping solution for 15 to 25 minutes, washed again and blocked for 1hr in 5% milk in TBST. The subsequent steps of antibody incubation and detection were done as described above.

2.2.6 Transient Transfections

S2 control cells or S2 cells stably expressing WgGFP were transfected using either the Calcium Phosphate method or the FuGENE reagent (Roche).

For Calcium Phosphate transfections, cells were seeded in 35 or 60 mm dishes and at around 80% confluency were transfected with the Calcium Phosphate precipitate. For 60 mm dishes, 0.4 ml sterile 0.25 M CaCl2 was added to 20 μg DNA in a sterile eppendorf and this mix was added drop by drop to 0.4 ml of sterile swirling 2xHEBS (16 g NaCl, 0.7g Kcl, 0.4 g Na2HPO4 anhydrous, 2 g d-Glucose, 10 g Hepes to 1 litre final volume). The mix was incubated for 30 min at room temperature and added to the cells while shaking the dish. The precipitate was left up to 14 hours, cells were then harvested, spinned down at 1000 rpm for 2 min and the medium was discarded and substituted for fresh serum free medium (SFM, from Gibco) and induced with CuSO4.

The FuGENE 6 reagent was used following the product instruction manual, with the following specifications. In all transfections, 1μg of DNA was used for 3μl of FuGENE reagent for a 35mm dish with 2 ml of medium. When higher volumes of medium were used the same 1:3 ratio was applied. Cells were incubated with the complex overnight (15 hrs), spinned down at 1000 rpm for 2 minutes and fresh SFM medium was subsequently added again. Cells were induced in fresh dishes with CuSO4.

Expression was induced either with 500μM CuSO4 for 1 day or with 350μM for 2 days. Mock transfections were done following exactly the same protocols but using no DNA.

The constructs used for transfections were the following: CD8GFP, SecrHRP, HRP-Wg (produced by C. Alexandre), dlpHA (the cDNA in pUAS was a gift from S. Cohen and was subcloned in pMTV5 by C.Alexandre) and dalllyFLAG, all cloned in the pMTV5 vector.

2.2.7 Differential centrifugation

WgGFP and HRP-Wg cells were induced and allowed to express the protein for 2 days in 2.5% or serum free medium (Gibco) The culture medium was collected and spinned first for 3 min at 1000 rpm to remove cellular debris, then centrifuged again at 20 000g for 30 min at 4°C using a SS-34 rotor to remove possible contaminating heavier cellular membranes and finally at 135 520 g for 1hr at 4°C, using a TLA-55 rotor to separate soluble and insoluble secreted material. The pellets (P135) were solubilised in RIPA buffer (0.15M NaCl 1% NP-40, 0.5% DOC, 0.1% SDS, 50mM Tris pH 8.0 and one mini Complete tablet of protease inhibitors (Roche) added per 10 ml of solution) and processed for Western Blotting using standard protocols (Sambrook et al, 1989). Supernatant from the high speed spin (S135) was concentrated with Centriprep 30 or Centricon 30 concentrators (Amicon) as described in the product manual. Volume was reduced to match the volume used to ressuspend the corresponding pellet.

For the preparation of cell extracts, cells were pipetted up and down, separated from the supernatant by spinning at 1000 rpm for 3 min and solubilised in RIPA buffer.

2.2.8 Sucrose Density Gradient of WgGFP supernatant

A stable cell line expressing WgGFP and transiently transfected with CD8GFP was induced for 2 days in SFM medium (Gibco) and 20 ml of culture medium was collected, cleared by centrifugation for 30 min at 20 000 g and concentrated to 2 ml. The concentrated supernatant was mixed with 80% sucrose in TNE (0.1M Tris pH 7.5, 0.15M NaCl, 0.2mM EGTA) and overlaid with equal volumes of 30% and 5% sucrose in TNE. The gradient was spun for 15 hrs at 35K (154 000g) in an SW-40 Ti rotor. 1 ml fractions were collected starting either from the bottom or the top of the gradient and the density was measured for each sample, using a refractometer (Bellingham and Stanley Ltd.). The proteins were TCA precipitated and

ressuspended in 40µl of PBS. SDS-sample buffer was subsequently added and samples were loaded equally on a 10% SDS-PAGE gel.

2.2.9 TCA precipitation

For protein precipitation, Tricloroacetic acid (TCA, Sigma) was added to a final concentration of 10 %, incubated on ice for 15 minutes and spun at 14 000 rpm in a microcentrifuge for 20 minutes. The supernatant was discarded and the pellet was washed with 1 ml of 100% ice-cold acetone. The pellet was spun again for 10 minutes and allowed to dry after removal of the acetone. After this step, 25 to 40 µl PBS was added, depending on the amount of precipitated protein and the pellets were shaken at 37° C for several hours. If necessary, to facilitate ressuspension of the pellets, Tris pH 9.0 was added.

2.2.10 Sucrose density Gradient of HRP-Wg supernatant

A stable line expressing HRP-Wg was subjected to the same procedure as described above for WgGFP. The gradient was spun at the same speed and 1 ml fractions were collected from the top of the gradient. The sample densities were measured using a refractometer and all samples were stained, according to the product manual, with o-phenylenediamine (OPD) dihydrochloride (Sigma), a soluble substrate for horseradish peroxidase conjugates. The reaction was either stopped with 3N HCl and read spectrophotometrically at 492nm or left to proceed in aliquots in an ELISA plate and the HRP activity measured kinetically at 450nm in an ELISA reader, using the GENESIS software in an ELISA reader. The correlation of density to concentration and % by weight was done by using a Beckman information sheet (Techniques of Preparative, Zonal and Continuous Flow Ultracentrifugation).

2.2.11 Tx-114 Phase Separation

For Triton X-114 Phase Separation the supernatants with secreted HRP-Wg protein or of control non-expressing cells were collected, cleared by centrifugation at 20 000g for 30 min and concentrated 50x. Phase separation was adapted from Willert et al 2003 with the following differences.

The media were mixed 1:1 with ice cold 4.5% Triton X114 (from a 10% stock) in a 150mM NaCl, 10mM Tris-HCL pH7.5 buffer and incubated on ice for 5 minutes. The samples were then warmed to 33° C for 5 minutes, mixed and spun for 2 minutes at 13 000 rpm in a microcentrifuge, at room temperature (25° C/ 26 ° C). The aqueous (top) phase was separated from the bottom (detergent) phase and 50 μl aliquots were diluted 5x in the reaction buffer and used for HRP activity detection.

Detection of the HRP activity was done using OPD as described above.

The reading programmes used were the following: 1st: readings every 20 seconds for a total of 5 minutes with 5 seconds mixing intervals. 2nd: readings every minute for a total of 15 minutes with 5 seconds mixing intervals.

2.2.12 PI-PLC treatment

S2hsWg(+) and (-) cells were heat shocked for 40 minutes to induce expression, and allowed to recover for 40 minutes. $2x10^6$ cells were treated with PI-PLC, as described (Tsuda et al., 1999). After the treatment, samples were centrifuged at 3000 rpm for 3 minutes to separate the cells from the supernatant. The supernatants were differentially centrifuged as described above and all samples were processed for Western Blotting.

2.2.13 Detergent Treatment

S2hsWg (+) and (-) cells were allowed to grow in 60 mm dishes to confluency, heat shocked for 45 minutes and allowed to recover for 1 hour. The medium was substituted for SFM and cells were allowed to express the protein for another 2

hours. Cells and conditioned medium were collected. The medium was cleared at 20 000 g for 30 min. at 4° C. These were treated with different combinations of cold detergents. The solutions used were the following: A- 1mM Sodium Deoxycholate (Sigma), 0,1 % Nonidet P-40 (BDH), 0.01 % SDS, 150mM NaCl, 50mM Tris-HCL, pH 8.0, B- Triton X-100 1% (Sigma), 150mM NaCl, 50mM Tris-HCL pH 8.0, C- Octyl β-D-glucopyranoside 60 mM (Sigma), 150mM NaCl, 50mM Tris-HCL pH8.0. The samples treated with the detergent solutions and control samples (Wg conditioned media treated just with buffer and control media not containing Wg also treated only with buffer) were incubated on ice for 30 min. After treatment, the cells were centrifuged at 100 000 g for 1 hr, as before and the cell extracts (CE), sup 100 and P 100 were processed for Western blotting. The final volume of the P100 fraction was adjusted to 4x the volume of sup 100 or CE.

2.3 Molecular biology

2.3.1 Small scale preparation of DNA

The Qiagen Spin miniprep kit (Qiagen) was used for all small-scale plasmid preparations, according to the manufacturer's protocol.

2.3.2 Large scale preparation of DNA

The Qiafilter MaxiPrep kit (Qiagen) was used for all big-scale plasmid preparations, according to the manufacturer's protocol.

2.3.3 Nucleic acid quantification by spectrophotometry

Nucleic acid quantification was performed by spectrophotometry at λ = 260 nm, where an optical density (OD) unit corresponds to 50μg/ml of double-stranded DNA or to 40μg/ml single-stranded RNA. The ratio between the readings at λ= 260 nm

and λ= 280 nm provided an estimate of the purity of the nucleic acid preparation (pure preparations of DNA should have OD_{260}/OD_{280} ratio of 1.8).

2.3.4 Agarose gel electrophoresis

Nucleic acid size determination and/or separation were performed by agarose gel electrophoresis. Gels were prepared by dissolving agarose in 0.5x TAE (20 mM TRIS acetate, 1 mM $Na_2EDTA.2H_2O$ (pH 8.5)) to a final concentration of 0.8 – 1.2% (w/v), depending on the expected size of the DNA fragments, and 0.5 mg/ml ethidium bromide. Nucleic acid samples were mixed with 2x gel loading buffer (2x TAE, 50% v/v glycerol, 0.25% w/v bromophenol blue) and, in the case of RNA, a specific loading buffer with RNase inhibitor was used (Ambion). Electrophoresis was performed at 60 V until appropriate resolution was achieved. Ethidium bromide-stained nucleic acid was visualised using ultraviolet light (λ ~ 302 nm) and fragment size was estimated by comparison with the 1 kb ladder molecular weight markers (Gibco BRL) run in at least one of the gel lanes.

2.3.5 Gel extraction of DNA

For the extraction of DNA from agarose gels the QIAquick Gel Extraction Kit (Qiagen) was used according to manufacturer's protocol.

2.3.6 Ethanol precipitation of nucleic acids

EtOH precipitation of nucleic acids was carried out by adding 1/10 volume of 3 M sodium acetate (NaAc) (pH 5.2) and 2.5 volumes of cold 100% EtOH to the nucleic acid solution. This mixture was left at -20°C for approximately 20 min. Centrifugation was carried out at 20,000 x g for 20 min, the pellet was washed in 70% EtOH and spun again at the same speed for 15 min. After EtOH removal, the nucleic acids were left to air-dry at room temperature for approximately 10 min and re-suspended in TE (1 mM EDTA, 10 mM TRIS.HCl pH 8.0) or distilled water.

2.3.7 Restriction digestion of DNA

Restriction enzyme digests were performed at the recommended temperature for approximately 1h 30min using commercially supplied restriction enzymes and buffers (Boehringer Mannheim, New England Biolabs). The enzyme component of the reaction never comprised more than 10% of the reaction volume. For multiple enzymatic digests, the most compatible buffer for all the enzymes used was chosen as long as all the enzymes were predicted to digest at least 75% of the DNA in those conditions.

2.3.8 Messenger RNA purification from cells

Poly-T-coated Dynabeads, as part of a Dynabeads kit (Dynal), were used to purify messenger RNA from S2hsWg(+) and S2hsWg(-) cells, according to manufacture's instructions.

2.3.9 RT-PCR

First strand cDNA was synthesised from total RNA template using random hexamer primers or a poly-T primer and superscript reverse transcriptase (Gibco BRL). To this end 20μl aqueous reactions were prepared, containing 1μg of RNA, 1μl of 0.1mg/ml primer, 4μl 5x RTase buffer, 1μl 20mM dithiothritol (DTT), 0.5μl RNase inhibitor, 2μl 5mM dNTP mix and 0.25μl RT. A negative control devoid of RT was also prepared. Reactions were allowed to proceed at 42 °C for 30 min, after which cDNAs were used as templates for PCRs with specific primers. The PCR was carried out for 20 cycles with the following programme: 1min at 93°C, 1min at 53°C and 1 min at 72°C. The primers used to amplify gene fragments are the following: CAA GCG GAC AAT AGC CCT GCC CCC CAA GTG GCC and GCA AGT CCA CGC GAC CGT TGG CGA AGT AGG for *dly* and ATT TAC CCT GCT CTG CGG CTT CGT CGG CC and CGA GTG GGT CAT GTT CTC CGA AAT CTG CGC C for *dally. fused* (*fu*) was used as control using the following primers: GGT GGT GGC

CAT AAT GGT AAT CTC CAA GCG CGG and GCA GCC CAG GTA CCA CAT GTC CGC.

2.3.10 Construction of the tagged versions of dally

To produce a FLAG tagged version of dally, a Aat II site was created in pBluescript KS dally (a gift from S. Selleck, University of Arizona) for the insertion of FLAG at the N-terminus. The DNA was cut with Nhe and Xho and subcloned in pMTV5.

To insert dallyFLAG in the UAS vector , pBluescript KS dally was cut with EcoRI and Xho and subcloned in pUAS.

Chapter 3 – Regulation of Wingless Distribution *In Vivo*

CHAPTER 3 - REGULATION OF WINGLESS DISTRIBUTION *IN VIVO*

3.1 Wingless distribution in the embryonic epidermis

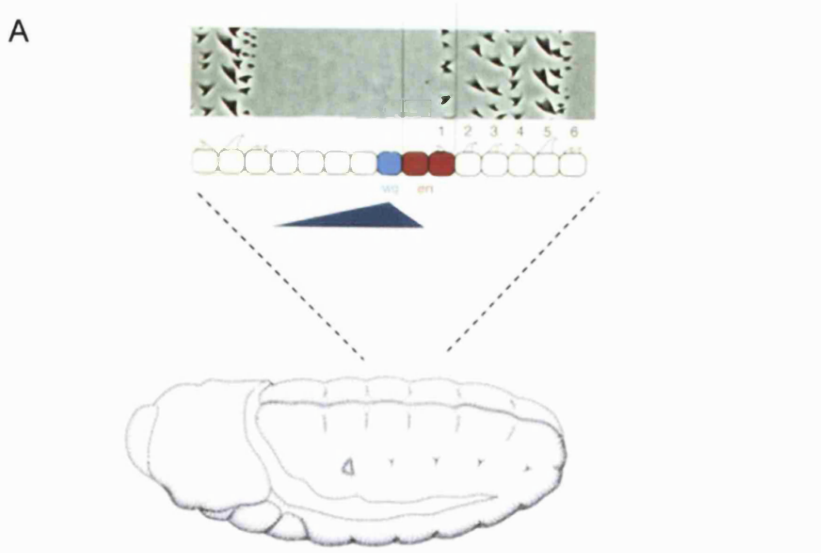
3.1.1 Detection method of Wingless at the cell surface

When a standard Wingless antibody staining is done, the signal from the intracellular protein is very strong, masking the signal from the protein at the cell surface. Since one of my aims is to understand how Wingless distribution is regulated at the cell surface I developed a method to detect only the cell surface protein. The procedure is an adaptation of a method devised by Strigini & Cohen (2000), for *Drosophila* wing imaginal discs, as described in "material and methods".

To verify that this procedure only detects extracellular antigens, a double staining for Wg and βgal was performed on embryos of the genotype *engrailed*-GAL4 UASnlacZ, that express nuclear lacZ in the *engrailed* cells. These embryos show a weak Wg staining and no nuclear lacZ staining (Fig. 3.1 B, D). In embryos of the same genotype stained using the conventional method, lacZ is easily detected (Fig. 3.1 C). Therefore, the extracellular staining protocol does not detect intracellular signal, even if very strong, as is the case of en-driven lacZ.

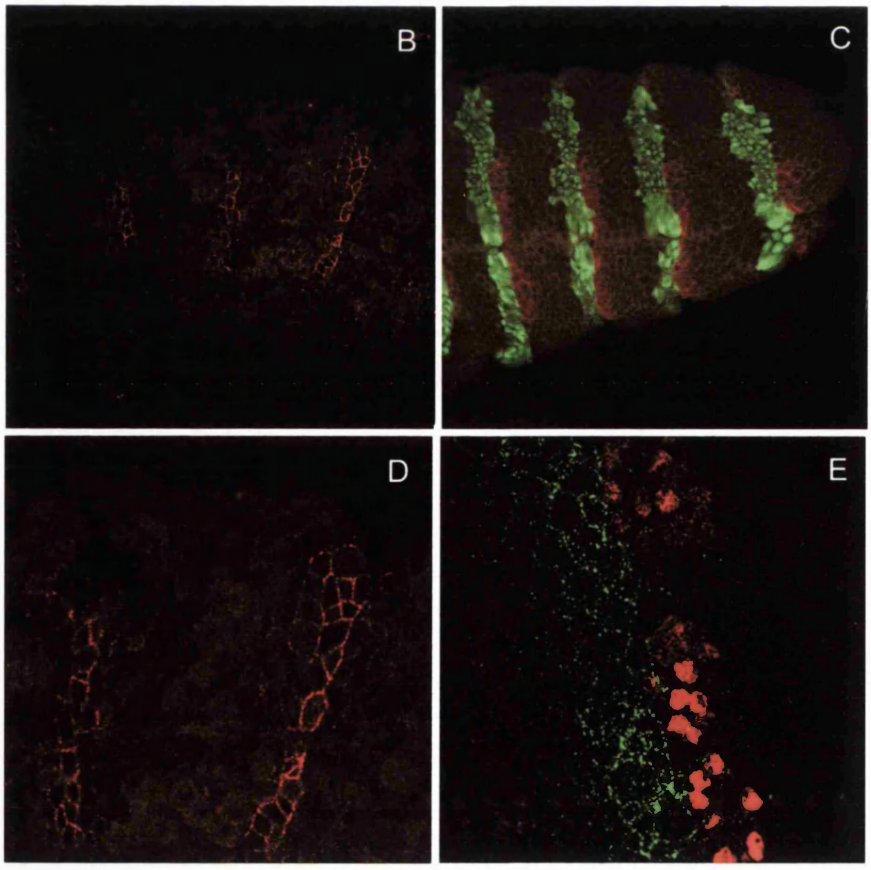
Extracellular staining viewed at high magnification reveals Wingless to be uniformly present at the cell surface, in 3 cell wide stripes (Fig. 3.1 D). Conventional staining with anti-En following staining for extracellular Wg shows that extracellular Wg is detected in wg expressing cells which line the en domain and in cells located 2-3 cell diameters at the anterior (Fig. 3.1 E).

Figure 3.1- Extracellular Wingless is detected 2 to 3 cells anterior to the source.
A- Diagram of a wild-type embryo at stage 11 showing the wingless and engrailed domains of expression and the Wingless domain of action, in blue. *wingless* later specifies naked cuticle, as represented.
Anterior is to the left and dorsal is up.
Embryos at stage 11 of genotype *en*-GAL4 UAS nlacZ, stained for lacZ (green) and Wingless (red) are shown in B,C and D. Detection of engrailed (red) and Wingless (green) in D. Figure B shows an embryo stained with the extracellular method and figure C shows an embryo stained with the conventional method.
D is a magnification of the embryo shown in B, showing extracellular Wingless in 3 cells in each segment.
In figure E is shown an embryo stained with the extracelular method for Wingless (green), followed by a conventional staining to detect engrailed (red), in which is clear the presence of Wingless 3 cells anterior to the source, adjacent to the en expressing cells. In all images, anterior is to the left.
Figure C shows a dorsal view of the embryo, all other images show ventral views.
Figure D and E show a two fold magnification.



Extracellular Wg En

Total Wg En



Extracellular Wg En

Extracellular Wg
Total En

3.1.2 Extracellular Wg is retained in expressing cells

The wingless secreted protein, in order to pattern the tissue, needs to travel 3 to 4 cells away from the source. As mentioned above, two mechanisms could account for this movement: in one, Wg spreads along the tissue and in the other, Wg is carried along by moving and dividing cells. Either mode is sufficient for the establishment of Wingless normal range of action (Pfeiffer et al., 2000).

It had been shown before that Wingless lingers in the secretory pathway for a long time, thus explaining in part the retention in expressing cells and inheritance of the Wg protein in daughter cells (Pfeiffer et al., 2000). I asked whether Wingless is also retained at the cell surface. To test this I used embryos of the genotype *wg-*, *engrailed*-GAL4 UAS-GFP-*wingless*, which are confined in a well defined expression domain. I observed that Wg is present almost exclusively at the surface of expressing cells, and that very little is detected in flanking cells (Fig 3.2 A), Some active protein must reach these flanking cells because they produce naked cuticle, under these experimental conditions (Fig 3.2 A, inset). However, the bulk of detectable Wg remains attached to the surface of expressing cells. Using the same staining procedure in wild-type embryos, extracellular Wingless is detected in a 3-4 cells stripe anterior to the source, which roughly reflects the wingless domain of action (Fig 3.2 B). This is the same domain where intracellular wg seems to linger in the secretory pathway.

I conclude that wg protein is retained both intracellulary and at the surface of expressing cells and their descendants.

3.1.3 Wg is lost in *sugarless* mutant embryos

It has been suggested that Wg binds heparan sulfate proteoglycans in culture (Reichsman et al., 1996), and genetic studies have revealed that in the absence of each of two known enzymes required for heparan sulfate biosynthesis (*sgl* and *sfl*)

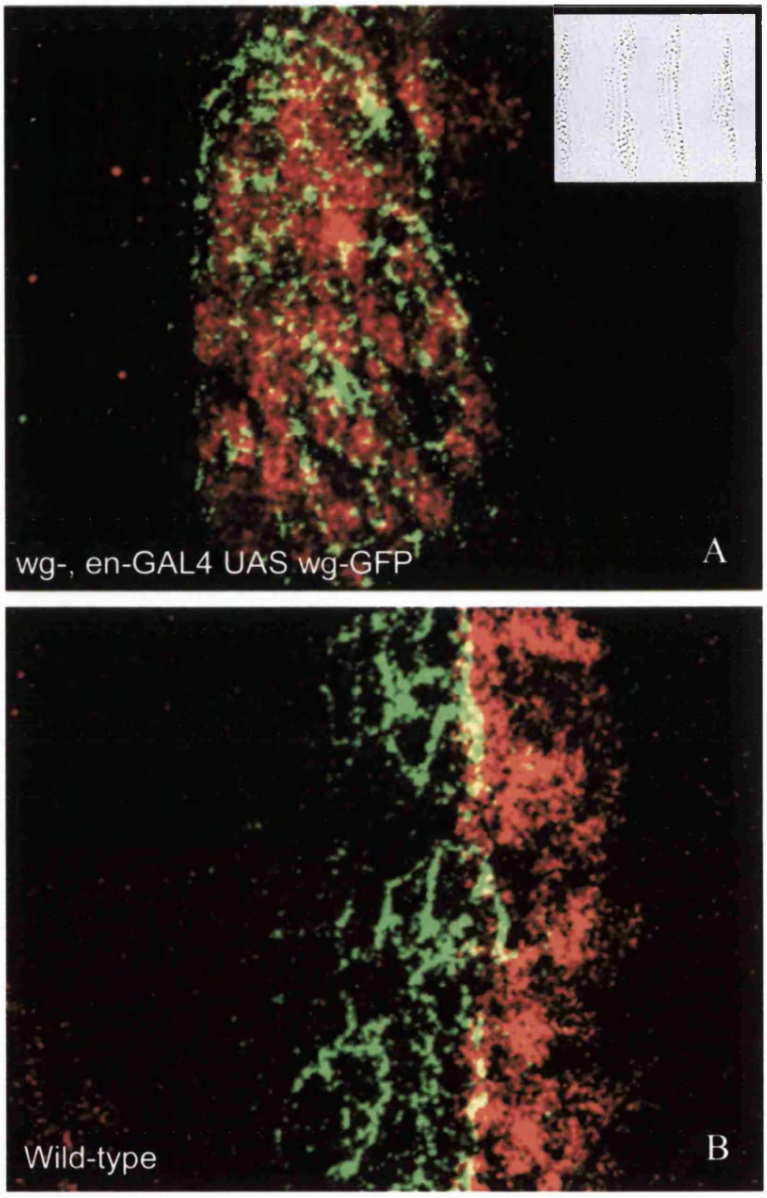


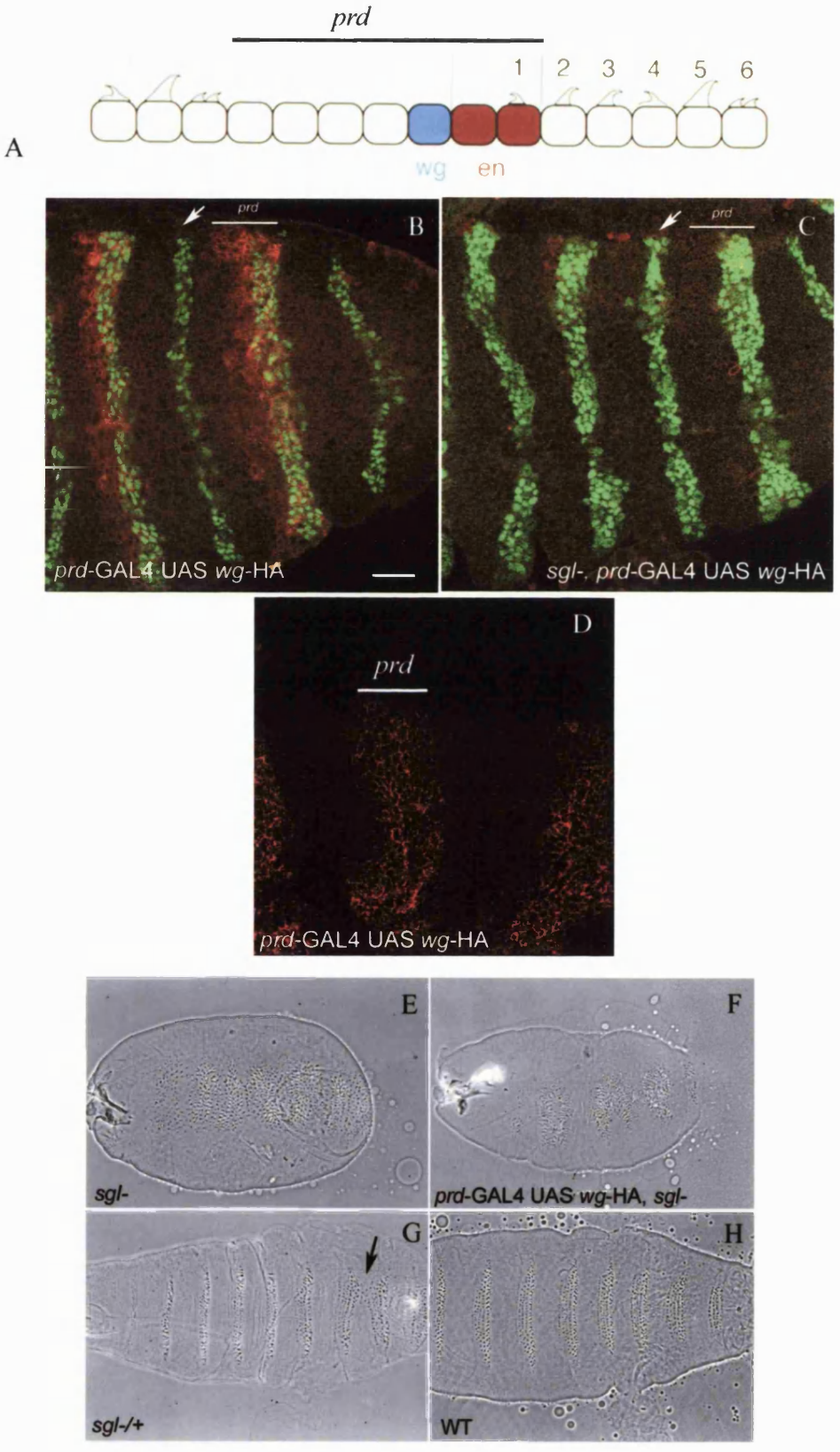
Figure 3.2- Extracellular Wg is retained in expressing cells.
Extracellular anti-Wg staining (green) followed by anti-En (red) staining in stage 11 embryos of genotype wg-, en-GAL4 UAS wg-GFP (A) Wg is retained in expressing cells and no Wg is detected outside this domain.
The same procedure was used to stain stage 11 wild-type embryos (B)
In both images anterior of the segment is to the left
Inset in A shows the cutice phenotype originated from the embryos of genotype: wg-, enGAL4 wgGFP. The naked cuticle is specified indicating that wg-GFP is secreted and transported to that domain.
(image of cuticle by S. Pfeiffer)

wg function is impaired (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999). These observations suggest that the tight association of Wg to the membrane could be due to binding to HSPGs. I investigated the possible role of the HSPGs in retention by analysing the distribution of Wg in *sugarless* mutant embryos.

The sgl gene is maternally contributed so in order to remove all activity it is necessary to produce germline clones. I used the FLP/FRTovoD system, which produces embryos of four genotypes: *prd*-gal4/+, *sgl*/UAS*wg*-HA*sgl*; hsflp/+, *sgl*/UAS*wg*-HA *sgl*; *prd*-gal4/+, *sgl*/TM3 and hsflp/+, *sgl*/TM3. Most embryos carrying only one copy of *sugarless* have a normal cuticle phenotype, with only a small number showing cuticular defects (Fig. 3.3 G, arrow). In the absence of *sgl* no naked cuticle forms indicating lack of wg signalling (Fig. 3.3 E). However, expression of exogenous *wg* in alternate segments in *sgl* mutant embryos leads to the formation of naked cuticle, indicating that *sgl* is not absolutely required for signaling (Fig. 3.3 F). I looked at the distribution of Wg in similar experimental conditions. Embryos mutant for *sgl* and in which exogenous HA-tagged Wg is expressed in the *paired* (*prd*) domain were selected. *prd*-gal4 was used as a driver because it is unaffected by changes in wg signalling. The *prd* domain comprises the *en* cells and four cells anterior to these (Fig. 3.3 A). Only the distribution of HA tagged proteins was considered and En detection was used as a spatial marker. I found Wg to be almost totally lost in embryos that are mutant for *sgl* (Fig. 3.3 C). Since such staining protocol reveals both the intracellular and cell surface signal, I conclude that Sgl affects the amount of Wg protein both in the secretory pathway and at the cell surface.

Because *prd*-gal4 induced transcription is unchanged in sgl mutants, the decrease of Wg protein in *sgl*-deficient embryos could be due to lack of retention in expressing cells or to a decrease in protein stability. As indicated before, in a *sgl* mutant, *en*

Figure 3.3 Wg is lost in *sugarless* mutant embryos
A- Diagram of the embryonic epidermis, showing *paired* (*prd*) pattern of expression.
B-D - Anti-En staining (green) and anti-HA (Wg) staining (red) in stage 11 embryos of genotype *prd*-gal4, *sgl*/ UASwg-HA*sgl* (C) and control embryos of genotype *prd*-gal4 UASwg-HA (B). extracellular anti-HA (Wg) staining in stage 11 control embryos (D).
Most intracellular and extracellular Wg is lost in *sgl*- embryos (C)
The arrow in C indicates partially rescued *en* expression in segments where *prd* is not expressed (compare with arrow in B)
All photographs are at the same magnification and the scale bar represents 35um.
E-H - Cuticle phenotypes of embryos originated from the cross:
hsflp/+, *sgl* /UASwg-HA *sgl* (E), *prd*-GAL4/+, *sgl* /UASwg-HA *sgl* (F), *prd*-GAL4/+, *sgl*/TM3 or hsflp/+, *sgl*/TM3 (G). A wild-type cuticle is shown for comparison (H).
All photographs are at the same magnification of 20x.


A
prd
1 2 3 4 5 6
wg en
B
prd-GAL4 UAS wg-HA
C
sgl-, prd-GAL4 UAS wg-HA
D
prd
prd-GAL4 UAS wg-HA
E
sgl-
F
prd-GAL4 UAS wg-HA, sgl-
G
sgl-/+
H
WT

expression is switched off as a result of lack of Wingless (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997). I note here that exogenous Wg in *sgl* embryos leads to a partial rescue of en expression. En expression is even rescued in the segments where *prd* is not expressed, as also observed by Binari and co-workers (1997) (Fig. 3.3 C, arrows). This is consistent with the cuticle phenotype that shows rescue of naked cuticle, sometimes in all segments (Fig. 3.3 F). Hacker et al (1997) observed the same cuticle phenotype in embryos of the same genotype. This suggests that Wg protein is present and that its range is increased in *sgl*-deficient embryos. The fact that I do not detect secreted Wingless is likely due to its rapid dispersal in the perivitelline space. These results favour the hypothesis of a decrease in Wg protein levels due to lack of retention in the cell.

In a *sgl* mutant in which Wingless is not added exogenously, wg signalling is reduced because of protein dilution. As a result, hh expression is incompletely maintained and wg transcription decays rapidly leading to the canonical loss of naked cuticle (Fig. 3.3 E).

3.1.4 Dally pattern of expression

Two proteins of the glypican class of Heparan Sulfate Proteoglycans, Dally and Dally-like (Dlp), have been suggested to have a role in wingless signalling (Baeg et al., 2001; Lin and Perrimon, 1999; Tsuda et al., 1999)

Dlp is expressed in most of the wg domain of action, in the cell that expresses wg and three cells anterior to these, in each segment (Khare and Baumgartner, 2000).

Since the information regarding the pattern of *dally* expression in the literature was contradictory, I did an *in situ* hybridisation on embryos to assess *dally* expression at several stages of embryonic development. The embryos were probed for *dally* and *engrailed*. I found *dally* to be expressed in a dynamic manner. It is expressed uniformly at stage 5 starting to be expressed in stripes at stages 7/8 while at stage 9 *dally* is expressed in the Wg range of action (data not shown). At stages 10 and 11,

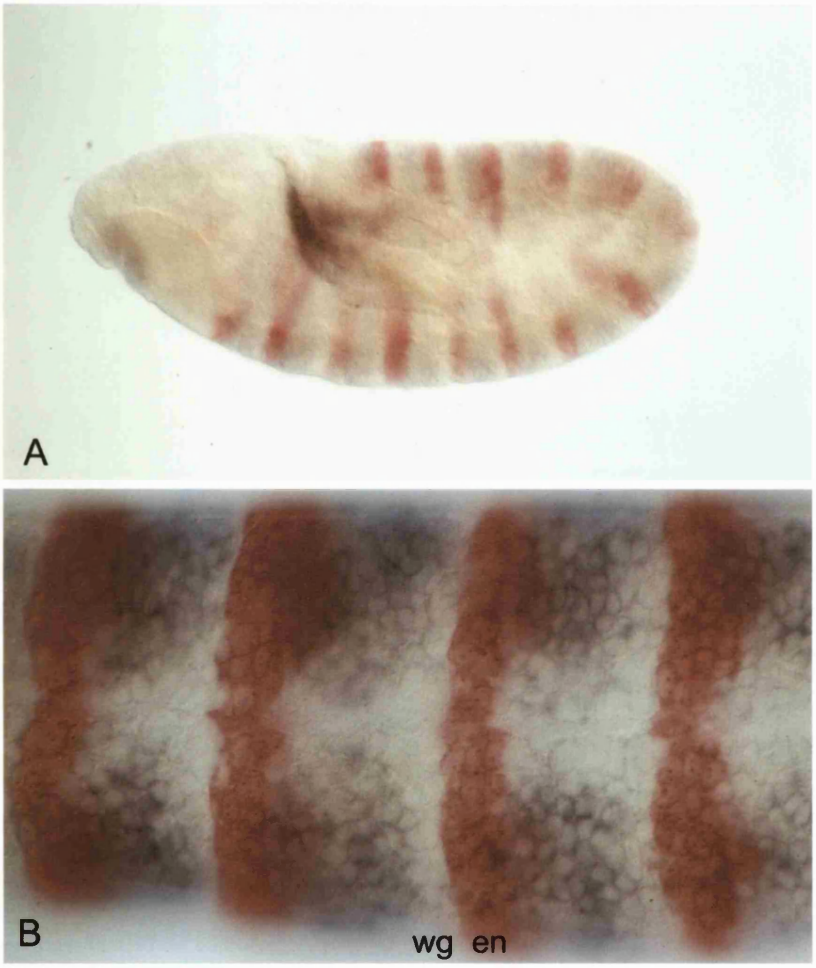


Figure 3.4- In situ detection of dally and engrailed mRNA.
A shows a whole embryo at stage 11. B is an enlargement of the ventral region of the same embryo.
It is evident the expression of dally uniformly in the epidermis, except in the wg and en expressing cells.
In both images, anterior is to the left and dorsal is up

when *wg* specifies naked cuticle, *dally* is expressed in all cells of the epidermis except those that express *wg* and *en* (Fig. 3.4). By late stage 12 *dally* expression fades away.

Thus, *dlp* is expressed in the right cells to participate in Wg retention while *dally* is not.

3.1.5 Misexpression of *dally* and *dally-like* in the embryonic epidermis

To understand the role of the proteoglycans in Wg distribution we misexpressed *dally* and *dally-like* in distinct spatial domains in the embryonic epidermis, using the GAL4-UAS system (Brand and Perrimon, 1993). It has been shown that overexpression of *dally* with a heat shock promoter produces naked cuticle in the central part of the segment (Tsuda et al., 1999). However, overexpression derived from heat-shock experiments is not always reliable, so I set out to confirm this result by using the GAL4 system. If *dally* contributes to *wg* signalling we would expect an increase in the extent of naked cuticle if we express *dally* uniformly in the epidermis, using armadillo-gal4. However, the cuticle phenotype of these embryos resembles that of wild-type (Fig. 3.5), although most embryos die. The same result is obtained when *dally* is expressed in the *wg*, *en, patched*, *ftz* and *paired* domains (data not shown) and most of the embryos hatch. (*wg*: 88% n=167 embryos, *ftz*: 78% n=187 embryos, *prd*: 67% n=178 embryos)

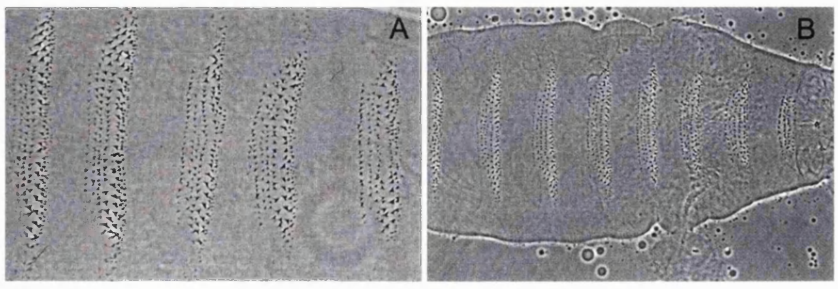


Figure 3.5- Cuticle phenotype of an *arm*-GAL4 UAS dally embryo.

A Cuticle phenotype of an embryo originated from a cross of *arm*-GAL4 flies with UAS *dally* flies. The cuticle shown has been selected as representative of the strongest phenotype found. Magnification is 40x. B shows a WT cuticle phenotype at a magnification of 20x.
In both cases anterior is to the left.

Again these results suggest that dally may only have a minor role on wg function.

To assess the role of *dally-like* in Wingless distribution we misexpressed this gene in the *wingless* and *engrailed* domains. Again, if *dally-like* (*dlp)* contributes to wingless signalling we would expect a small increase in the amount of naked cuticle when *dlp* is expressed in the *en* cells. On the other hand, if Dlp is involved in retaining Wg in the expressing cells we could imagine that misexpressing the gene would lead to a dominant negative phenotype. However, when *dlp* is misexpressed in these domains the embryos have a wild-type cuticle phenotype (data not shown).
By looking at the distribution of Wingless when *dlp* is overexpressed in the wg or en cells, it is clear that Wg accumulates at the surface of the cells that overexpress *dlp*, whether they are receiving or producing the signal. In both cases, Wingless accumulates at the cell surface, with virtually no staining inside the cell, which can be seen in the control embryo as a fuzzy signal (compare Fig. 3.6 A, B with C). All visible vesicles seem to be around the cell and most colocalize with Dlp (seen in yellow).
This accumulation seems to be only in the cells that express *dlp* and the expression of the proteoglycan does not have an effect on Wg in adjacent cells, as can be seen by the distribution of Wg when *dlp* is overexpressed in the engrailed cells (the engrailed domain of expression is seen in blue). In the cells that do not express *dlp*, Wg is as wild-type (Fig. 3.6 A, arrow).
As seen in Fig.3.6 A, posteriorly, Wg accumulates in all engrailed expressing cells, although wild-type Wg at stage 11 is only present in the most anterior engrailed cell.

An explanation for this is that the accumulation is likely to have occurred at an earlier stage when the range of Wg is still symmetric. Alternatively dlp may prevent Wg degradation.

Also, when analysing the Wg distribution in cells that overexpress *dlp* in the wg expressing domain, I find that Dlp is not restrained to the wg expressing cell and that Wg spreads in the normal range, 3 to 4 cells anteriorly to the source, but shows accumulation at the cell surface (Fig. 3.6 B). The observation that Dlp does not spill from the en domain when overexpressed there (Fig. 3.6 A), suggests that Dlp and Wg travel together carried by expressing cells and their daughters, by one of the mechanisms of signal dispersal described before.

These results suggest that Dlp might have some binding affinity to Wg and that it leads to its accumulation when expressed in the same cells. Another possibility is that *dlp* expression leads to binding of Wg to another protein, which would also lead to the accumulation at the membrane. These results are consistent with the possibility that Dlp could retain Wg at the cell surface of expressing cells.

In embryos of genotype *wg*-GAL4 UAS-*dlp* the cuticle is correctly specified. Thus, increased retention is compatible with a normal range of signalling. This is consistent with the finding that membrane-tethered Wg rescues a *wg* mutant (Pfeiffer et al., 2000).

In the embryos in which *dlp* is expressed in the engrailed cells the specification of the cuticle is not affected because Wg has a wild type distribution anterior to the source. In a small proportion of the cases, the cells adjacent to row 1 have ectopic denticles (Fig. 3.6 A'). These are the result of lack of wg signalling in the first engrailed cell where Wg is normally active. Thus, *dlp* has a mild dominant negative effect on signalling even though it leads to the accumulation of Wg at the cell surface. I will return to this point when I discuss the results obtained in imaginal discs.

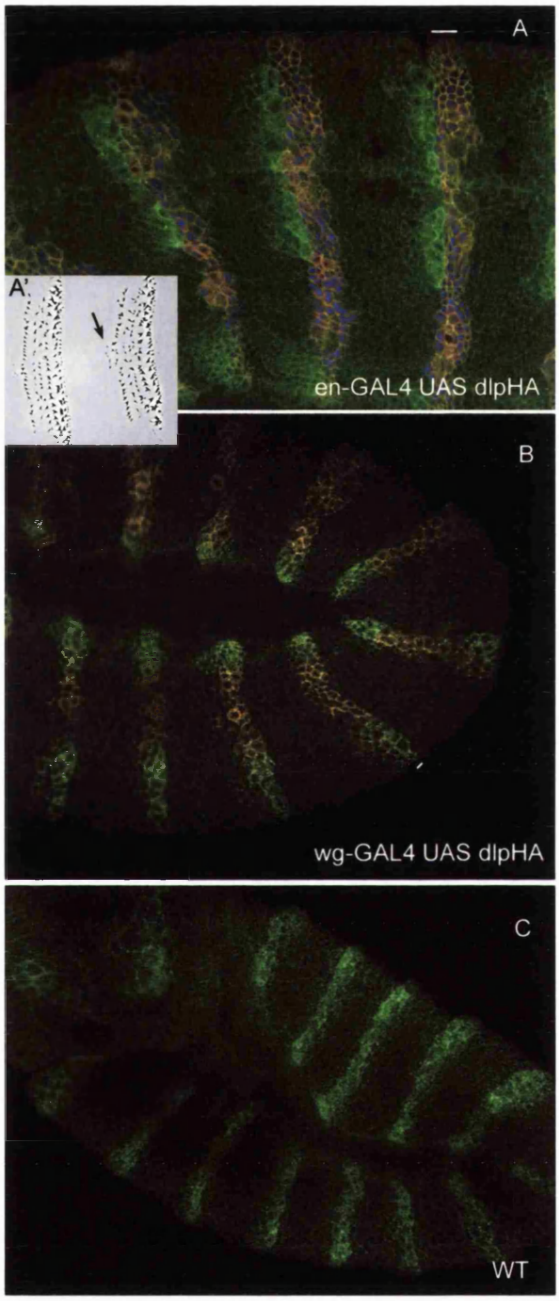


Figure 3.6- Wingless is distributed mostly at the cell surface of cells that overexpress dlp
A and B show stage 11 embryos of genotype en-GAL4 UAS nlacZ UASdlpHA (A)
and wg-GAL4 UAS dlpHA (B). C shows a stage 11 control wild-type embryo.
In A, Wg is stained in green, HA (Dlp) in red and lacZ (En) in blue
In B, Wg is stained in green and HA (Dlp) is stained in red.
In C, a wild-type embryo is shown, stained for Wg.
The images represent single confocal sections. In all images, anterior is to the left
and dorsal is up
Wg accumulates at the surface of cells when dlp is overexpressed,
either in the en (A) or wg (B) expressing cells.
Note the signal fuzinness and scatered Wingless vesicles on the WT embryo (C),
that are not present in the embryos that overexpress dlp.

3.2 Wingless distribution in wing imaginal discs

To further analyse the role of *dally* and *dlp* in regulating Wg distribution we chose the wing imaginal disc as our experimental system. This system has some advantages over the embryonic epidermis. Cell movement does not contribute to transport and the wg and hh pathways are not dependent upon each other, as it does in the embryo. This is especially important since it has been recently reported that *dlp* is required for *hh* signalling (Lum et al., 2003).

Moreover, in this tissue Wg travels at a distance of up to 10 cells symmetrically from the source, allowing for the study of long-range transport.

3.2.1 Wg is retained in expressing cells of wing imaginal discs

Since, in the embryonic epidermis, Wg is mostly retained in expressing cells we asked if Wg behaves the same way in this tissue. In the wing imaginal disc, Wg spreads approximately 10 cells away from expressing cells, which are located along the dorso-ventral boundary. I used the GAL4 system to misexpress WgGFP in two different spatial domains in the wing, *apterous* (*ap*) and *decapentaplegic (dpp)*. *apterous* is expressed in the dorsal compartment of the wing pouch and *decapentaplegic* is expressed along the anterio-posterior axis, in the anterior compartment. Exogenous WgGFP, observed by the GFP signal, is mainly detected in the expressing cells, as seen in the embryonic epidermis (Fig. 3.7 A, B). Wingless vesicles can also be detected some cell diameters away from the expressing cell, travelling ventrally, away from the apterous domain (Fig. 3.7 A, arrows) and posteriorly, away from the dpp domain (Fig 3.7 B, arrows), indicating that not all Wingless is retained in the cells that make it.

3.2.2 Misexpression of *dally* in wing imaginal discs

As mentioned above, the Heparan Sulfate Proteoglycans are likely to play a role in the regulation of Wingless distribution. However, overexpression of *dally* has no

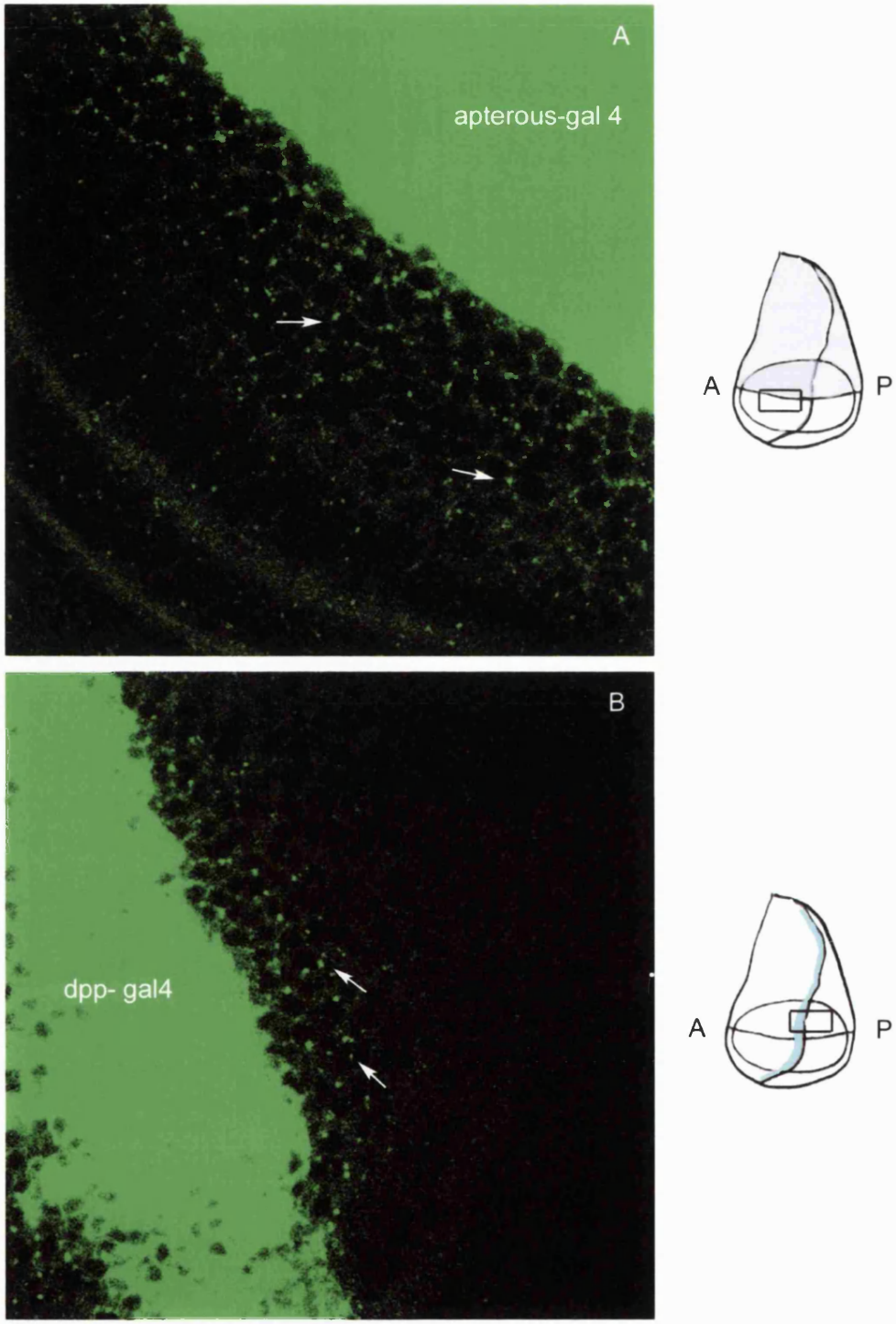


Figure 3.7 Wingless is retained in expressing cells in wing imaginal discs
WgGFP expressed in the apterous domain (A) and the dpp domain (B), detected by the GFP signal. arrows indicate vesicles outside the expression domain. In both pictures dorsal is up and anterior to the left.
The diagrams indicate the domains of expression of ap and dpp and the boxes correspond to the picture area.

apparent effect on Wg in imaginal discs (Baeg et al., 2001; Strigini and Cohen, 2000). Strigini and Cohen (2000) addressed this question by overexpressing *dally* in the patched domain, along the anterior-posterior boundary, and did not observe an alteration of the normal range of Wg or its localisation within the cells. In the third instar wing imaginal disc, *dally* is expressed at the dorso-ventral boundary in a stripe of 10 to 14 cells, partially overlapping the wg domain, and at the anterior-posterior boundary in a stripe of 2 to 4 cells, as seen with a *dally* enhancer trap insert (*dally* ^{P2}) (Jackson et al., 1997).

To understand better the role of this gene, I overexpressed *dally* in the *wingless* or the *dpp* domain, using the GAL4 system. Since good antibodies are not available for Dally we constructed a transgenic line that produces FLAG tagged Dally. The dpp domain was visualised by using a *dpp*-GAL4 UASGFP line.

When *dally* is misexpressed either in the wingless (Fig. 3.8) or the dpp domain (not shown) there is no obvious effect on Wingless distribution. A higher magnification image shows that Wingless is in vesicles and is transported at a normal distance from the source (Fig. 3.8 C). Consistent with this, the wing phenotype of the adult flies is as the wild-type (not shown).

3.2.3 Misexpression of dally-like in the wing imaginal disc

Although it has not been possible yet to understand the effect that *dally* might have on Wingless, there has been some evidence that *dally-like* (*dlp*) could influence its range.

It has been reported that overexpression of dlp, either in the *ptc* or *en* domain, leads to an accumulation of Wingless at the cell surface (Baeg et al., 2001). The *patched* domain consists of a 3-4 cell-wide stripe along the anterior-posterior axis, while *engrailed* is expressed in the posterior compartment of the wing pouch. Interestingly, overexpression of *dlp* in the wing margin, with the C96-Gal4 driver, seems to induce

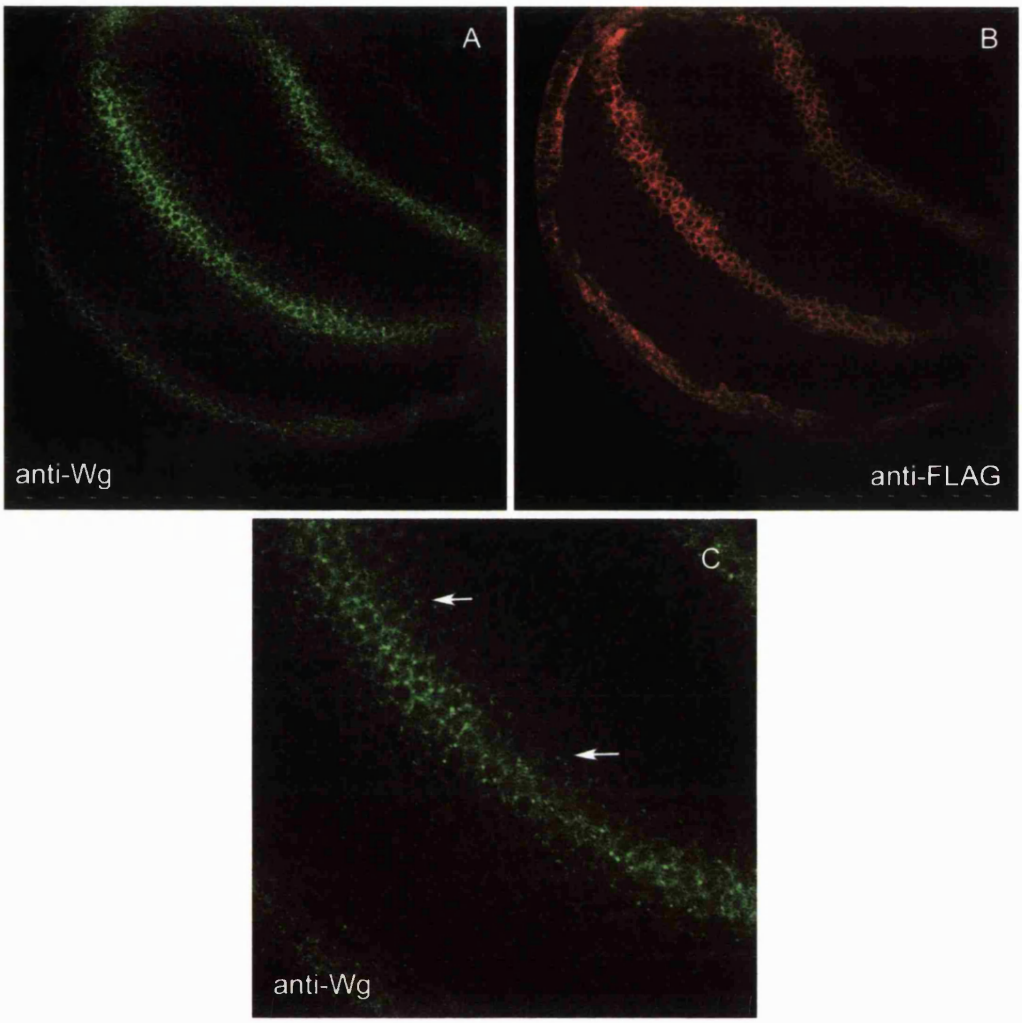


Figure 3.8 Misexpression of dally in the wg cells does not affect Wg distribution
dallyFLAG was misexpressed in the wg domain and third instar wing discs were stained for Wingless in green (A) and for FLAG (Dally) in red (B).
C is a magnification of A, showing Wg vesicles away from the source.
In all images anterior is to the left and dorsal is up. All images represent single confocal sections.

loss of wingless signalling, as observed in the adult wing phenotype (Baeg et al., 2001).

In third instar wing imaginal discs, the pattern of dlp expression is complementary to that of wg: high far away from the source, lower in cells adjacent to the wg expression domain and absent from the wg producing cells (X. Franch-Marro, pers. comm.). This expression pattern is similar to that of Dfz2, the signalling receptor, in the wing (Cadigan et al., 1998)

To further analyse the role of *dlp* I misexpressed it in different domains, using the GAL4 system and analysed its effects on Wg distribution in third instar imaginal discs. Since good antibodies are also not available for Dlp I used a transgenic line that produces HA tagged Dally-like (Baeg et al., 2001). The dpp domain was visualised by using a dpp-Gal4 UAS-GFP line, as before.

3.2.3.1 Misexpression in the dpp domain

decapentaplegic (dpp) is expressed in the anterior compartment along the anterior-posterior boundary in the wing pouch, in a domain that crosses the wg domain at the medial part of the pouch. This area of crossing normally gives rise to the distal tip of the adult wing blade.

To misexpress dlp in the dpp domain I crossed *dpp*-Gal4 UAS-GFP/Cyo flies to UAS *dally-like*-HA/Cyo flies. This cross produces larvae of four genotypes, two which are *dpp*-Gal4 UAS-GFP /Cyo and *dpp*-Gal4 UAS-GFP /UAS*dally-like*-HA, thus providing the control and the overexpression situation, respectively.

Since, in the embryo, *dlp* seems to prevent Wg release from wg expressing cells I was particularly interested to find out whether this is true also for the imaginal discs.

Overexpression of *dlp* leads to accumulation of Wg, seemingly at the cell surface (Fig. 3.9 B, arrow). However, such accumulation occurs both in cells that express Wg and in flanking cells. This could be explained in three ways. One is that in the wing disc dlp does not prevent Wg release from the expressing cell. Another

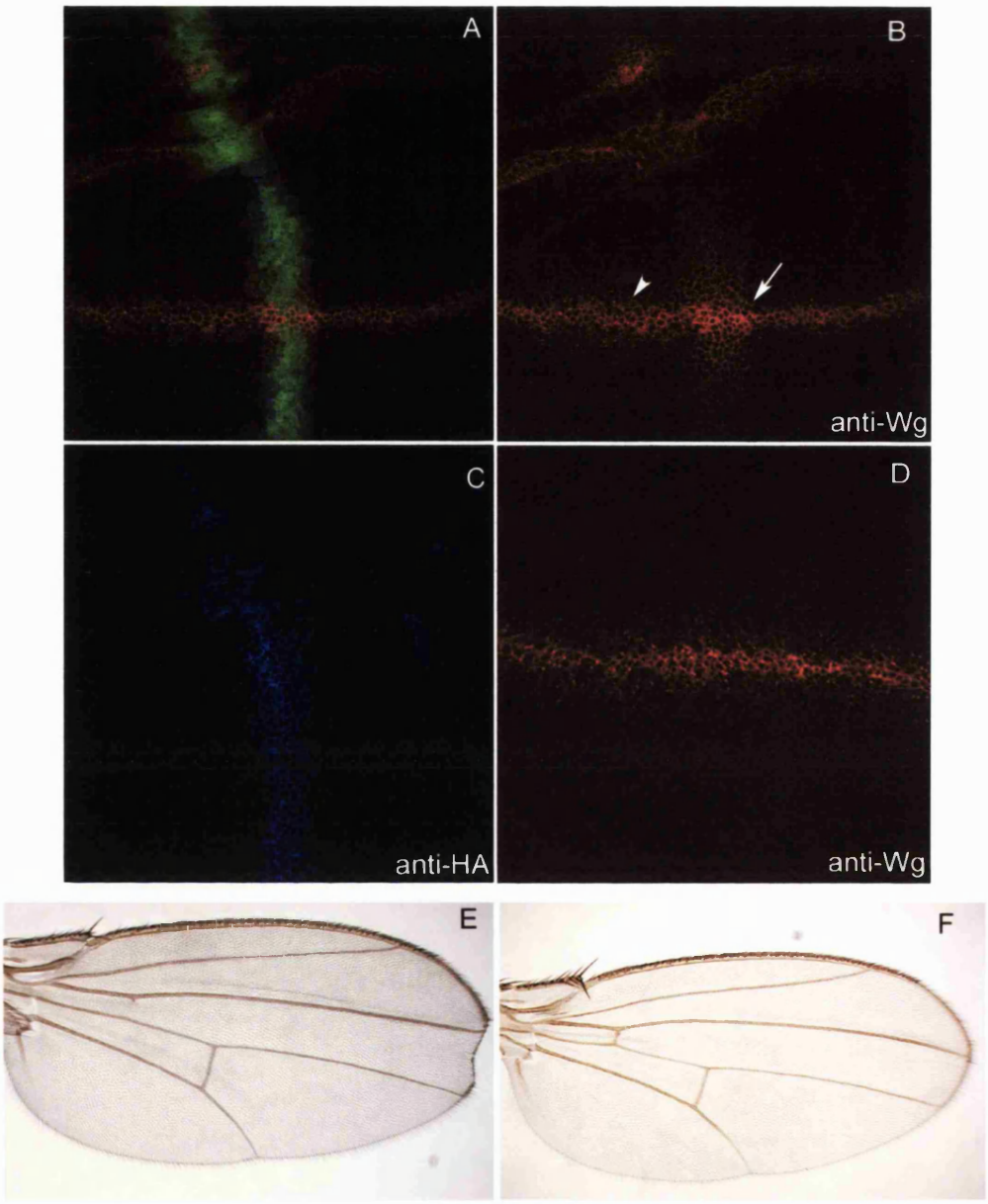


Figure 3.9 Wingless accumulates in cells expressing dlp
A, B and C show stainings of a 3rd instar wing imaginal disc in which dlp was misexpressed in the dpp domain, of genotype dpp-GAL4 UASGFP /UAS-dlp. The imaginal discs were stained with anti-Wingless (red) and anti-HA (blue) and the dpp domain was detected by GFP fuorescence
D shows a staining for Wg in a control disc of genotype dpp-GAL4 UASGFP/Cyo.
A shows a merge picture of the three channels. Wingless staining is shown in B and D (control) and HA staining is shown in C.
In all images anterior is to the left and dorsal is up.
E and F show wing phenotypes of flies in which dlp was misexpressed in the dpp domain (E) and control wild-type flies (F).

possibility is that the Wingless that is detected further from the source has been released before dlp expression, since wg starts to be expressed earlier, in the 2nd instar larval stages. A third possibility is that stabilised Wg comes from around the expression domain. These possibilities will be discussed below (see Appendix I).

In any case, it is clear that dlp overexpression leads to decreased signalling, as can be seen by the wing phenotype of the adult flies. The distal tip of the wing (where the domains of wg and dpp expression meet) is notched, a diagnostic of reduced wg signalling (Fig. 3.9 E).

3.2.3.2 Misexpression in the wingless domain

To further investigate whether overexpression of *dlp* reduces the release of wg from expressing cells, I used the wg-GAL4 driver.

Indeed, all detected Wingless accumulates in the cells that overexpress *dlp*, and no vesicles are detected outside this domain (Fig. 3.10 B, C). Wg seems to bind strongly to Dlp at the cell surface.

To assess whether the accumulated Wg is able to activate the signalling pathway, I assayed the effect of *dlp* overexpression on the expression of *distal-less* (*dll*), a target gene of Wingless that requires intermediate levels of the signal in order to be activated, in a range of approximately 10 cells from the wg source. I recombined *dll* lacZ with *dlp*-HA, and stained for β-gal (Dll), following a cross to wg-GAL4.

Wg signalling is still activated although at a much-reduced level (Fig. 3.10 B, C, compare with control staining, A), suggesting that some Wg is still released, although at a level below detectability. Consistent with a reduction in signalling, the wing phenotype shows loss of anterior bristles, which are normally specified by high levels of wg signalling. Moreover, there is a general reduction of the wing size, consistently with an early reduction of wg function. (Fig. 3.10 D and inset)

Surprisingly, I also observe some ectopic bristles in cells flanking the margin (Fig. 3.10 E, and inset, arrows). Thus, wg signalling could be prevented in cells that

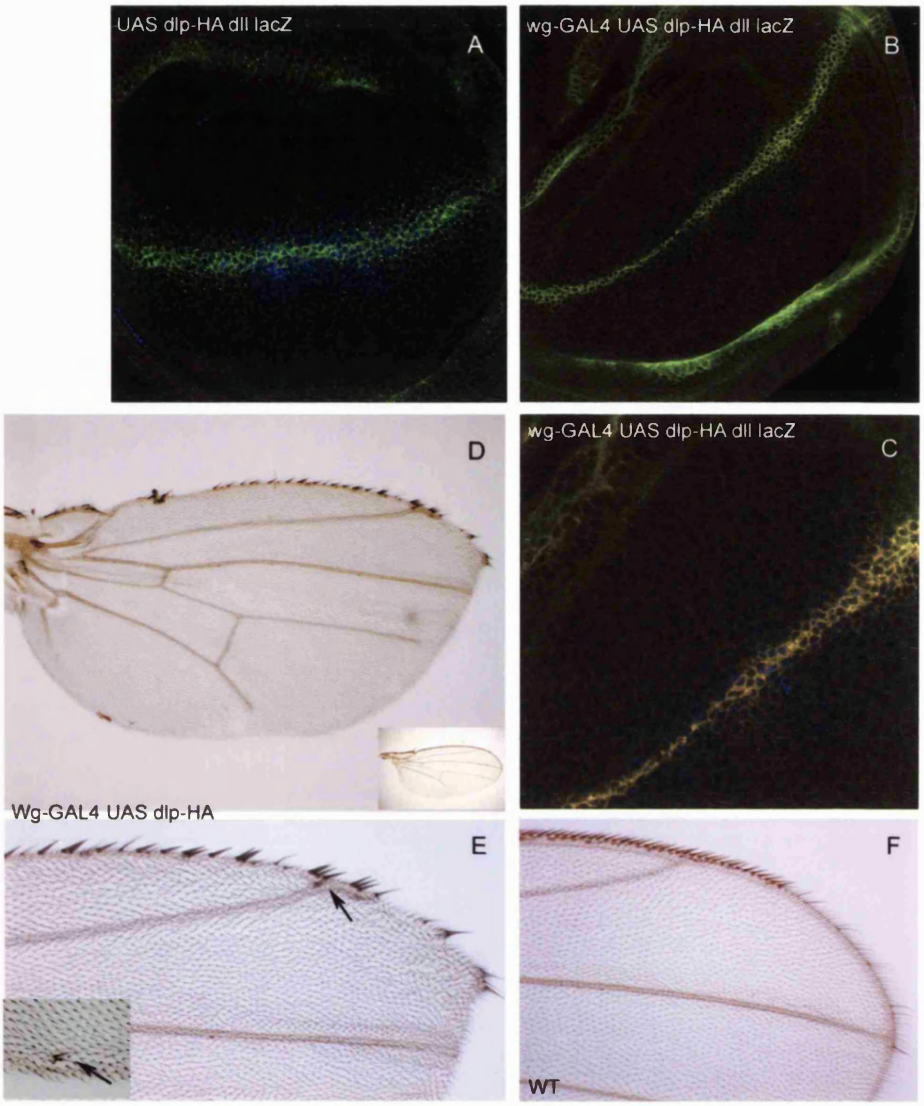


Figure 3.10 Wingless accumulates at the cell surface and wg signaling decreases when dlp is overexpressed in the wg cells
Images A, B and C show a 3rd instar wing imaginal disc of larvae of the following genotypes: UASdlp-HA dll-lacZ/Cyo (A) and wg-GAL4/ UAS dlp-HA dll-lacZ (B, C). C is a 2x magnification of B. Wg is shown in green, HA in red and Dll in blue. Wg accumulates in cells that express dlp, as seen by the merge (yellow, C) and signaling is decreased as seen by Dll reduced expression (blue, B, C)
D and E show wing phenotypes of adults of the genotype
wg-GAL4/ UAS dlp dll lacZ.
Note the reduction of the wing and loss of bristles at the wing margin.
A WT wing is shown for comparison (inset)
E is a detail of the adult wing shown in D to show ectopic bristles (arrows) in the anterior and posterior (inset) areas of the wing. The loss of bristles at the margin is evident at this magnification. Detail of a WT margin is also shown (F).

overexpress dlp, while it could be allowed (or stimulated) in flanking cells (see General Discussion).

3.2.3.3 Misexpression in the apterous domain

In order to address further the effect of *dlp* on the release and transport of Wg, I expressed it in the apterous domain. Apterous is expressed in the dorsal compartment, including half of the wg expression domain. Since wg normally spreads symmetrically into the ventral and dorsal compartments, the ventral compartment can be used as a control for any effect of dlp in the dorsal compartment.

As expected, when *apterous* drives the expression of *dlp*, Wg accumulates in that domain (Fig. 3.11 C). As outlined above, the distribution of Wg on the ventral side is expected to be as wild-type, since apterous is not expressed in that region. However, this is not the case. No Wg vesicles are detected in the ventral compartment, even though it contains a whole row of wg-expressing cells that do not express *dlp*. Although Wg is not detected ventrally *dll* expression is weakly activated (Fig. 3.11 D), suggesting that some Wg that is not detected under the imaging conditions used may be able to travel and signal.

As expected, *dll* is drastically reduced in the dorsal compartment (Fig. 3.11 D), consistent with the results described above. The adult wing shows loss of stout anterior bristles (Fig. 3.11 F, arrows). These bristles are specified by *achaete (ac)* expression (Couso et al., 1994). *ac* is a target gene that requires high levels of Wg and the absence of these bristles is then consistent with the reduction in wg signalling.

These results suggest that *dlp* overexpression prevents or reduces the release and subsequent transport of the Wingless signal. It is also interesting to note that even

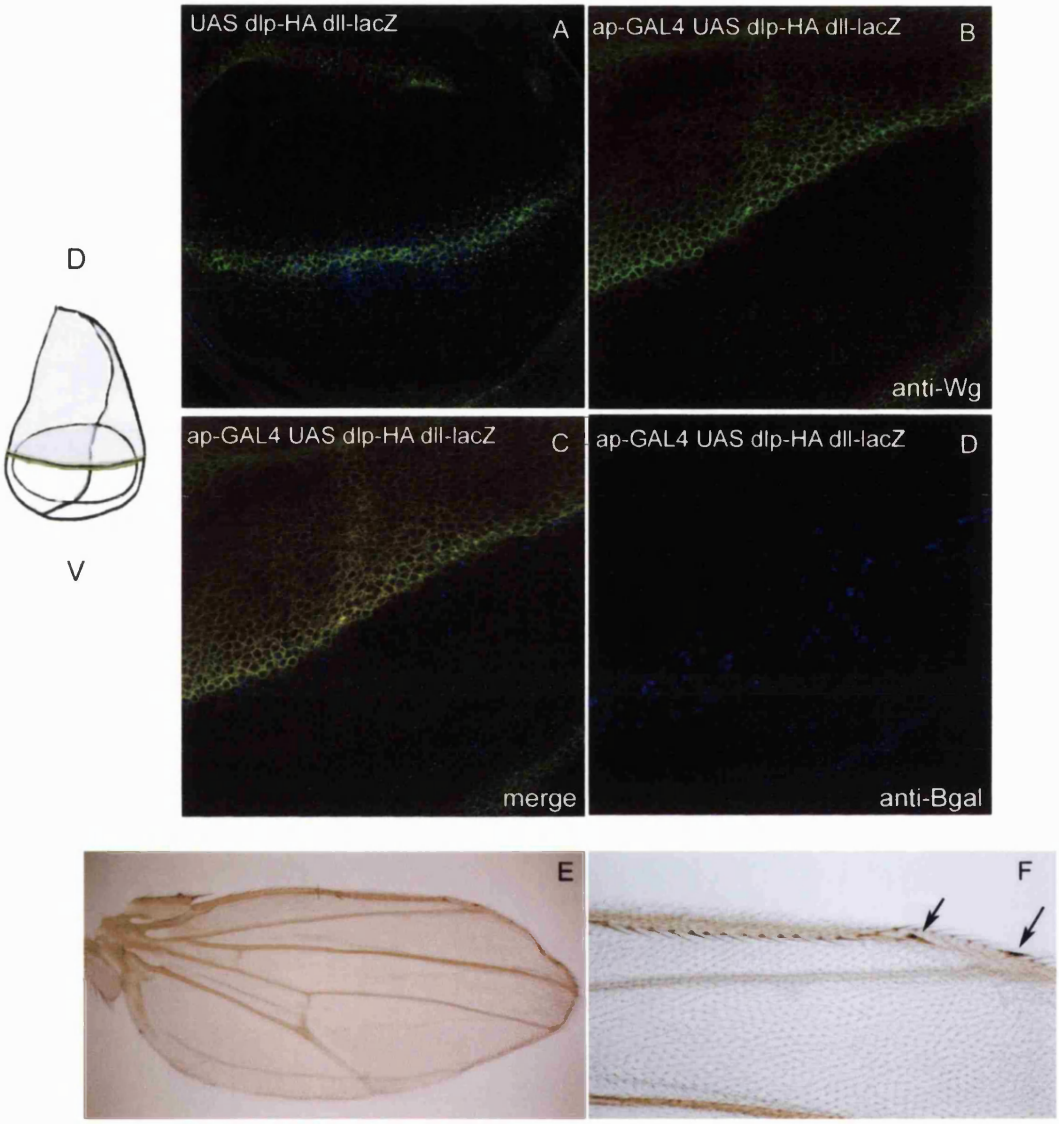


Figure 3.11 Wingless accumulates in the cells that overexpress dlp.
B-D show different images of a triple staining on a 3rd instar wing imaginal disc of genotype ap-GAL4/UASdlp-HA dll-lacZ. A shows the same staining in a control disc of genotype UASdlp-HA dll-lacZ/Cyo. the discs were stained for Wg (green), HA (Dlp, red) and b-gal (Dll, blue).
Wg accumulates in cells that express dlp, as seen by the overlaping staining of red (HA) and green (Wg) (C) and in the single staining for Wg (B).
This accumulation leads to decreased wg signaling, as seen by a decrease of Dll in the dorsal domain, in blue (D) and by the wing phenotype that shows loss of stout anterior bristles at the wing margin, in which only two are seen (arrows, F)
F is a detail of the adult wing shown in E.
In all images, anterior is to the left and dorsal is up.
A diagram is shown for reference, indicating the ap (light blue) and wg (green) domains of expression.
Images B-D have a magnification of 1.5x.

though overexpression of *dlp* leads to an increase in wg level, this is accompanied by a reduction in signalling within dlp- overexpressing cells.

Chapter 4- Wingless Release from Expressing Cells

CHAPTER 4 - WINGLESS RELEASE FROM EXPRESSING CELLS

Introduction

In the previous chapter I have shown that most Wingless is retained in the cells that make it and that the heparan sulfate proteoglycans contribute to this retention.

Lipid modification (Willert et al., 2003) could also contribute to membrane association and reduced rate of release.

Nevertheless, at some point, expressing cells need to release the signal in order to pattern the tissue. The mechanism of release is unknown. It is also unclear whether accessory proteins or chaperones might be used during the release process.

It has been suggested that Wg could travel through tissues in association with membrane structures called argosomes (Greco et al., 2001)

Alternatively, Wingless could also be released transiently from the membrane with the help of another protein, as has been suggested for Hh (Burke et al., 1999).

In this chapter I will start investigating the release of Wg, using a biochemical approach in cultured S2 cells.

4.1 Wingless is secreted in an insoluble form

It has been long known that Wingless is secreted in a soluble form into the supernatant of expressing cells (van Leeuwen et al., 1994).

To investigate whether Wingless is also secreted in association with membranes as suggested by the argosome model, I collected conditioned media from S2WgGFP or S2hsWg cells and centrifuged once at 1000 rpm for 3 min to remove cellular remains. The supernatants were centrifuged again at 20 000 g to remove heavier membranes and were then spun at high speed, 135 000 g, for 1hr. The resulting supernatant (S 135) contains soluble Wingless while the pellet (P 135) is expected to contain insoluble Wg. As can be seen, Wingless, expressed under the heat-shock

promoter, is detected in the pellet, suggesting that a fraction of Wg is released in the medium in an insoluble form (Fig 4.1 A, lane 4). The same result is obtained with WgGFP (data not shown). Note that a faint band is also detected in the soluble fraction (Fig 4.1 A, lane 6).

Thus, Wg is secreted both in a soluble and an insoluble form. The relative amounts of the two fractions will be discussed later. In this cell line, Wg is detected in three bands of different molecular sizes of 52, 55 and 57 kDa. These isoforms had been reported before and are thought to represent different levels of glycosylation with the fast migrating band representing Wg that has not been glycosylated and the higher forms having increasing number of N-glycans (Kadowaki et al., 1996; Tanaka et al., 2002). I find soluble Wg in what seems to be isoform II and the insoluble Wg as isoforms II and III (Fig 4.1 A).

To find out if the presence of Wg in this pelletable fraction is specific, I tested for the presence of another membranar protein, CD8, fused to GFP. Because CD8 is an integral protein, it is not expected to be present in the medium. I expressed CD8 GFP transiently in the WgGFP cell line and analysed the presence of both proteins in the pellet. Since both proteins are expressed under the metallothionein promoter the inducing agent is the same. Also, the detection was done in the same blot using the same antibody and ECL plus detection reagents, so that actual expression levels could be compared. I find WgGFP in the pellet, as before, while CD8GFP is not present, even though the level of expression, as detected in the cell extract, was similar (Fig. 4.1 B). No bands are detected in control medium from S2 cells, indicating that the antibody is specific and detects only the GFP signal (Fig. 4.1 B, lanes 1 and 5).

To ensure that CD8-GFP was properly targeted to the membrane, I assessed the localisation of the protein in transfected cells and found it to be localised to the cell surface. (Fig. 4.2 B). A bright-phase image shows that the cells are healthy and that there is no GFP signal in nearby non-transfected cells (Fig. 4.2 A,C).

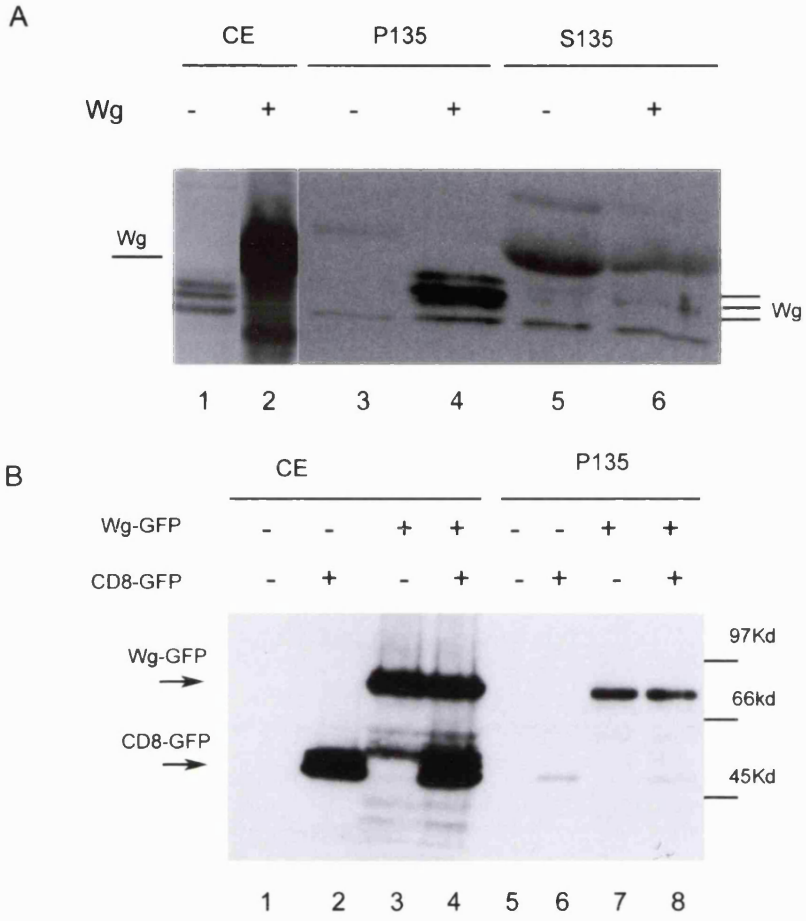


Figure 4.1 Wingless is also secreted as an insoluble form
A - Immunoblots of S2hsWg(-) cells (lane 1) and S2 cells expressing Wg, S2hsWg(+) (lane 2) and of conditioned medium collected from non expressing (lanes 3 and 5) and wg expressing cells (lanes 4 and 6).
Lanes 1 and 2 represent the cell lysates. Lanes 3 and 4 represent P135 and lanes 5 and 6 correspond to S135 of non expressing and Wg expressing cells, respectively. The membranes were incubated with anti-Wg antibody. Exposure times were:1 min (lanes 1 and 2) and 5 min (lanes 3-6).
Wg is detected in the pellet (lane 4) and it is also visible as a faint band in the soluble fraction (lane 6)
B - Immnunoblots of S2-WgGFP cells either transfected with CD8-GFP or mock transfected.
Lanes 1 to 4 represent the cell extracts (CE) and 5 to 8 represent P135.
Wg-GFP and CD8-GFP are expressed in similar levels but only Wg-GFP is detected in the pellet after differential centrifugation.
The membrane was probed with anti-GFP.

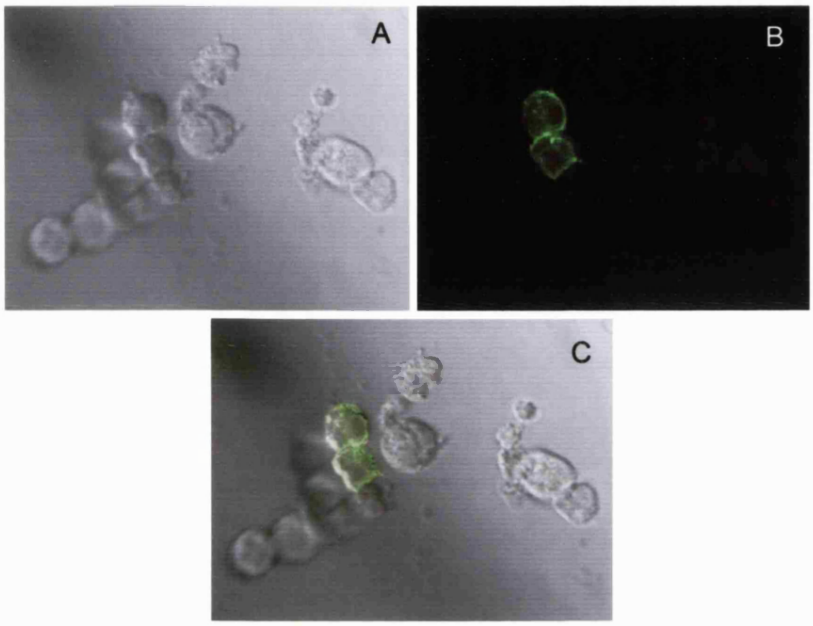


Figure 4.2. CD8-GFP is localised mainly at the membrane
A shows a bright field image of S2 cells transfected with CD8GFP. B is a confocal picture of the same cells and C shows a merge. CD8-GFP is at the membrane and no signal is seen in adjacent non transfected cells.
The images have a 2x magnification.

These results suggest that the presence of Wingless in the pellet (P135) is specific to Wingless and not due to random membrane shedding from the cell.

4.2 Wingless is partially solubilized by Triton X-100

To further understand the nature of the pelletable fraction and assess if Wg is membrane associated I tested several detergents on their ability to solubilise it.

Treatment of cellular material with detergents disrupts most lipid-lipid and also most lipid-protein interactions, so that most proteins are solubilised. However, if a protein resists solubilisation by mild non-ionic detergents, such as Triton X-100, at 4 °C it is usually thought to be part of a membrane microdomain. These domains, a minor fraction of cell membranes, can be isolated as detergent-resistant membranes, and have been called lipid rafts (Schuck et al., 2003; Simons and Ikonen, 1997).

Therefore, if a protein resists solubilisation with Triton X-100 at 4 °C it is conventionally regarded as being associated with lipid rafts. However, if it does not, it could either be associated with other membrane domains or not associated with the plasma membrane at all.

I induced the expression of Wg in S2hsWg(+) cells and collected the cleared conditioned medium containing the secreted Wg for treatment with different detergents, in parallel. After treatment the media were differential centrifuged, as described in materials and methods. S2 cells that do not express wg were used as control. Detergents such as SDS are able to disrupt nearly all non-covalent interactions in proteins, so it is not surprising that Wg is solubilised when treated with SDS 0.1% at 4 °C for 30 min. The protein is detected in the soluble fraction while Wg from conditioned medium that was treated only with buffer is only detected in the pellet (Fig. 4.3 A). More importantly, I find that not all Wg is solubilised when treated with 1% Triton X-100 (Fig. 4.3 A, B). Although Wg is reduced in the pellet (P 135) and detected as a very faint band in the supernatant (sup 135, asterisk) there is still

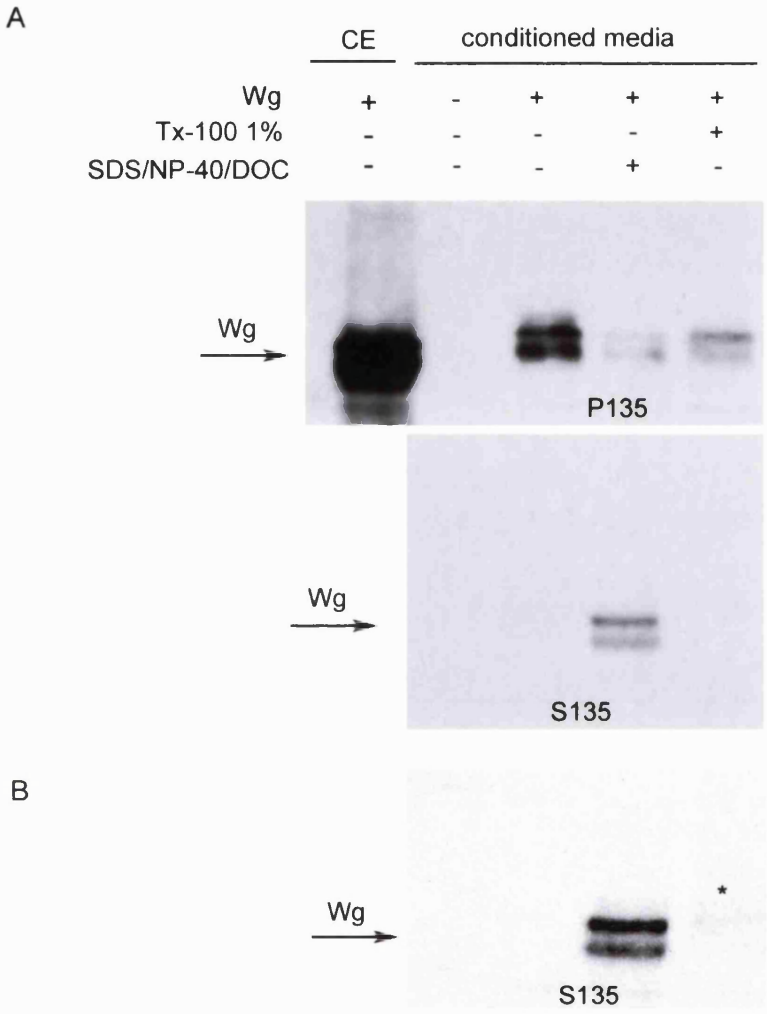


Figure 4.3 Treatment with Triton X-100 partially solubilises Wingless
A- immunoblot of S2hsWg(+) cells, indicating wg expression (CE) and treated conditioned medium collected from the same cells.
The upper panel shows the P135 (insoluble) centrifuged after treatment with the detergents at 4 oC, while the lower panel shows the soluble fractions of the same samples (sup 135). Wg is solubilised when general detergents (SDS, NP-40, DOC) are used but it is only partially solubilised when treated with Tx-100 1%. Wg was detected with mouse anti-Wg and the film was exposed 5 min.
B - shows a higher exposure (30 min) of the soluble fraction, where a very faint band corresponding to Wg is seen when the medium is treated with Tx-100 (asterisk), indicating that some Wg is solubilised.
The soluble fraction is 4x more diluted than the insoluble and all samples were equally loaded in the gel.

some Wg that resisted solubilisation (Fig. 4.3 B). All samples, in each fraction, were loaded equally in the gel, as seen by Ponceau staining (not shown).

This result suggests that some Wg might be associated with membranar domains such as lipid rafts. However, as will be shown below, this fraction is not clean so it would be important to confirm that this is the case by treating the supernatant with Triton X-100 at 4 °C and subject the sample to sucrose gradient centrifugation. If Wg would be detected in the low-density fractions it would be reasonable to assume that it is associated with microdomains in the membrane.

4.3 Electron Microscopy of secreted Wingless

The work with detergents leads to the preliminary suggestion that Wg could be released in association with membrane structures. This requires confirmation by EM.

As a first exploratory assessment of the supernatant by electron microscopy, I used supernatant from cells that were transiently transfected with HRP-Wg, secreted-HRP (secr-HRP) or mock transfected, these last two used as a control. The supernantants were spun 1000 g for 5 minutes and purified using magnetic beads coated with an anti-Wg antibody. The beads containing bound material were stained for HRP and analysed by electron microscopy. I find some structures that resemble membrane vesicles in the supernatant from HRP-Wg expressing cells. Moreover, these structures contain HRP activity (Fig. 4.4, arrows). However I can also detect membrane vesicles in the supernatant from secr-HRP expressing cells. Presumably, these vesicles bind unspecifically to the beads.

In the above experiment, soluble Wg is also present and it could compete with "vesicular Wg" for binding to the beads. This could account for the relatively small number of " vesicles" found in the sample containing HRP-Wg. Another possibility is that expression of HRP-Wg from transiently transfected cells is relatively low. This experiment needs to be repeated with a stable cell line that expresses HRP-Wg.

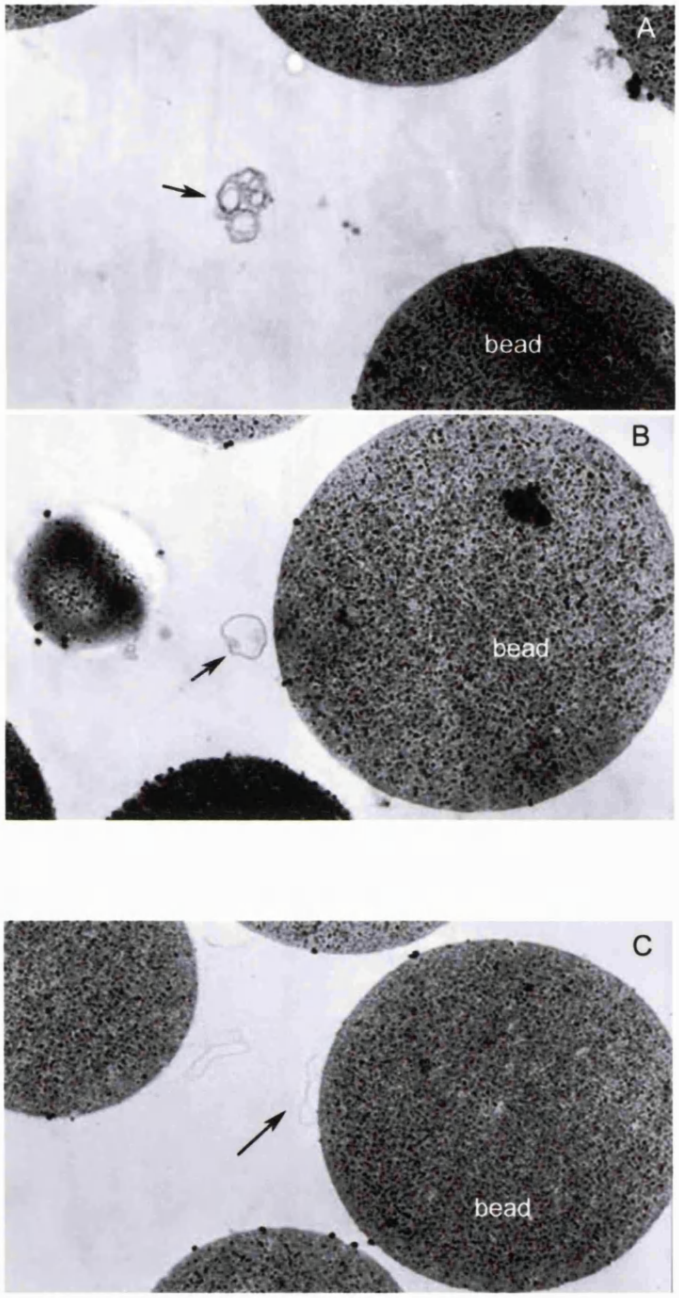


Figure 4.4 HRP-Wg and secr-HRP supernatant seen by electron microscopy
The two upper panels (A, B) show two samples of supernatant (low spin) from HRP-Wg expressing cells, immunoisolated with anti-Wg by the use of magnetic beads. The arrows indicate membranes that seem to be HRP-positive (Wg).
The lower panel (C) shows a sample of supernatant from secr-HRP expressing cells, in which the same procedure was used.
The arrow shows a HRP-negative membrane.
All pictures were taken at a magnification of 10 K.

Another problem with these results as they stand is that HRP-positive structures do not appear to bind closely to the beads.

Overall, my initial result warrants further work to improve on the aim of detecting Wg-containing structures by EM of supernatant. I will later come back to this issue and suggest a way to visualise the secreted insoluble Wingless by electron microscopy.

4.4 Wg-GFP floats in a sucrose density gradient

To further address the question of whether the secreted insoluble Wingless is of membranar nature, I used a standard assay to separate low-density agglomerates such as membranes, from high-density complexes. A discontinuous sucrose gradient was overlaid over conditioned medium containing Wingless-GFP that had been cleared at 20 000 g for 30 minutes and concentrated.

Wingless is detected in fraction 1 at a density of 1.25 g/ml (12%) (Fig 4.5) (for density values see Appendix II). The relatively high density of this fraction is due to evaporation of the liquid in the tube in this particular experiment, also explaining the shift of the soluble (high density) fractions. This result (in which Wg floats at a density of 11%-12%) was reproduced in two separate experiments.

CD8GFP, transiently transfected in the WgGFP cell line, is not found in this fraction or in any of the fractions, as expected for a protein that is not secreted, even though the expression level was similar (Fig. 4.5, lane 1). Thus, the presence of Wingless in a floating form is specific and not due to random release of membrane material. This assay complements the differential centrifugation assay used before. In both assays I showed that Wingless is released in an insoluble form either as a protein-lipid complex or as part of a membranar vesicle.

Although no quantification was attempted it is clear the Wingless that is detected in the low-density fractions is much less than what is detected in the differential centrifugation assay. Conversely, the amount of soluble Wg detected in the floatation assay is much higher than that deduced from ultracentrifugation.

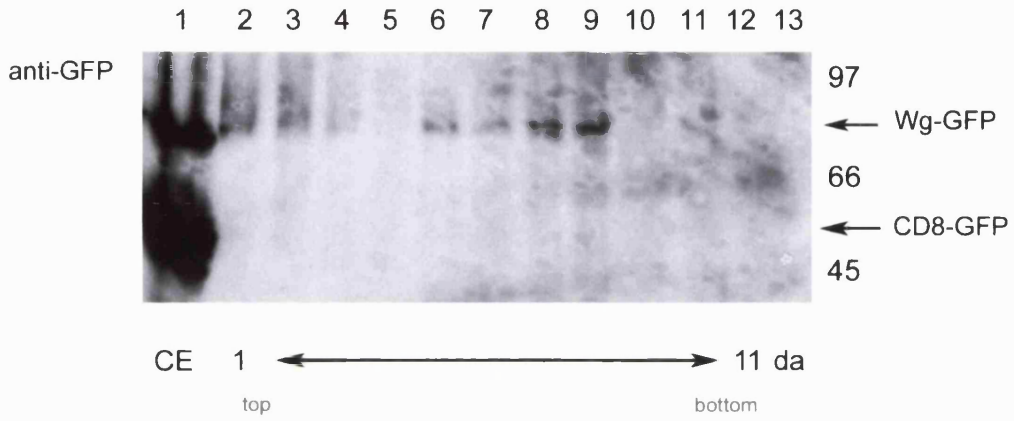


Figure 4.5 Wg-GFP is detected in low density fractions.
Immunoblot of supernatant from S2 cells expressing WgGFP and transiently transfected with CD8GFP, floated through a sucrose density gradient and probed with anti-GFP antibody.
The fractions were taken from the top of the tube, so 1 to 5 represent low density while 6 to 12 represent high density. Lane 1 represents the cell extract. Lane 2 to 12 correspond to the fractions of the density gradient and lane 13 represents a sample of dally-FLAG, as a negative control for the GFP antibody.

I suggest that the sucrose gradient assay separates the lipid bound, low-density components from soluble protein and precipitates, while the differential centrifugation assay separates out the soluble material from precipitates and membrane-associated proteins. This is important because a combination of both assays give us a clean sample of both the soluble and the low-density insoluble secreted Wingless.

This will be useful to assess the activity of each form in signalling, for visualisation of the insoluble form in electron microscopy and assessment of binding partners by immunoprecipitation (see Future Work).

4.5 HRP-Wg floats in a sucrose density gradient

As discussed above, it will be important to visualise membrane-associated Wg by electron microscopy.

An improvement over my initial attempt would be to use the floating fraction from the supernatant of HRP-Wg expressing cells as starting material. In this way we will have a clean sample that should have the majority of the insoluble low-density Wingless and that can be easily seen in EM by way of the HRP staining. As a prelude to this approach, I asked whether HRP-Wg is present in a low-density fraction, like GFP-Wg.

Media from S2 cells expressing Wingless-HRP and control non-transfected cells was collected and spun at 20.000g to remove any debris. The media was then subjected to a discontinuous sucrose density gradient and 1ml fractions were collected from the top. Aliquots of each sample were stained with a soluble substrate of HRP. After the staining reaction a clear signal was detected in the denser fractions 10 to 12 and also in fraction 4. The latter fraction had a density of approximately 11% sucrose, equivalent to 1.14 g/ml (see Appendix II for density values). The colorimetric reaction was read in an ELISA plate reader for a duration of 1 hr in intervals of 5 minutes with intervals of mixing. The resulting kinetic trend indicates a slope for fractions 4 and

10-12 that corresponds to a positive reaction rate (not shown). The reaction rate values were measured for 30 min with 5 minutes interval (1st reading) and the results are shown in Figure 4.6. The measurements clearly reveal higher HRP activity in the S2 HRP-Wg samples 4, 11 and 12 (in purple), while no activity is detected in the control line S2 that does not contain HRP (in blue).

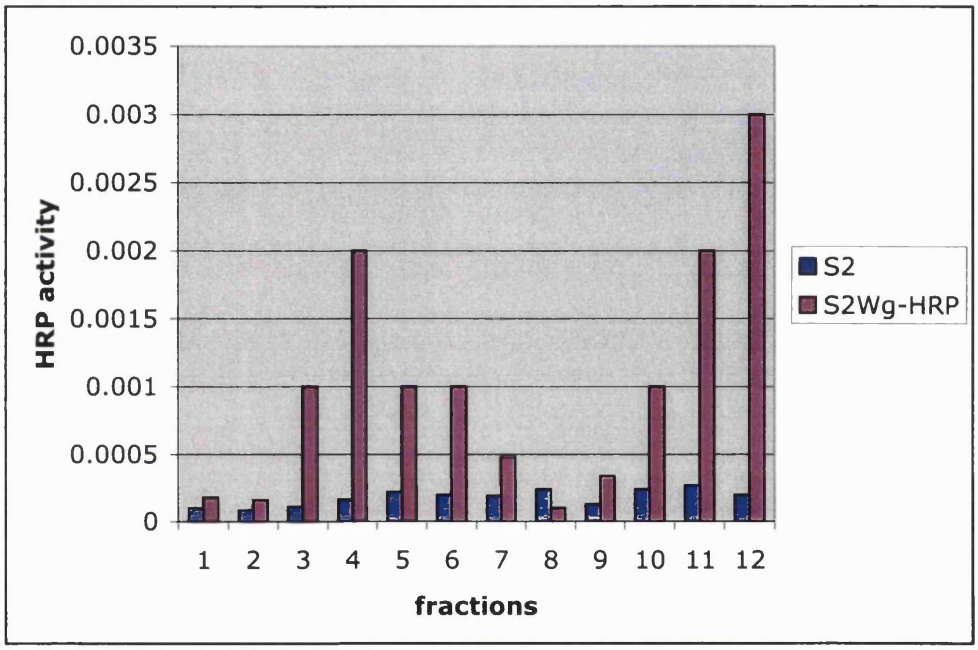


Figure 4.6 HRP-Wg is detected in low density fractions

Values of the HRP reaction rates at the first reading time, in supernatants from S2 and S2HRP-Wg cells subjected to a sucrose density gradient. Fractions 1 to 12 were taken from the top of the tubes, so that fraction 1 has the lowest density and fraction 12 the highest.

This result, with HRP-Wg, is identical to the one obtained with WgGFP, suggesting that the HRP tag is not deleterious (HRP-Wg has much reduced activity *in vivo*). Interestingly, it has been shown that exosomes, secreted from activated platelets or human B cells and separated in a similar gradient, are enriched at 1.09 g/ml-1.22 g/ml densities (Heijnen et al., 1999; Raposo et al., 1996; Wubbolts et al., 2003), with a peak at around 1.16 g/ml. Also, insoluble membranes purified from Drosophila embryos were observed between the densities of 6% and 15% in an Optiprep™

discontinuous sucrose gradient (Rietveld et al., 1999). It is then reasonable to suggest that Wingless detected in this fraction is in membranar exovesicles, although a situation in which Wingless is merely bound to lipids in a complex is still possible.

To distinguish between these two possibilities the key experiment will be to visualise Wingless in this fraction by electron microscopy.

4.6 Some HRP-Wg partitions to the detergent phase in a TX-114 assay

The hydrophobic nature of secreted Wnt proteins was recently shown with a phase separation assay (Willert et al., 2003). This assay relies on the property of non-ionic detergents, such as the Triton X series, to replace most lipid molecules in contact with the hydrophobic domain of an integral membrane protein. This leads to the formation of a soluble protein-detergent mixed micelle. The detergent Tx-114 forms a phase separation at a cloud point of 20 ° C, a temperature that is convenient for the separation of native proteins (Bordier, 1981). Above the cloud point, the detergent forms micellar aggregates with integral membrane proteins and as temperature increases a clear phase separation forms, with a phase depleted in detergent (aqueous phase) and the other enriched in it (detergent phase). Highly hydrophobic proteins remain in the detergent phase.

I used this assay to test if Wingless behaves like a highly hydrophobic protein, as does Wnt-3a. I chose to use the HRP-Wg fusion protein in this assay because it is easily detected with the HRP substrate.

I found HRP-Wg in both the detergent and the aqueous phase (Table 4.1). As expected, the HRP reaction does not take place in supernatant from cells that do not express HRP-Wg.

	S2		S2 HRP-Wg	
	Aqueous	Detergent	Aqueous	Detergent
1st reading	0.006	0.017	0.180	0.052
2nd reading	0.002	0.002	0.008	0.042

Table 4.1 Some secreted HRP-Wg partitions to the detergent phase in a Tx-114 assay

Values of the HRP reaction rates at two different reading times, in supernatants from S2 and S2HRP-Wg cells after a Tx-114 phase partitioning assay. Blue indicates a positive rate

The programmes used for the readings were as follows: 1st: for a total of 5 minutes with readings every 20 sec, with 5 sec intermixing. 2nd: for a total of 15 min with readings every 1 min and 5 sec intermixing.

The presence of Wingless in the detergent phase indicates that it is highly hydrophobic and/or is bound to a highly hydrophobic membrane protein. Willert and colleagues reported that secreted Wnt-3a is hydrophobic due to palmitoylation and that this modification occurs on an essential cysteine, so bearing in mind that Wg is hydrophobic and that this stretch of cysteines is highly conserved in the Wnt family of genes it is likely that Wingless-HRP, and Wg, is also palmitoylated.

Wingless is also present in the aqueous phase, likely reflecting hydrophilic, soluble Wg, in which the lipid is not exposed.

Chapter 5 – Regulation of Wingless Release

CHAPTER 5 - REGULATION OF WINGLESS RELEASE

Introduction

As seen in the previous chapter, Wingless is secreted in both a soluble and an insoluble form, possibly in exovesicles. Since I have also shown in chapter 3 that Wingless is retained in expressing cells in vivo by HSPGs and since Wingless binds HSPG's in S2 cells (Reichsman et al., 1996), I was interested in assessing what role the proteoglycans might have on the release of the insoluble form. One could imagine a model in which proteoglycans, and possibly also the palmityl group, targets Wg to the a membrane domain that allows the release of Wingless in a vesicle. Accordingly, reduction of proteoglycans levels could facilitate the release of Wingless as a soluble form, at the expense of the insoluble form.

5.1 dally and dally-like are expressed in S2 cells

To first test this hypothesis it was essential to assess if *dally* and *dlp* are expressed in the S2 cells.

By RT-PCR I found that both genes are expressed (Fig. 5.1). The gene *fused* (fu) was used as a positive control.

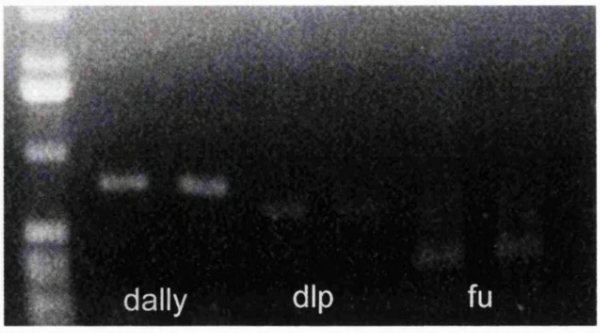


Figure 5.1 *dally* and *dlp* are expressed in S2 cells

An RT-PCR, using RNA purified from S2 cells, shows the expression of the two genes. dally was amplified as a 800 bp fragment and dlp as a 700 bp fragment. A DNA marker in which the stronger band represents 1.6 Kb is shown for size comparison.

5.2 Effect of dally on wingless release in S2 cells

To ask whether dally has an effect on the release of Wg I used the differential centrifugation assay with S2Wg-GFP and S2 as control cells.

5.2.1 Overexpression of *dally*

5.2.2.1 Dally is released to the medium

dally-FLAG was transiently transfected in cells expressing Wg-GFP and S2 cells. Cells were probed with anti-FLAG to check the level of expression. I then assayed for Dally release, which could conceivably occur either as part of a vesicle (maybe associated with Wingless) or by cleavage of its GPI anchor. In the first case one would expect it to be insoluble, and in the second case, soluble.

I find that Dally is released to the medium and that is detected both in the soluble and insoluble fractions (Fig. 5.2.1). Also the protein detected in the soluble fraction has a smaller molecular size that is consistent with GPI anchor removal due to cleavage.

5.2.2.2 Secreted insoluble Wg is reduced when dally is overexpressed

When dally is overexpressed, the level of Wingless in the insoluble fraction (P135) is highly reduced (Fig 5.2.2). This suggests that Dally might be retaining Wg in the cells. It would be interesting to investigate if the level of soluble Wg in the supernatant was also lower. In this experiment, Wg was not easily detected in the soluble fraction, due to the presence of some serum (5%) in the conditioned medium, which overloaded the gel not allowing a clear result.

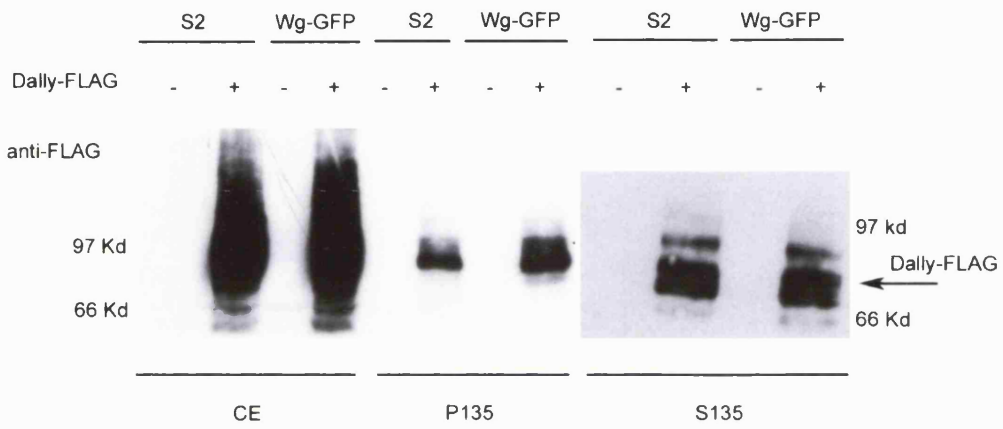


Figure 5.2.1 Dally is released to the medium in S2 cells
immunoblot of S2 or S2Wg-GFP cells transfected with dally-FLAG
dally is highly expressed in the cell extract (CE), with a carachteristic smear
that corresponds to sugar modification. dally is released to the medium
in soluble (sup 135) and insoluble (P135) forms. The soluble form has
a smaller molecular size.

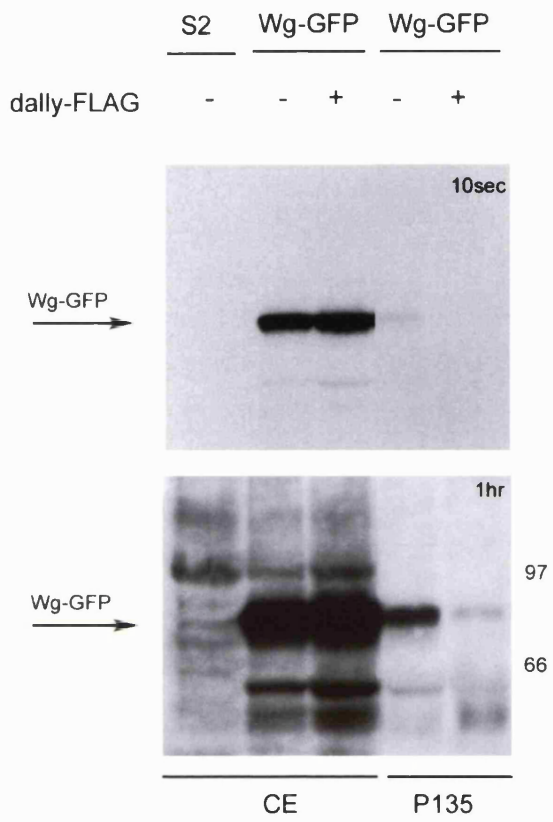


Figure 5.2.2 Secreted insoluble Wg is reduced when dally is overexpressed
Immnunoblots of S2 or S2Wg-GFP cells, either mock transfected or transiently transfected with dally-FLAG and probed with GFP (Wg)
The figure shows the cell extract (CE) and the pellet originated from the high speed spin (P135).
The level of Wg released as an insoluble fraction (P135) decreases when dally is overexpressed
Two different exposure times are shown: 10 seconds (upper panel) and 1 hr (lower panel).

5.3 Released Dally-FLAG floats in a sucrose gradient

If Wg is released in a membrane associated form it is reasonable to think that it does so bound to membrane proteins such as Dally and Dally-like, since these are attached to the outer leaflet of the plasma membrane by a GPI-anchor and since GPI-anchored proteins have been shown to be present in exosomes (Denzer et al., 2000). Therefore, Dally could be released in the medium in the same low-density fractions as Wg.

I tested the release of Dally by expressing dally-FLAG in S2 cells and collecting conditioned medium after 2 days of expression. The supernatants were cleared of debris by centrifuging at 20 000 g for 30 minutes and subjected to a discontinuous sucrose density gradient. The fractions were removed from the top of the tube and analysed by western blotting.

Dally-FLAG is present in fraction 4 (Fig. 5.3) at a density of 1.3510, which corresponds to 12% /1.25 g/ml of sucrose. This is the same fraction in which I found Wg in earlier experiments (see Appendix II for density values). It is interesting to note that Dally is also detected at high levels in the soluble fractions with a what seems to be a smaller molecular size, suggesting that it could be cleaved.

5.4 Effect of dlp on Wg release in S2 cells

I used the differential centrifugation assay to ask if dally-like (dlp) has a role in Wg release from expressing cells.

5.4.1 Overexpression of dlp

dlp-HA was transiently transfected in Wg-GFP expressing cells and the effect on Wingless release is shown in Fig. 5.4 B. Wingless seems to be more secreted since a higher level of Wingless is detected both in P135 and in S135. This is especially obvious in the soluble fraction. This result was unexpected because dally-like induces the accumulation of Wg at the cell surface in wing imaginal discs.

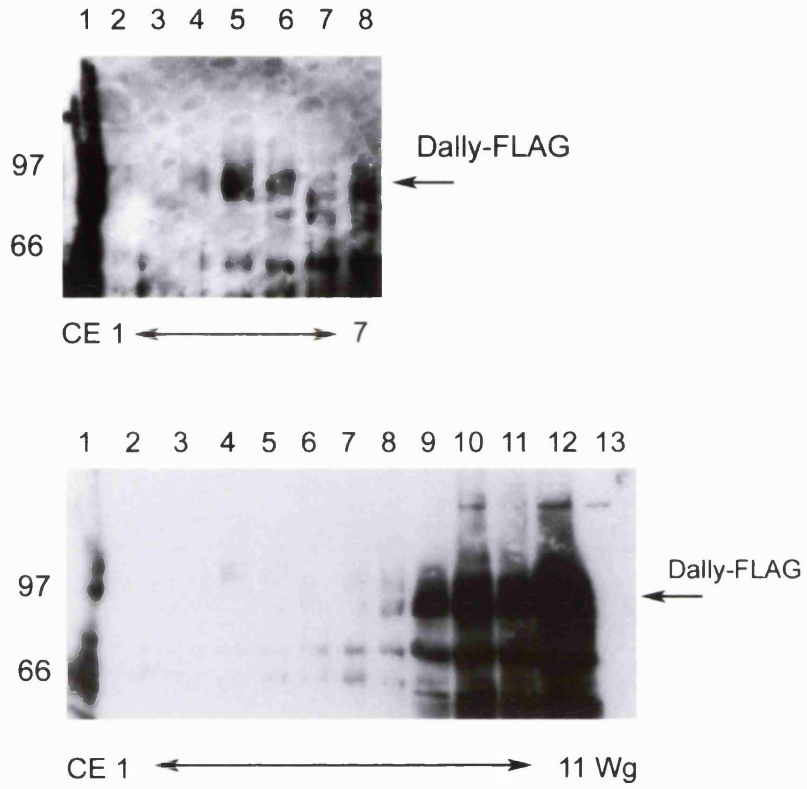


Figure 5.3 Dally-FLAG is detected in low density fractions.
Immunoblot of supernatant from S2 cells expressing
WgGFP and transiently transfected with dally-FLAG, floated through a
sucrose density gradient and probed with anti-FLAG antibody.
The lower panel shows the immunoblot with an exposure time of 10 seconds,
and the upper panel a part of the same blot after an exposure time of 5 minutes.
The fractions were taken from the top of the tube, so 1 to 5 represent low density
while 6 to 12 represent high density. lanes 1 represent the cell extract (CE)
lanes 2 to 12 represent the sucrose fractions and lane 13 was loaded with
a sample containing Wg-GFP, as a negative control for anti-FLAG.
Dally-FLAG is detected in the low density fraction 4 (lane 5. upper panel)

Although the detection of Dlp by western blotting was not successful (the antibody did not detect the HA tag by western blotting), expression was confirmed by immunofluorescence, whereby Wg was detected by the GFP signal and Dlp-HA by anti-HA. Dlp was detected at the membrane, as expected but did not always co-localise with Wg. Co-localisation is seen sporadically in small domains at the cell surface (Fig. 5.4 A, arrowheads).

5.5 The role of GPI anchorage on Wg release

If Wg is retained by a glypican at the cell surface, I expect release of the glypicans to increase the amount of Wg released in the medium. This was tested with an enzyme that specifically cleaves GPI anchors (PI-PLC). Three combinations of transfections were looked at: wg alone, wg and dally-FLAG and mock transfection. To test the efficacy of the enzyme, I looked at the behaviour of Dally, using anti-FLAG.

After PI-PLC treatment, Dally is no longer found in the insoluble fraction (P135). Instead, nearly all Dally is found in the soluble fraction (S135), consistent with GPI cleavage by the enzyme. The size of soluble Dally is smaller that what is detected in the cell extract (CE) or in the pellet (P135), as expected (Fig.5.5 A). The membrane was then stripped and reprobed for Wg. As shown in Fig. 5.5 B, Wingless release is not affected by PI-PLC treatment. The levels of secreted protein are similar in treated and untreated cells (Fig 5.5 B, lanes 7,8 and 11, 12).

There are three possible explanations for the fact that Wingless release is not affected by PI-PLC. One is that the binding to GPI anchor proteins is not required for Wg release. Alternatively, association of Wg with other proteins or to the membrane by another type of anchorage is sufficient to maintain Wg attached to membranar structures.

A third possibility is related to the procedure itself. This experiment was performed in S2hsWg cells at 37 °C, following a published procedure. However in these circumstances the protein is constantly being expressed so the enzyme may not be

able to act on all Wingless protein as it is expressed. This experiment should be repeated under similar conditions but at a lower temperature (25 °C).

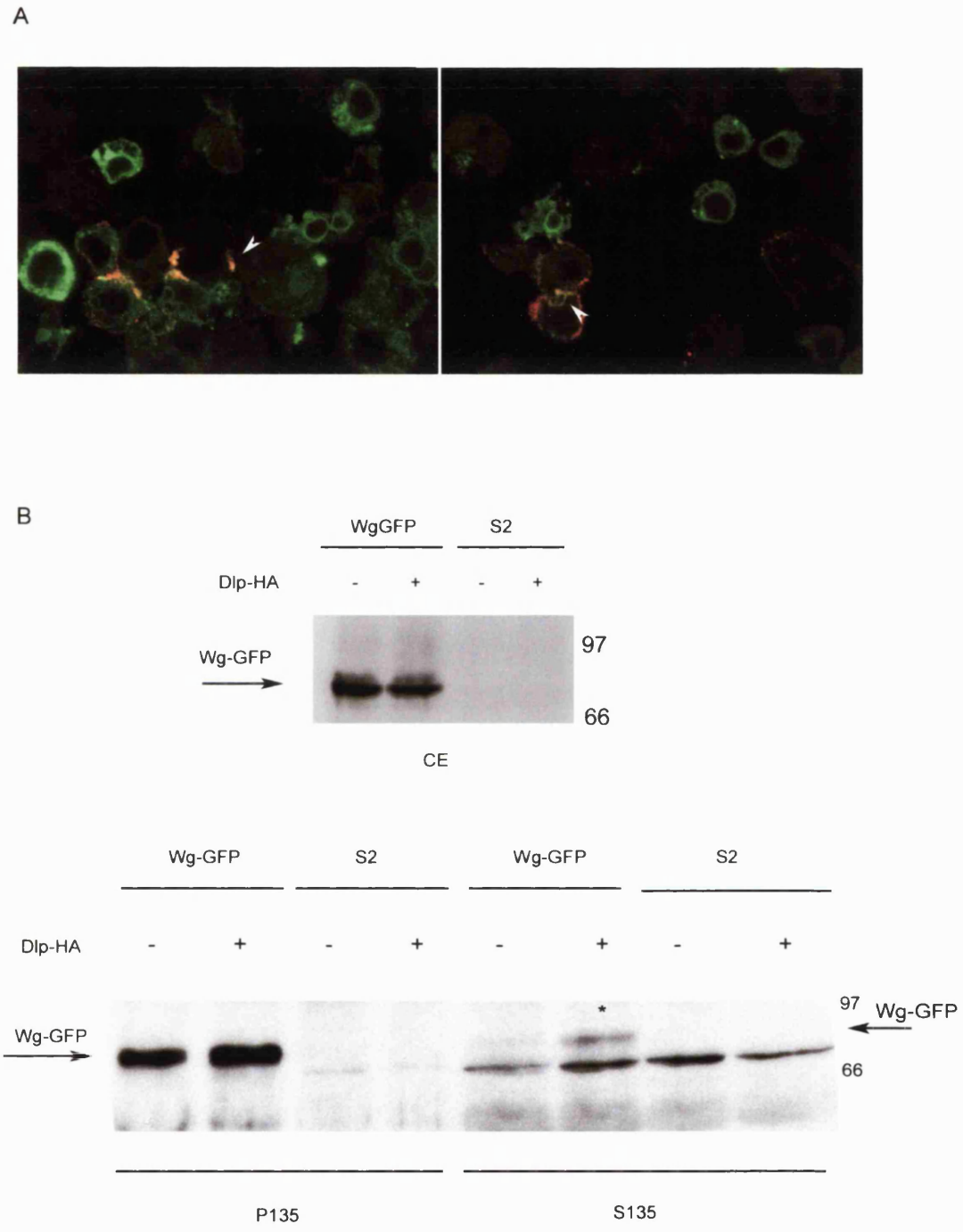


Fig 5.4 Overexpression of dlp in S2Wg-GFP cells
A- staining of S2 and S2Wg-GFP cells trasnsfected with dlp-HA
GFP (Wg) is shown in green and dlp was stained for HA, in red.
Dlp is seen at the cell surface of the cells and only occasionally co-localises with Wg (arrowheads)
B shows immnunoblots of cell extracts (CE) and supernant subjected to differential centrifugation from the same cells. After the high-speed spin, Wg is detected at higher levels both in P135 and S135 when dlp is overexpressed. It is especially obvious in the soluble fraction (S135, asterisk)
The blots were probed with anti-GFP and the exposure time was of 20 sec for the lower panel and 1 min for the upper panel.

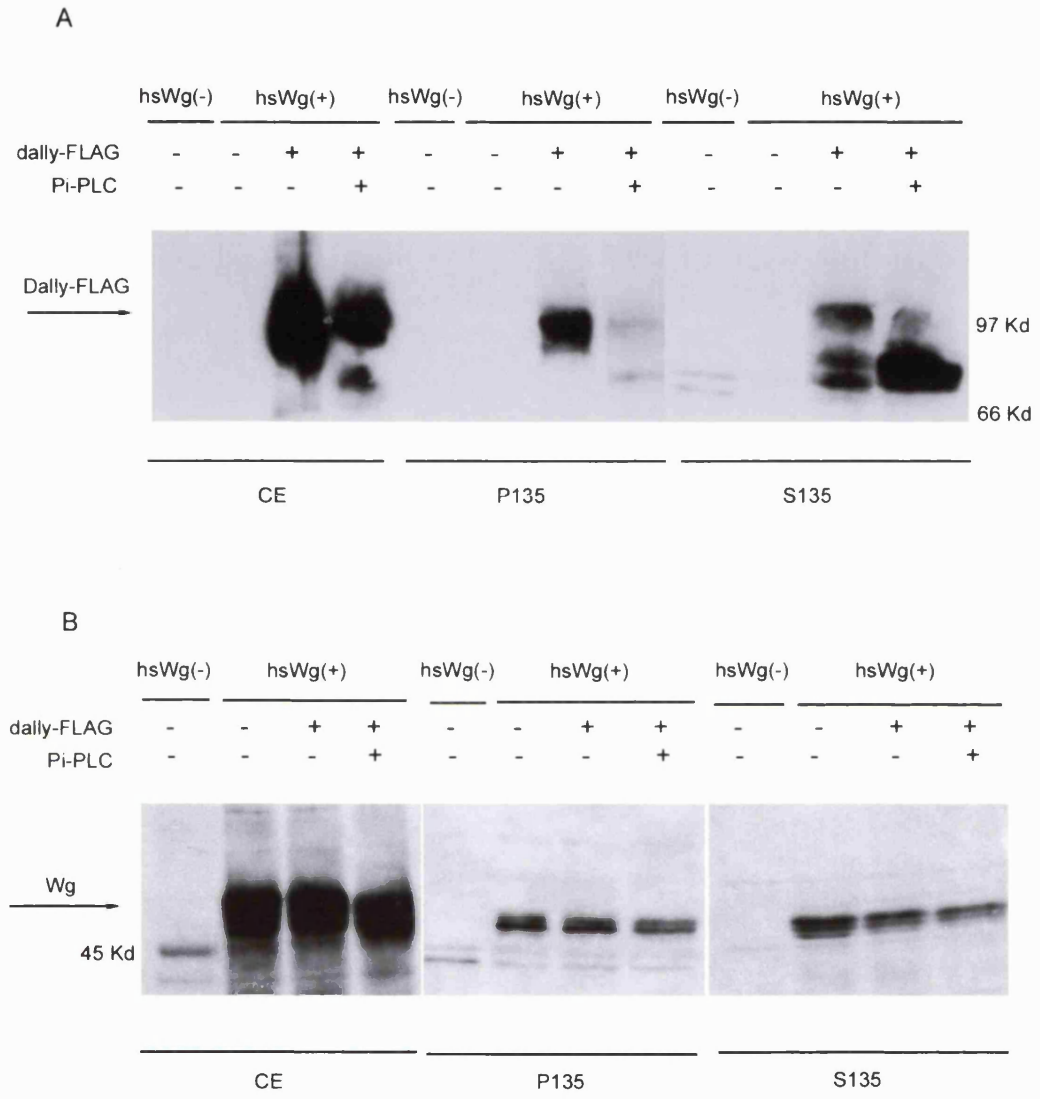


Figure 5.5 Release of Wingless upon PI-PLC treatment
A - Immunoblots of cell extracts (CE) and supernatants treated with PI-PLC or untreated and subjected to differential centrifugation, from S2hsWg(+) and (-) cells transfected with dally-FLAG or mock transfected. The membrane was probed with anti-FLAG (dally) Insoluble dally-FLAG shifts to the soluble fraction after treatment with Pi-PLC
B - The same membrane reprobed for Wg. Wg release is not affected by the treatment with PI-PLC

Chapter 6 – General Discussion and Future Work

CHAPTER 6 - GENERAL DISCUSSION AND FUTURE WORK

The mechanism of transport of a morphogen with such varied functions as Wingless is of general interest and has been extensively debated over the last two decades. Wingless, a secreted protein, was generally assumed to be released from expressing cells in a soluble, free form. However, information regarding the post-translational modifications of the Wingless protein and its association with heparan sulfate proteoglycans led to a very important question: how is a protein that is so tightly bound to the membrane secreted in order to act at the distance?

In this study I used two approaches that I considered complementary to address this question. I used the embryo and wing imaginal disc as models of short and long-range transport, taking advantage of the wealth of work done on these systems over the past years. To investigate these questions in more detail and to allow the isolation of Wingless, I used the S2 cells as my tissue culture system. This system carries some advantages, namely the use of RNA interference (RNAi), which will allow me, in the long term, to knock down the genes encoding the proteoglycans, and the information already available concerning the hs-Wg cell line and secretion of Wg.

What is the role of the HSPGs on Wingless release and transport?

Previous work had suggested that heparan sulfate proteoglycans could be important regulators of Wg distribution (Binari et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999). Embryos mutant for *sgl* or *sfl*, genes that encode two enzymes involved in processing of the HSPG, show a cuticle phenotype similar to that of a *wg* mutant. The more recent observation that overexpression of Dlp leads to the stabilisation of Wg suggests a role for this molecule in the formation of the Wg gradient (Baeg et al., 2001). These observations led to the proposal of two possible models of action: one is that HSPGs act as co-receptors. The second model suggests that the HSPGs do

not act directly in the activation of the pathway but capture Wg in receiving cells, allowing the presentation of the ligand to the receptor.

By optimising a detection method for proteins at the cell surface I was able to show that extracellular Wg, is mostly retained in expressing cells and that relatively little is present outside the expression domain. At least in the embryo, heparan sulfate proteoglycans seem to be required for this retention. In their absence Wg is no longer retained intracellulary or extracellulary. This suggests that besides its suggested role of capture at the cell surface of receiving cells, HSPGs might be also responsible for the retention of Wg in secreting cells. This is consistent with the observation that heparinase treatment of wing discs removes most Wg from the cell surface as well as from intracellular locations (Greco et al., 2001)

Consistent with a role in retention, overexpression of Dlp leads to strong Wg accumulation at the surface of expressing cells, reducing its release and long range transport.

In a wild-type situation Dlp might be required for the concentration and retention of Wg in the expressing cell until some other protein or some active process releases it from the membrane. Thus, the observed down regulation of *dlp* expression in wg expressing cells could be required for release and subsequent transport of Wg. When *dlp* is overexpressed, Wg accumulates strongly and is no longer allowed to be transported away efficiently.

Further test of this putative role of *dlp* will require that one assess the distribution of Wg in *dally/dlp* mutant clones in wing imaginal discs.

The effect of knocking-down *dally* and *dlp* has already been performed by dsRNA injection in embryos (Desbordes and Sanson, 2003), By contrast with the situation in *sgl* mutants, no perturbation of wg activity was seen. However, RNAi may not completely knock out maternally stored RNA, leaving open the possibility that *dally*

and *dlp* could be involved in wg function. Alternatively, other proteoglycans could be required for wg function.

Intriguingly, overexpression experiments suggest a negative role of *dlp* on wg signalling, at least within cells that overexpress it (cell autonomous dominant-negative effect). This result goes against the proposed model that HSPG's, e.g. Dlp, are co-receptors or present the ligand to the receptor (Baeg and Perrimon, 2000; Lin and Perrimon, 1999). Accordingly to either suggestion, one would expect *dlp* overexpression to increase wingless signalling. How dlp prevents wg signalling remains an open question.

One possibility is that Wingless bound to Dally-like is physically separate from the receptor and that overexpression of *dlp* does not allow the engagement of Wg to its receptor. A second possibility is that overexpression of dally-like outcompetes the binding of Wg to its receptor. A third possibility is that another protein is needed to transfer Wg from Dlp to the signalling receptors. and that in this situation its activity is also not sufficient to remove most bound Wg. One candidate is the enzyme identified in quail, Q-sulf1. Q-sulf1 is an extracellular HS specific sulfatase, which contributes to Wnt signalling. It could remove sulfates from HSPGs, thus allowing Wg to be released and bind its receptor (Ai et al., 2003; Dhoot et al., 2001).

Although dlp is a cell-autonomous inhibitor of wg signalling I also show that cells overexpressing dlp stimulate wg signalling in adjacent cells (provide they themselves do not overexpress dlp). Thus, Wg that is stabilised by dlp seems to be able to access signalling receptors in neighbouring cells.

The role of dally in wg function remains unclear since over expression of dally has only a minor influence on Wg distribution and function.

Further progress in our understanding of *dally* and *dlp* will require the assessment of Wg distribution in mutant clones of *dally* and *dlp* in wing imaginal discs. This awaits the isolation of null mutants in both genes.

My work with tissue culture cells showed that Wg is secreted both as a soluble and insoluble forms and that overexpressed dally causes a decrease in Wg release. Conversely, overexpression of dlp leads to a higher level of secreted Wg.
These results seem to contradict my results *in vivo*, which indicate that dlp has a retention role while dally does not. Expression of different cofactors/regulators in the two systems could account for the different results. However, before drawing any conclusion, my overexpression results in S2 cells should be reproduced. Moreover, to further uncover the role of dally and dlp in Wg release it would be interesting to analyse the release of the protein in circumstances in which the endogenous *dally* and *dlp* are knocked-down by RNAi.

How is Wingless released from expressing cells?

I considered three main hypotheses at the onset of this work: either Wingless is released free or as part of a protein complex or associated with a membrane vesicle. The fact that Wg is lipid-modified makes it unlikely that is released mostly as a soluble form. Indeed, I find that much Wingless is released as an insoluble form, and that some of it could be membrane associated. The observation that some membranar structures are present in the low spin supernatant of expressing cells, as seen by electron microscopy, is consistent with the presence of some Wg in exovesicles. A control transmembrane protein (CD8-GFP) does not associate with low-density structures, suggesting that this form of Wg packaging could be specific.
Note that only a fraction of Wg associates with low-density structures. Therefore, additional modes of release must operate.

To confirm the vesicular nature of "low-density" Wg, EM visualisation will be needed. A way to achieve this and circumventing the problems encountered in my experiment, I would need clean starting material. One approach would be to isolate the low-density fraction from HRP-Wg expressing cells. Fraction choice would be checked by OPD (substrate for HRP) staining. This fraction would be used for immunoisolation with magnetic beads and subsequent sectioning for EM.
It would also be of interest to assess the biological activity of this fraction, which could be achieved using an armadillo accumulation assay in S2 cells.

If Wg is secreted as an exovesicle it is tempting to think that it might be bound to proteoglycans. The finding that I do not see a reduction in the fraction that contains the membrane associated Wg upon PI-PLC treatment suggests that Wg does not need to be bound to a GPI anchored protein to remain associated to the membrane.
One possibility is that palmitoylation is sufficient for membrane association so that when the HSPG anchor is removed Wg is still able to bind the membrane through the palmitate. However, Dally is released by PI-PLC treatment and therefore retention of Wg at the cell surface would require concomitant dissociation from Dally.
Before investigating this result further, it needs to be reproduced with a different means of inducing Wg expression. As mentioned, PI-PLC activity was assayed at 37° C, at which temperature Wg is continuously produced by hsWg cells. Thus, under these experimental conditions new Wg is continuously produced and this could interfere with the result.

The idea that a secreted protein such as Wingless could be released in a vesicular form is relatively novel, but it is in accordance with the recent finding that Wingless is lipid modified (Willert et al., 2003) and with previous data that demonstrates its tight association with membranes (Reichsman et al., 1996). It had already been suggested that Wingless might use vesicular carriers as means of release from the

expressing cell (Greco et al., 2001), although no attempt of isolation has been reported.

The biochemical isolation of "vesicular Wg" can lead to the identification of other proteins that might be involved in this process. These could, subsequently, be studied *in vivo* and provide valuable information about the release and transport of Wingless in a wild-type situation.

I find that some Dally is released in a membrane-associated form. It is possible that Wg and Dally are present in the same structure although this was not directly addressed in this study.

In addition, a shorter form of Dally is released in the medium of expressing cells. This could represent Dally that had its GPI-anchor cleaved. The shedding of glypicans from the cell surface has been reported although no role has been ascribed to the cleaved form (reviewed by Bernfield et al., 1999).

I find that Wg is bound tightly to the cell surface by HSPGs and possibly also by palmitoylation. Also I showed that Wg is released both in a soluble and insoluble form. I favour a model in which Wg is secreted as a soluble form by having its palmitate covered by another protein or by Wg itself. Wingless is also released in an insoluble form as an exovesicle. Upon release from the cell surface, either by the action of an enzyme or by budding of a vesicle, Wg is transported to the next cell, accumulating at the basolateral surface of receiving cells (see Figure 6.1).

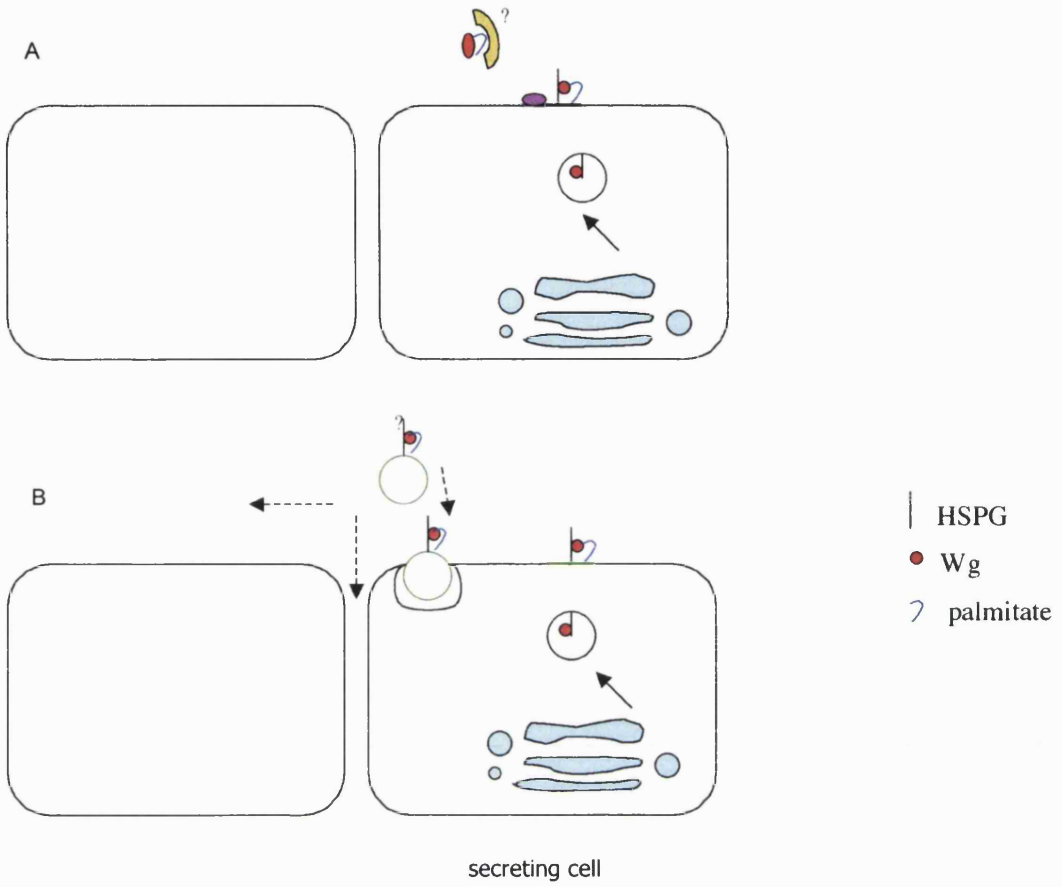


Figure 6.1 Model of Wg release from secreting cells

Wingless is bound to the cell surface through palmitoylation and HSPGs and is released as a soluble and as an insoluble form.

A- In the first case (soluble), Wg is released from the cell surface, possibly by the action of an enzyme (represented in purple), retaining its palmitate. The lipid is covered by the protein itself or by another protein(s) (represented in yellow), rendering it soluble and able to be transported to the receiving cells.

B- In the second case, Wg is released in a membrane-associated form, attached through the palmitate and possibly through binding to the GPI anchored HSPGs. After released the membrane-associated Wg could be either endocytosed by the secreting cell and move to the receiving cells by transcytosis, endocytosed only by the adjacent cell, or transported by diffusion.

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Appendix I

During the course of the investigation of the effect of dlp on Wg distribution I observed that Dlp overexpression in the dpp domain leads to Wg accumulation mostly in the wg expressing cells. It is possible that, in cells that express dlp, all Wg accumulates as soon as it reaches the surface. However I still detect some Wg stabilised further away from the source.

In the wing, Wg starts to be expressed very early, in the 2nd instar, and at this time the protein fills most of the wing pouch. dpp is expressed later, in the 3rd instar. Taking this into account it is possible that some of the Wg that is present in the dpp domain is protein that had been secreted before the start of dlp expression.

Another possibility is that indeed not all Wg accumulates and some protein is able to escape and travel further and accumulate in cells expressing dlp further away.

In the next set of experiments I tried to address this question.

Misexpression of dlp in the wg and apterous domains

To address whether Wg further away in the dpp domain was Wg present before the onset of expression of dlp or some Wg that was able to travel at the distance we misexpressed dlp using simultaneously the wg and the apterous (ap) drivers. Apterous was used because it comprises a larger domain in which the effects are more visible, but otherwise it has the same relevance for the question as if using dpp. The rationale behind this experiment is the following. Since Wg is expressed earlier than dpp or apterous I reasoned that if Wingless present outside the domain is due to Wingless that had been present there before dpp expression, by expressing dlp in the wg and ap cells combined the accumulation of wg would only take place in the wg cells, and wg would not be able to travel further at the distance.

In this scenario dlp expression driven by wg alone should have the same effect on Wg distribution as when dlp is expressed with wg and ap together and expression of

dlp driven by apterous alone would lead to accumulation of Wg in the apterous domain, as seen with dpp.

When dlp is expressed in the wg domain alone all detected Wingless accumulates in the cells that express dlp (Fig. A1C). However when dlp is expressed simultaneously in the wg and ap cells, the Wingless protein accumulates in the wg cells, along the dorso-ventral boundary, but is also detected in the apterous domain, which comprises the dorsal compartment, including the dorsal half of the wg domain (Fig. A1B).

These results argue in favour of the hypothesis that some Wg is able to travel at the distance, not all being trapped.

As expected, when apterous alone drives the expression of dlp, Wg accumulates in that domain (Fig. A1 D). In this situation I would expect the Wg distribution ventrally to be as wild-type, since apterous is not expressed in that region. However, that is not the case. This could be because the level of dlp is very high and if Wg has high affinity to it, it would be trapped even if coming from adjacent cells.

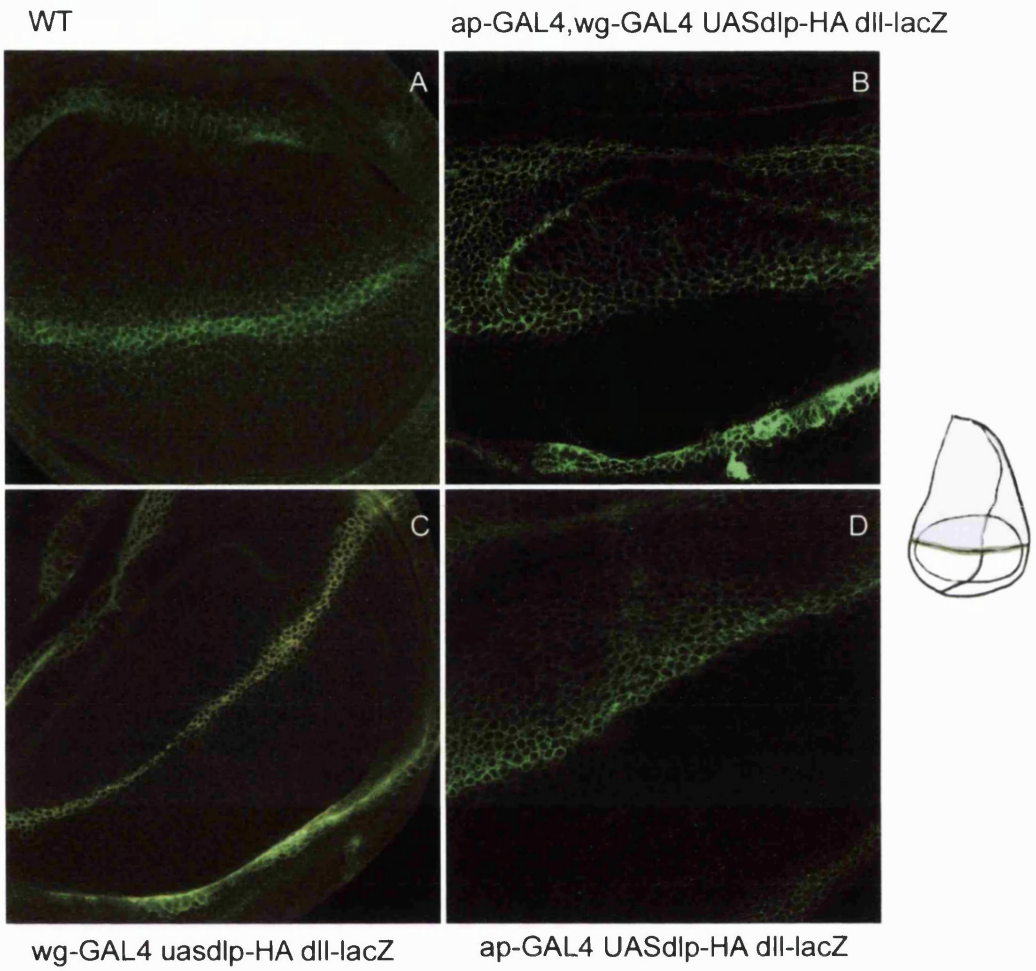

Figure A1 Some Wg is released from expressing cells when dlp is overexpressed
Third instar wing imaginal discs stained for Wg in green.
A shows a WT imaginal disc. B-D show imaginal discs in which dlp was overexpressed in different domains, ap,wg (B), wg (C) and ap (D).
Wg is detected only in expressing cells when dlp is overexpressed in the wg domain
When dlp is overexpressed in ap and wg cells simultaneously, Wg is detected outside the wg expressing cells.
A diagram representing the ap (light blue) and wg (green) domains of expression is also shown.
In all images, dorsal is up and anterior is to the left.

Appendix II

Density Values of S2 and S2HRPwg samples (see Fig. 4.6)

S2

Fractions	Refractive index	g/ml of solution (aprox.)	% sucrose by weight (aprox.)
1	1.3540	1.47	14 %
2	1.3540	1.47	14 %
3	1.3580	1.75	16.5 %
4	1.3681	2.4	22.5 %
5	1.3712	2.7	24.5 %
6	1.3721	2.75	25 %
7	1.3731	2.8	25.5 %
8	1.3732	2.8	25.5 %
9	1.3740	2.88	26 %
10	1.3750	2.95	26.5 %
11	1.3770	3.1	27.5 %

Table A 2.1 Values of refractive index and concentration data for S2 samples

All values based on measurements of sucrose at 20 °C (MW= 342.3), using density and index data from the International Critical Tables.

S2HRP-Wg

Fractions	Refractive index	g/ml of solution	% sucrose by weight
1	1.3430	0.7	7
2	1.3440	0.8	7.5
3	1.3445	0.8	7.8
4	1.3485	1.10	10.6
5	1.3580	1.75	16.5
6	1.3678	2.45	22
7	1.3710	2.7	24.5
8	1.3730	2.8	25.5
9	1.3740	2.88	26
10	1.3760	3.0	27
11	1.3770	3.1	27.5
12	1.3780	3.15	28.5
13	1.3795	3.25	29

Table A 2.2 Values of refractive index and concentration data for S2HRP-Wg samples.

All values based on measurements of sucrose at 20 °C (MW= 342.3), using density and index data from the International Critical Tables.

Density Values of S2WgGFP (+CD8-GFP) and S2 (+ dally-FLAG) samples

(see Fig. 4.5 and Fig. 5.3)

S2WgGFP (+ transfected CD8-GFP)

Fractions	Refractive index	g/ml of solution	% sucrose by weight
1	1.3510	1.25	12
2	1.3515	1.34	12.4
3	1.3550	1.52	14.5
4	1.3660	2.2	21.5
5	1.3710	2.7	24.5
6	1.3720	2.75	24.9
7	1.3730	2.8	25.5
8	1.3730	2.8	25.5
9	1.3758	3.0	27
10	1.3760	3.01	27
11	1.3780	3.1	28.5

Table A 2.3 Values of refractive index and concentration data for S2WgGFP (+CD8GFP) samples.

All values based on measurements of sucrose at 20 °C (MW= 342.3), using density and index data from the International Critical Tables.

S2 (+ transfected dally-FLAG)

Fractions	Refractive index	g/ml of solution	% sucrose by weight
1	1.3430	0.7	7
2	1.3435	0.71	7
3	1.3451	0.83	8.1
4	1.3510	1.25	12
5	1.3620	2	19
6	1.3700	2.59	24.6
7	1.3720	2.75	24.9
8	1.3725	2.76	25.1
9	1.3745	2.9	26.3
10	1.3751	2.95	26.5
11	1.3755	3.0	27
12	1.3770	3.1	27.5

Table A 2.4 Values of refractive index and concentration data for S2 (+dally-FLAG) samples.

All values based on measurements of sucrose at 20 °C (MW= 342.3), using density and index data from the International Critical Tables.