WINGLESS TRANSPORT IN THE EMBRYONIC EPIDERMIS OF DROSOPHILA

Thesis submitted in accordance with the requirements of the UNIVERSITY OF LONDON for the degree of DOCTOR OF PHILOSOPHY

Sven Pfeiffer
Division of Mammalian Development
National Institute for Medical Research
The Ridgeway, Mill Hill
London NW7 1AA
(Registered at University College London)
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
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<tr>
<td>AEL</td>
<td>after egg laying</td>
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<tr>
<td>AP</td>
<td>antero-posterior</td>
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<tr>
<td>Arm</td>
<td>Armadillo</td>
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<td>Arr</td>
<td>Arrow</td>
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<tr>
<td>β-Cat</td>
<td>β-Catenin</td>
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<tr>
<td>Cer</td>
<td>Cerberus</td>
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<tr>
<td>C-terminal</td>
<td>carboxy terminal</td>
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<tr>
<td>D</td>
<td>diffusion coefficient</td>
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<tr>
<td>Dally</td>
<td>division abnormally delayed</td>
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<tr>
<td>DFz</td>
<td><em>Drosophila</em> Frizzled</td>
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<tr>
<td>Dkk</td>
<td>Dickkopf</td>
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<tr>
<td>Dll</td>
<td>Distalless</td>
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<tr>
<td>Dly</td>
<td>Daily-like protein</td>
</tr>
<tr>
<td>DN</td>
<td>dominant negative</td>
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<tr>
<td>Dpp</td>
<td>Decapentaplectic</td>
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<tr>
<td>Dsh</td>
<td>Dishevelled</td>
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<tr>
<td>DV</td>
<td>dorso-ventral</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>Egfr</td>
<td>EGF receptor</td>
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<tr>
<td>En</td>
<td>Engrailed</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FFT</td>
<td>fast Fourier transform</td>
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<tr>
<td>FRP</td>
<td>Frizzled-related protein</td>
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<tr>
<td>Fz</td>
<td>Frizzled</td>
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<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GPI</td>
<td>glycosyl phosphatidyl inositol</td>
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<tr>
<td>HA</td>
<td>haemagglutinin</td>
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<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>His2AvD</td>
<td><em>Drosophila</em> H2A.F/Z histone</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparan sulfate proteoglycan</td>
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<tr>
<td>kD</td>
<td>kilo Dalton</td>
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ABSTRACT

The wingless gene encodes a secreted glycoprotein of the Wnt family. Wingless is required for many cell fate decisions during Drosophila development (Klingensmith and Nusse, 1994) and has been proposed to function as a classic morphogen (Bejsovec and Martinez Arias, 1991; Struhl and Basler, 1993, Lawrence and Struhl, 1996; Zecca et al., 1996 and Lawrence and Struhl, 1996). Morphogens are defined as localised factors that pattern fields of cells in a concentration dependent manner (Wolpert, 1981). The mechanisms through which morphogens in general, and Wingless in particular, build their activity gradients have been unclear, with evidence for both restricted diffusion and intracellular transport (reviewed in Gumbiner, 1998; Pfeiffer and Vincent, 1999; Strigini and Cohen, 1999; Howes and Bray, 2000 and The and Perrimon, 2000). This thesis describes investigations of Wingless movement across the Drosophila embryonic epidermis.

In chapter 2 I demonstrate an alternative mode of transport for Wingless. In embryos, wingless is transcribed in narrow stripes of cells abutting the source of Hedgehog. I show that these cells or their progeny leave the domain of wingless expression towards the anterior. As they do so, they no longer receive the Hedgehog signal and stop transcribing wingless. They can, however, retain inherited Wingless protein in secretory vesicles and carry it over a distance of up to 4 cell diameters from the site of transcription.
Experiments with a membrane-tethered form of Wingless show that this mechanism is sufficient to account for the normal range of Wingless. However, if the contribution of protein inheritance is removed, Wingless can still reach distant target cells by diffusion/transport. I suggest that both transport mechanisms operate in the wild type.

In chapter 3 I develop a method for the detailed study of epidermal cell movement in living embryos, inspired by the findings of chapter 2 that demonstrate the importance of cell lineage for intrasegmental patterning. I apply these methods to the study of cell movement during germband retraction, mainly because this is the time when Wingless specifies epidermal cell fates. Although it is assumed that there is no mitotic activity during this stage, I find a surprising amount of cell divisions. A patterned behaviour appears to be present in the orientation of mitoses.

In chapter 4 I use a biologically active GFP-tagged Wingless (GFP-Wingless) to study Wingless trafficking. I find that GFP-Wingless (as well as Wingless) remains tightly associated with secreting cells and this may explain the short range seen in embryos. At the anterior of each stripe of wingless expression, where Wingless specifies bald cuticle, extracellular Wingless is readily endocytosed and can be recycled back to the cell surface (the first direct observation of ligand recycling in live embryos). I suggest that, within this region of the ectoderm, endocytosis and recycling serve to sustain high level signalling.
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HOW TO USE THE CD-ROM

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The CD-ROM in the back of the thesis contains QuickTime movie files that are referred to in Chapters 3 and 4. The “Read Me” text file explains the minimum hardware requirements, how to use the CD-ROM and a trouble shooting guide. Installers for QuickTime 3.02 and Netscape Communicator 4.5 are also included on the CD-ROM.

Watching the movies using a browser:

Insert the CD-ROM into your CD drive and open it. Among the items on the CD-ROM, you will see the installation folders for Netscape and QuickTime, and files called “Read Me” and “Movies.html”. If your computer does not already have versions of Netscape Navigator/Communicator and QuickTime that are appropriate for using the CD-ROM, please install the versions provided before proceeding.

There are two ways to start the presentation:

(a) If you use an appropriate version of Netscape Navigator/Communicator as your default browser, double-click on “Movies.html”.

(b) If you normally use a different browser, open Netscape Communicator/Navigator first, then open the “Movies.html” file from within the browser.

If you have any problems, please see the troubleshooting tips in the “Read Me” file on the CD-ROM.

Watching the movies using a QuickTime player:

All movies are stored in the "movies" folder as QuickTime files. They can be played back using a QuickTime 3 (or higher) player. The legends can be found in the corresponding "legends" folders as text files.
Chapter 1: Introduction

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Introduction
The development of multicellular organisms involves a complex series of events that appear to be precisely choreographed. A single cell, the zygote, gives rise to millions of cells that form an organism with many different structures such as limbs, eyes and the brain. This immense achievement raises many questions that have intrigued biologists for centuries: How do cells become different from each other? What controls the behaviour of individual cells so that they form organised structures?

The elaboration of complex patterns during development depends on signals that embryonic cells exchange with each other. Many of these signals are mediated by molecules that can act over a long range, many cell diameters away from their source. Not much is known about how these molecules achieve their long-range action. This thesis describes experiments designed to help understand how a specific signalling molecule -Wingless- achieves its long-range action during Drosophila embryonic development.

Drosophila Embryonic Development
In my description of early embryogenesis I use the terminology of Foe and Alberts (Foe and Alberts, 1983) who staged embryos according to mitotic cycle number. For stages
after cellularisation (after mitotic cycle 13) I switch to the terminology of Wieschaus and Nusslein Volhard (Wieschaus and Nusslein-Volhard, 1986).

Segmentation

Once fertilised, the zygotic nucleus undergoes 13 cycles of nearly synchronous mitotic divisions, creating a syncytium (a detailed description of many aspects of mitosis during early Drosophila embryonic development can be found in Foe et al., 1993). Throughout the first 6 mitotic cycles the nuclei reside in the interior of the embryo, but they begin to migrate towards the periphery of the embryo during the seventh mitotic cycle. By mitotic cycle 10 most nuclei have reached the cortex and form an evenly spaced monolayer at the surface of the embryo. These ~6000 "syncytial blastoderm" nuclei undergo three additional rounds of mitosis prior to cellularisation. They begin to cellularise 30min after their 13th mitosis, forming a "cellular blastoderm".

The segmentation of the Drosophila blastoderm is a hierarchical stepwise process involving a number of transcription factors (reviewed in Pankratz and Jackle, 1993 and Perrimon, 1994). Concentration gradients of maternally contributed "co-ordinate gene" products polarise the embryo along the antero-posterior axis. These gradients are fully established by mitotic cycle 9. They are then used to drive the differential transcription of zygotic genes, the gap genes. Gap genes are under direct control of the co-ordinate genes and are activated in subsets of maternal gradients, subdividing the embryo into
broad bands of gap gene expressions during mitotic cycle 10. These overlapping bands constitute a pattern of transcription factor expression from which the periodic expression of "pair rule" genes is derived at the end of mitotic cycle 12. The expression pattern of gap and pair rule genes is generated by defined cis-acting elements that respond either to shallow transcription factor gradients of maternal origin or to comparatively steep gradients formed by gap proteins.

With the onset of cellularisation at stage 5 (Wieschaus and Nusslein-Volhard terminology, (Wieschaus and Nusslein-Volhard, 1986) pair rule gene activity divides the blastoderm into metameric units that will become morphologically visible as parasegments at mid-stage 10. The anterior boundaries of the pair rule genes fushi-tarazu and even-skipped initiate the 14 segmental stripes of expression of the segment polarity genes, in particular wingless (wg), engrailed (en) and hedgehog (hh). wg expression is initiated at the posterior and en and hh expression is initiated at the anterior margin of each parasegmental primordium. Parasegments are out of register with the future segments of the larva because en, which is always expressed at the posterior end of segments, is expressed at the anterior end of each parasegment (Martinez-Arias and Lawrence, 1985). After stage 9 expression of pair rule genes decays, while expression of the segment polarity genes is maintained. Segment polarity gene expression provides positional information to each cell within a metameric unit.
Intrasegmental Patterning

At stage 5 expression of the secreted glycoprotein Wingless is activated in a narrow stripe of cells at the anterior of the parasegmental boundary (Baker, 1987). The cells just posterior to each \( wg \) stripe express the transcription factor \( En \) (DiNardo et al., 1985; Fjose et al., 1985; Kornberg et al., 1985) and the secreted protein Hh (Mohler and Vani, 1992; Lee et al., 1992). After the decay of pair rule gene expression at stage 9 the stable maintenance of \( en \) expression depends on paracrine \( Wg \) signalling (DiNardo et al., 1988; Bejsovec and Martinez Arias, 1991; Heemskerk et al., 1991) whereas stable \( wg \) expression requires signalling from Hh (Hidalgo and Ingham, 1990) whose expression is autonomously maintained by \( En \) (Mohler and Vani, 1992; Lee et al., 1992).

At stage 10 the expression of \( en \) and \( wg \) become independent of each other (Bejsovec and Martinez Arias, 1991). Once the interface between \( en \) and \( wg \) expressing cells is stabilised it acts as a signalling centre to generate cell diversity across the parasegment. A number of distinct cell types become apparent at the end of embryonic development when the ventral epidermis is decorated by a segmentally repeated pattern of denticles (DiNardo et al., 1994). Each segmental unit contains 13-14 cells. Six of these rows contain denticle-secreting cells while the remaining cells make bald cuticle. Six classes of denticles (numbered 1-6) are distinguishable by their shape, size and polarity (Bejsovec and Wieschaus, 1993).
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The Role of Wingless in Intrasegmental Patterning

Wingless signalling becomes evident in two ways. In the short term, Armadillo (Arm) the fly homologue of β-Catenin (β-Cat), is stabilised in Wg receiving cells (Riggleman et al., 1990; Peifer et al., 1994) while at the end of embryogenesis, cells which have received the Wg signal secrete bald cuticle. Embryonic lethal wg mutations show a replacement of the regions of naked cuticle with a continuous lawn of denticles and a loss of segmentation (Nusslein-Volhard and Wieschaus, 1980). Wingless signalling induces bald cuticle by repressing shavenbaby (svb) a transcriptional regulator of the cytoskeleton (Payre et al., 1999). Repression of svb leads to the suppression of the outgrowth of actin bundles. At stage 14, actin bundles form in cells that did not receive the Wg signal. The pattern of these actin bundles foreshadows that of the larval denticle belts (Dickinson and Thatcher, 1997). Wg and Hh orchestrate the patterning of the denticle belts by regulating the expression of Veinlet/Rhomboid (Ve/Rho) and Serrate (Ser) (Alexandre et al., 1999). Ve/Rho is an indirect activator of the EGF receptor (Egfr) and Ser is an activator of Notch (N).

Germ Band Extension

Segment polarity gene expression commences shortly after cellularisation. Once cellularisation is completed, gastrulation ensues (reviewed in Costa et al., 1993 and Leptin, 1995). First the ventral furrow invaginates along most of the ventral midline,
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bringing the prospective mesoderm into the embryo. At about the same time the cephalic furrow forms near the anterior end. Next the posterior endoderm is formed through the invagination of posterior midgut and hindgut primordia. At the same time the germ band on the ventral side of the embryo elongates around the posterior pole and onto the dorsal side. The germ band, which consists of ectoderm and underlying mesoderm, will give rise to segmented parts of the body. By the time germ band extension is complete, every cell behind the cephalic furrow has assumed a new position.

The cells that give rise to the germ band originate from ventral positions along the posterior two thirds of the blastoderm. Over a period of about two hours the germ band undergoes more than a two-fold increase in length while halving its width (Irvine and Wieschaus, 1994). Germ band extension can be divided roughly into two phases. The fast phase occupies the first 45min during which time the germ band completes most of its extension. There is no cell division in the ventral neurogenic region at this time. Cell rearrangement is the sole driving force. During the slow phase, which follows the fast phase, the germ band attains its maximal anterior extent, mainly driven by cell division (Hartenstein and Campos-Ortega, 1985 and Campos-Ortega and Hartenstein, 1985).

Time-lapse video microscopy of living embryos reveals extensive cell rearrangements during the fast phase of germ band extension (stage 8, 3.10-3.40hrs after egg laying, AEL). Dorso-ventral columns of neighbouring cells condense into patches,
while anterior-posterior lines of cells lengthen and drift apart until formerly adjacent cells are separated by up to 3 intercalated cells (Irvine and Wieschaus, 1994). A metameric unit, as defined by the interface of en and wg expression, grows in size along the anterior-posterior axis from 3-4 cells at blastoderm to 6-7 cells at the end of this first phase of germ band extension.

The slow phase of germ band extension begins at the onset of mitotic cycle 14 in the ventral ectoderm. After cellularisation a new mitotic pattern replaces the earlier synchronous one. Groups of cells, termed mitotic domains by Foe (Foe, 1989), enter mitosis in close synchrony with each other, but out of synchrony with cells from other mitotic domains. The pattern of mitotic domains in the ventral ectoderm is complex and although these domains have fixed positions relative to landmarks of gene expression such as en, they do not correlate in a simple manner with these landmarks (Foe, 1989). Cells entering mitotic cycle 14 in the ventral ectoderm can be detected as early as 3.45hrs AEL and as late as 5hrs AEL. From stage 10 until mid-stage 11 a great deal of mitotic activity occurs throughout the epidermal primordium, with cells undergoing two more rounds of cell division. Towards the end of stage 11, mitotic activity begins to decline and the cells of the ventral ectoderm enter G1 where they arrest. During proliferation cells become smaller and each parasegment measures 15-16 cells in the anterior-posterior axis at the end of stage 11.
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**Germ Band Retraction**

At the end of stage 10 (5.30 hrs AEL), the germ band is fully extended, with about half of it's length on the ventral side and the other half bent back along the dorsal side of the embryo (Campos-Ortega and Hartenstein, 1985 and Hartenstein and Campos-Ortega, 1985). It remains stationary for about 70min and at about 7.20hrs AEL (stage 12) begins to retract along the same path that it extended. The process of germ band shortening is initiated in the thorax and spreads posteriorly. Germ band retraction has not been studied as carefully as extension. It seems that retraction is not driven by a simple reversal of the cell intercalations that took place during extension. During retraction the packing of cells is altered such that the length of a segment is transformed from about 15-16 cells at the end of stage 11 to 13-14 cells at the end of stage 12 (Martinez Arias, 1993).

**The Canonical Wingless/Wnt Signalling Pathway**

The 'canonical' Wg/Wnt pathway involves the posttranslational stabilisation of Arm/β-catenin. Certain Wnt receptors can also signal through the “planar polarity” pathway which is independent of the Arm/β-catenin cascade. The planar polarity pathway has recently been reviewed in Mlodzik, 2000; Bray, 2000; Mlodzik, 1999 and Shulman et al., 1998.

**wnt Genes**

wnt genes are implicated in a wide variety of biological processes. The first wnt gene, mouse wnt-1, was discovered as a proto-oncogene (Nusse and Varmus, 1982).
Since then wnt genes have been shown to be important regulators of a large number of developmental decisions. For example Wnt signalling in Xenopus has been implicated in embryonic axis formation and Wnt-1 activity in mice is required for forebrain development. Inappropriate activation of the Wnt pathway contributes to mouse and human cancers (reviewed in Peifer and Polakis, 2000; Polakis, 2000 and Cadigan and Nusse, 1997). The Drosophila wg gene, the orthologue of wnt-1, is one of the best-characterised wnt family members. During embryonic development Wg plays a critical role in patterning epidermal segments and the midgut epithelium, in the development of the Malphigian tubules, in the formation of the Stomatogastric nervous system, in the specification of a subset of neuroblasts and in imaginal disc patterning (reviewed in Siegfried and Perrimon, 1994 and Klingensmith and Nusse, 1994). The Wg/Wnt signalling pathway has been recently reviewed in Wodarz and Nusse, 1998; Dierick and Bejsovec, 1999; Arias et al., 1999 and Bejsovec, 1999.

**Wingless/Wnt Signalling Upstream of the Receptor**

*porcupine (porc)* function is required in cells that produce the Wingless signal. In *porc* mutant embryos Wg protein is confined to its narrow expression domain instead of spreading to adjacent cells as occurs in the wild-type (van den Heuvel et al., 1989; van den Heuvel et al., 1993). *porc* encodes a multi-transmembrane protein predominantly found in the endoplasmic reticulum (ER, Kadowaki et al., 1996). Co-expression of Porc
with Wg leads to increased N-linked glycosylation of Wg, consistent with a role of pore in processing of Wg (Kadowaki et al., 1996). Glycosylation may be important for folding, secretion and biological activity of Wg (Smolich et al., 1993).

Wnts have been shown to interact with other secreted proteins, such as Wnt inhibitory factor-1 (WIF-1; Hsieh et al., 1999), Cerberus (Cer; Piccolo et al., 1999), FrzA (Xu et al., 1998) and secreted Frizzled-related proteins (sFRP or FRP; Uren et al., 2000). Immunoprecipitation experiments show that FrzA binds to soluble Wg protein from cell culture supernatants (Xu et al., 1998). sFRP-1 has been shown to bind directly to Wg and to modulate Wingless signalling (Uren et al., 2000).

**Interactions between Wingless and the Receptor Complex**

The receptors for Wg signals are members of the Frizzled (Fz) protein family, seven-pass transmembrane proteins distantly related to guanine-nucleotide-binding (G)-protein coupled receptors (Bhanot et al., 1996; Yang-Snyder et al., 1996 and He et al., 1997). In *Drosophila* three Fz receptors have been characterised. Expression of Drosophila Frizzled-1 and 2 (DFz1 and DFz2 respectively) imparts cultured cells with the ability to bind and to respond to Wg (Bhanot et al., 1996). In the embryo DFz1 and DFz2 are redundant receptors for activating the Wg-Arm cascade (Bhat, 1998; Kennerdell and Carthew, 1998; Muller et al., 1999 and Bhanot et al., 1999). DFz3 by contrast acts as an
Two *Drosophila* glypicans genes, *division abnormally delayed (dally)* and *dally-like protein (dly)*, as well as other genes involved in heparan sulfate proteoglycan (HSPG) synthesis, play a role in increasing the efficiency of Wg signal transduction (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001; Binari et al., 1997; Hacker et al., 1997 and Haerry et al., 1997). HSPGs appear to increase the local concentration of Wg for its receptor (see also below).

Low-density lipoprotein (LDL) receptor-related proteins (LRPs) have recently been implicated in the Wnt pathway (Wehrli et al., 2000; Tamai et al., 2000 and Pinson et al., 2000). Wnt proteins can form a complex with the cysteine-rich domain of Fz and with LRP5/6 suggesting a dual receptor complex as a consequence of Wnt binding (Tamai et al., 2000). The intracellular tail of LRP5/6 can bind to Axin (which controls β-Cat in a Wnt dependent manner), perhaps inactivating it and thereby releasing β-Cat (Mao, J. et al., 2001). It appears that LRP5/6 has a second function: it binds Dickkopf (Dkk) which interferes with Wnt signalling in *Xenopus* (Glinka et al., 1998 and Mao, B. et al., 2001). Dkk binds to LRP5/6 through a part of the receptor that is not needed for interactions with either Wnt or Fz (Mao, B. et al., 2001). Binding to Dkk might alter the conformation of LRP5/6, so it can no longer interact with Wnt and Fz.
Mutational analysis in flies shows that arrow (arr), the Drosophila LRP6 homologue, is required in cells that receive the Wg signal. In the absence of arr, neither Dfz1 nor Dfz2 can activate the Wg pathway (Wehrli et al., 2000). A remaining question is whether Dkk is involved in Wg signalling in Drosophila. There is no recognisable dkk gene in the Drosophila genome (http://www.stanford.edu/~musse/wntwindow.html) but there might be unrelated proteins with a similar function.

**Wingless Signalling Downstream of the Receptor**

In canonical Wg/Wnt activated signalling Fz receptors stabilise Arm. In the absence of stabilisation Arm is susceptible to destruction via a cascade mediated by two protein complexes (reviewed in Polakis, 1999). Protein complex one contains Zeste-white 3 (Zw3) kinase, which phosphorylates Arm and Dishevelled (Dsh). Phosphorylated Arm binds to a second protein complex containing the F-box protein Slimb (Slmb, reviewed in Maniatis, 1999). This second protein complex adds a ubiquitin tag to Arm, targeting it for destruction by proteasomes. Activation of the Wg receptor activates Dsh, which somehow prevents the degradation of Arm. Cytoplasmic Arm accumulates and then enters the nucleus where it forms a complex with transcription factors of the TCF family (reviewed in Eastman and Grosschedl, 1999). This complex activates Wg responsive genes such as distalless (dll) and vestigial (vg; Neumann and Cohen, 1997 and Zecca et al., 1996). In the absence of Arm, TCF proteins repress Wg target genes. Binding of
Arm antagonises this repression. The Arm/TCF complex can also repress transcription: it prevents the expression \textit{svb} (Payre et al., 1999).

**Wingless Transport in the Embryonic Epidermis**

**Distribution of the Wingless Protein**

As expected for a secreted molecule, the distribution of Wg protein is wider than that of \textit{wg} mRNA. In the embryo, transcription of \textit{wg} is in single cell-wide stripes while the protein is detected over 3-4 cell diameters (Gonzalez et al., 1991, and Pfeiffer et al., 2000). A significant amount of the Wg protein is detected in intracellular vesicles (van den Heuvel et al., 1989). Early (around stage 9) Wg protein is equally distributed on both sides of the \textit{wg} transcription domain. Later (around stage 11) there is a marked asymmetry: Wg protein spreads anteriorward in each segment but does not travel very far in the posterior direction (Gonzalez et al., 1991; van den Heuvel et al., 1989 and Sanson et al., 1999). The functional range of Wingless action mirrors that of its protein distribution: bald cuticle is induced over up to 5 cell diameters anterior to the \textit{wg} transcription domain while only one row of cells to the posterior secretes bald cuticle.

In the wing imaginal disc, \textit{wg} is transcribed in a narrow stripe of cells at the dorsoventral (DV) compartment boundary. It acts up to 20-30 cells away from its site of synthesis and triggers a transcriptional response of target genes such as \textit{dll} and \textit{vg} in a dose dependent
manner (Neumann and Cohen, 1997 and Zecca et al., 1996).

The mechanism by which Wg protein reaches its target cells is unclear, but several models have been proposed (reviewed in Pfeiffer and Vincent, 1999; Howes and Bray, 2000 and The and Perrimon, 2000). A priori, one can imagine at least three modes of transport within an epithelium. One mode would be free diffusion in the extra-cellular space according to Fick’s law as is seen with small, uncharged particles in isotropic media in the absence of long-range forces. A second mode could be obstructed diffusion in the extracellular space. In obstructed diffusion, movement of particles is influenced by interactions with the environment. A third possibility would be active, energy-dependent transcellular transport. An example for transcellular transport is transcytosis. The term transcytosis was initially used to refer to transport along the baso-apical axis (reviewed in Mostov, 1994). In the case of Wg transcytosis would occur within the plane of the epithelium (Dierick and Bejsovec, 1998), I will therefore use the term "planar transcytosis".

**Diffusion through the Extracellular Space**

Early models of long-range morphogen signalling assumed a localised source from which the morphogen freely diffuses. Depending on the chosen boundary conditions, a steady-state concentration gradient with desired properties will ensue (Crick, 1970 and Lewis et al., 1977). An argument against free diffusion of morphogens in epithelia is that
uncharged large solutes do not seem to diffuse freely. Xia et al. measured diffusion coefficients of a small caged dye (8-((4,5-dimethoxy-2-nitrobenzyl)oxy)pyrene-1,3,6-trisulfonic acid, DMNB-HPTS) and 10,000 MW DMNB-caged fluorescein dextran in solution and in the lateral intercellular space (LIS) of Madin-Darby Canine Kidney (MDCK) cells. They found a 1.6-fold reduction in diffusivity of dextran in the LIS when compared to diffusion in free solution. No restriction was found for the smaller DMNB-HPTS (Xia et al., 1998). The restriction to diffusion of the dextran within the LIS may be due to molecular hindrance. One would therefore predict that the diffusion of Wg, with a MW of 52,000 (468aa) is also restricted in epithelia. Moreover the Wg protein adheres to cell membranes (Bradley and Brown, 1990; Chakrabarti et al., 1992 and Reichsman et al., 1996; and see below), which should prevent its free diffusion.

Despite these theoretical barriers, Strigini and Cohen presented evidence for rapid movement of extracellular Wg in wing imaginal discs (Strigini and Cohen, 2000). To study the formation of the extracellular Wg gradient Strigini and Cohen used a temperature sensitive allele of shibire (shi<sup>ts</sup>), the only Drosophila dynamin identified to date (van der Bliek, 1999). In shi<sup>ts</sup> mutant discs Wg secretion is greatly reduced, but is rapidly reinitiated when flies are shifted back to permissive temperature. Anti-Wg antibody stainings indicate that an extracellular Wg gradient covering 50 μm forms in
approximately 30min. However, since Strigini and Cohen collected different discs at a few different time-points to analyse the time course of Wg gradient formation, their observations can not address the question whether Wg diffuses freely or whether its diffusion is obstructed.

If the spread of Wg is regulated by interacting molecules, the localised expression of these molecules could cause an asymmetric distribution of Wg as is seen in the embryonic. Kerszberg and Wolpert studied theoretically how the binding of a diffusing morphogen to its membrane-bound receptor influences the distribution of the morphogen (Kerszberg and Wolpert, 1998). In their study Kerszberg and Wolpert find that in the presence of morphogen receptors, simple diffusion fails to establish a graded morphogen distribution. Instead the binding of the diffusing morphogen to its receptor leads to a flat, saturated distribution of receptor-bound morphogen. Kerszberg and Wolpert then propose an alternative way whereby a graded distribution of a morphogen would arise in the presence of a receptor. In their model, receptor molecules would transport the bound morphogen. The key assumption they make is that there is a difference in binding affinities for different oligomeric receptor states. The morphogen would bind to a receptor monomer, leading to heterodimerisation of that receptor. If the heterodimer has lower affinity for the morphogen, the ligand would be forced to hop onto an unoccupied receptor if spatially permitted. A morphogen molecule could therefore move along cell
membranes bound to the heterodimer and could be transferred between cells by hopping from a heterodimer to a monomer. If the lifetime of the heterodimer is sufficiently long, morphogen diffusion becomes directed, because already visited receptors cannot be occupied.

In conclusion, it has been shown that Wg spreads in the extracellular space of the wing disc epithelium, however the mechanism of this spread remains unknown. It appears that interacting molecules modulate the extracellular spread of Wg. I will now discuss several candidate molecules that could interact with Wg and how this interaction might modulate the range of Wg.

**Modulation of Wingless Diffusion by Interacting Molecules**

It is not clear yet whether Wg is transported by its receptor in a manner envisaged by Kerszberg and Wolpert. However, there is experimental evidence that in wing imaginal discs the range of Wg is lengthened in the presence of its receptor DFz2 (Cadigan et al., 1998 and Rulifson et al., 2000). One should point out here that these findings are based on overexpression experiments and that they should be confirmed by studying the behaviour of Wg protein in clones lacking DFz2 function. It is also important to note that these experiments do not address whether the signalling Wingless receptor is part of the transport machinery or whether there are distinct Wg receptor species involved in
signalling and transport.

Another set of molecules that interact with Wg in the extracellular space are glycosaminoglycans (GAGs). GAGs are unbranched, highly negatively charged polysaccharides consisting of repeating units (sometimes up to a hundred) of disaccharides (reviewed in Kjellen and Lindahl, 1991 and Lindahl et al., 1998). These sugar chains are attached to a core protein to form a proteoglycan. Proteoglycans are abundant on cell surfaces and play important roles in development (reviewed in Selleck, 2000; Perrimon and Bernfield, 2000 and Lander and Selleck, 2000). Heparin and heparan sulfate GAGs (which form the sugar chains of HSPGs) have been implicated in Wingless signalling/transport (reviewed in Cumberledge and Reichsman, 1997 and Baeg and Perrimon, 2000). Heparin binds Wg as well as other Wnts (Reichsman et al., 1996 and Bradley and Brown, 1990) and this binding is responsible for the tight association of Wg to the surface of wg expressing cells in cell culture (Reichsman et al., 1996). *in vivo* evidence for the role of GAGs in Wingless function comes from flies mutant for a gene encoding UDP-glucose dehydrogenase (UDP-GlcDH). This mutation has been named *sugarless* (sgl, Hacker et al., 1997), *kiwi* (Binari et al., 1997) or *suppenkasper* (Haerry et al., 1997). UDP-GlcDH is required for the biosynthesis of glucuronic acid, a precursor of all GAGs and flies lacking UDP-GlcDH are therefore not expected to have any GAGs. This has been confirmed by immunoblotting embryo extracts with anti-heparan
antibodies (Haerry et al., 1997). Flies mutant for UDP-GlcDH die during late embryogenesis and have a segment polarity phenotype reminiscent of wg mutants.

Two models have recently been proposed for the role of HSPGs in Wg function. One is that HSPGs sequester Wg thereby regulating the overall protein distribution and local concentration. In sgl mutant embryos, the domain of en expression is temporarily widened suggesting a longer range of action for Wg. But en expression subsequently decays suggesting insufficient signalling perhaps due to dilution of the signal (Haerry et al., 1997). A second model is that proteoglycans function as co-receptors. In tissue culture, Wg activity can be inhibited by treating cells with GAG lyases or with sodium perchlorate, a competitive inhibitor that blocks the sulfation of GAGs. Adding back heparin to chlorate-treated cells restores Wg activity to control levels, implying a role for GAGs in Wingless signal transduction (Reichsman et al., 1996). However, embryos lacking UDP-GlcDH respond to overexpressed Wingless, showing that GAGs are not absolutely required for the response to Wingless (Hacker et al., 1997; Binari et al., 1997 and Haerry et al., 1997). By contrast, the embryonic arr mutant phenotype is only rescued by overproduction of Dsh, but not by overproduction of the Wg ligand (Wehrli et al., 2000).

Another gene that is required for HSPG biosynthesis is sulfateless (sfl, N-deacetylase/N-sulphotransferase; Lin and Perrimon, 1999). In the absence of Sfl activity, GAG
chains of all HSPGs are either not synthesised or they are not properly modified (Lin and Perrimon, 1999 and Tsuda et al., 1999). sfl mutant clones show a dramatic decrease in extracellular Wg suggesting that the binding of Wg to sfl mutant cells is impaired (Baeg et al., 2001). Recently the Drosophila genes dally and dally-like (dly) have been shown to encode the protein core of the HSPGs involved in Wingless signalling (Lin and Perrimon, 1999; Tsuda et al., 1999 and Baeg et al., 2001). They encode glycosyl-phosphatidyl inositol (GPI)-linked glypican molecules. Embryos with reduced levels of dally and dly mRNA by virtue of RNA interference show a wg mutant phenotype. Overexpression of Dly in wing imaginal discs results in increased accumulation of extracellular Wg, suggesting that the interaction of Dly with extracellular Wg somehow tethers Wg protein to the cell surface (Baeg et al., 2001). Baeg et al. propose that the function of HSPGs might be to limit Wg extracellular diffusion. An alternative model is that HSPGs prevent the degradation of Wg by extracellular proteases.

In conclusion, HSPGs have been shown to interact with Wingless signalling. DFz2 and HSPGs seem to play a role in organising the extracellular Wg distribution. The precise mechanism by which HSPGs regulate Wingless signalling or how HSPGs modulate Wg movement through the extracellular space remains to be determined. So far I have discussed several aspects of Wg movement through the extracellular space. I will now discuss evidence in favour and against a model for Wg transport that proposes an
intracellular route for the movement of Wg.

**Planar Transcytosis**

The model of planar transcytosis proposes that Wg is actively transported through cells. According to this model Wg would be taken up by cells flanking the site of synthesis, transported through these cells in intracellular vesicles and then released, thus presented to more distant cells. The model of planar transcytosis is supported by the observations that endocytosis appears to be involved in Wg transport.

Evidence for the involvement of endocytosis in Wg transport first came from studies of *shi* mutant embryos. *shi* encodes a dynamin (van der Bliek and Meyerowitz, 1991) which is required for clathrin-mediated endocytosis (Poodry and Edgar, 1979). In *shi* mutant embryos, Wg no longer appears to act at a distance, according to the assay available (Bejsovec and Wieschaus, 1995). The ability to assay Wg action in embryos mutant for *shi* is limited because of the pleiotropy of the *shi* mutation and hence the inability to keep mutant embryos healthy for very long. Currently, the only practical immediate readout for Wingless signalling is the stabilisation of Arm protein. In wild type embryos, *wg* is transcribed in single-cell-wide stripes and this leads to the stabilisation of Arm over 3-4 cell diameters to either side of these stripes (Riggleman et al., 1990 and Peifer et al., 1991). In *shi* mutants, Arm is stabilised only at the site of *wg* transcription and in cells flanking the transcription domain, suggesting that Wg is not transported (Bejsovec and
Wieschaus, 1995). The stabilisation of Arm in shi mutant cells does, however, indicate that they are able to respond to Wg. One caveat here is that the shi mutation could conceivably compromise the sensitivity to Wg rather than its transport so that only cells receiving high levels of Wg would show detectable Arm stabilisation.

In a more recent paper Moline et al., 1999 used a dominant-negative form of Shi (Shi^{DN}). When Shi^{DN} is expressed as an UAS transgene Shi function is perturbed in defined regions of the embryonic epidermis and the cuticle pattern can be assessed, which was not possible with the shi^{ts} allele. Wg distribution within the domain of transgene expression is limited and Wingless signalling is impaired in cells surrounding the expression domain (Moline et al., 1999). This effect could be rescued by co-expressing Wg with Shi^{DN}, suggesting that Wg can still signal in the absence of most of the Shi activity. Moline et al. interpreted their data as defective transport of Wg through the domain where Shi function was perturbed. However, one can argue that if Wg secretion were impaired, Wg distribution would be affected much in the same way. Indeed, Shi has recently been implicated in Wg secretion in addition to Wg endocytosis. When Strigini and Cohen removed Shi activity in wing imaginal discs they could not detect any extracellular Wg. This taken together with their finding that Wg can move across clones
of shi mutant cells led them to suggest that Shi may be primarily required for Wg secretion rather than its transport (Strigini and Cohen, 2000).

The basis for the model of planar transcytosis has recently been challenged by yet another paper. One of the arguments for transcytosis was based on the disruptions of denticle diversity outside of the domain were Shi function was perturbed (Moline et al., 1999). In interpreting these results the authors assumed that Wg must act at a distance to specify denticle diversity. But recent data suggest that this is not necessarily true. Alexandre et al. have shown that the interplay between Hh and Wg produces short range signals such as Ser that pattern denticle belts locally (Alexandre et al., 1999). Moreover a membrane-tethered form of Wg expressed either in the en or wg domain rescues the denticle pattern of wg mutants (Sanson et al., 1999). This clearly indicates that Wg transport is not necessary for the patterning of the denticle belts. And therefore the perturbations in the denticle pattern seen in the Shi$^{DN}$ embryos can not be caused by disruption of Wg transport.

In conclusion, the model of transcytosis proposes that Wg is actively transported through cells in a process that requires endocytic components. The main arguments in favour of transcytosis have recently been challenged. Firstly, it was shown that Shi is involved in Wg secretion. And the effects of Shi on secretion can explain the results with Shi$^{DN}$ in
embryos without invoking planar transcytosis. Secondly, it was shown that the effects of Shi\textsuperscript{DN} on denticle patterning do not necessarily reflect perturbations of Wg transport. However one can not rule out transcytosis as a mechanism for Wg transport. I will now turn to a structure/function analysis of the Wg molecule. Such an analysis appears to further support the idea of active transport of Wg.

**Structure/Function Analysis of Wingless**

Recent work suggests that transport and signalling are separately mutable in the Wingless protein (Bejsovec and Wieschaus, 1995; Hays et al., 1997 and Dieriek and Bejsovec, 1998). Bejsovec and Wieschaus identified a mutant wingless allele, wingless\textsuperscript{CE7} whose protein product appears normally distributed during early stages (stage 8-10), but fails to stabilise Arm or maintain en expression (Bejsovec and Wieschaus, 1995). Thus, transport can occur without signalling and since wingless\textsuperscript{CE7} encodes a protein truncated at residue 367, the C-terminal region (368-468) appears dispensable for transport. Dieriek and Bejsovec identified three mutants which accumulate Wg protein in transcribing cells but not in neighbouring cells, suggesting a transport defect (Dieriek and Bejsovec, 1998). These mutant forms of Wg can activate the Wingless pathway in overexpression assays (this is particularly clear for one mutant, wingless\textsuperscript{NE2} which has a single point mutation, disrupting a conserved cysteine at position 247) and therefore, on
the face of it, signaling can occur in the absence of transport. However, NE2 mutants suffer from extensive loss of *en* expression. This is surprising since *en* maintenance is a short-range function of Wg (Vincent and Lawrence, 1994) and would thus be expected to continue in the absence of transport as long as Wg is secreted. One could argue that since NE2 ectopic expression of *wingless* induces naked cuticle, the mutant protein must be active and hence secreted. But it is also conceivable that the Wg activates its receptor intracellularly, perhaps within the secretory pathway. If such autocrine signalling can occur in the absence of protein export, one can not exclude the possibility that the Wg NE2 suffers from an export (as opposed to a transport) defect. Nevertheless, Dierick and Bejsovec conclude from their observations that signalling can occur in the absence of transport and that different classes of receptors might exist for transport and signalling.

Apparently consistent with this suggestion is the recent finding that embryos lacking both DFz1 and DFz2 can still transport Wingless away from its site of synthesis (Muller et al., 1999). Of course this transport may rely on remaining Fz receptors such as DFz3 which may be able to compensate for the lack of DFz1 and DFz2. Although in wg hypomorphic mutants DFz3 appears to attenuate the Wg signal, DFz3 is capable of transducing the Wg signal in cultured S2 cells (Sato et al., 1999).
In conclusion, certain Wg mutations appear to interfere with transport, rather than the signalling capabilities of the molecule. But since the assays for Wg activity and Wg transport that are currently available are limited, a thorough analysis of these mutations is impossible.

**Aim of the Project**

The aforementioned experiments have taught us a great deal about how Wg moves across an epithelium such as the embryonic epidermis. However, because the assays for Wg activity and Wg transport that are currently available (antibody stainings and cuticle preparations) are limited, a thorough analysis of the dynamics of Wg movement in the context of the developmental processes that occur during the time when Wg specifies epidermal cell fates is impossible. The development of new assays for Wg transport is needed to define the Wg transport pathway. The aim of my project was therefore to devise tools for the analysis of the dynamics of Wg distribution in living embryos and then to apply these tools to contribute to a better understanding of how Wingless moves.
Chapter 2: Wg-Expressing Cells Spread the Signal

CHAPTER 2: Wg-EXPRESSING CELLS SPREAD THE SIGNAL

Introduction

Much of the Wg protein is detected in intracellular vesicles (van den Heuvel et al., 1989 and Gonzalez et al., 1991). Vesicles outside the domain of transcription are assumed to contain Wg that has been taken up by non-expressing cells. In fact, internalisation of Wg has been proposed to be required for its transport: according to the model of planar transcytosis, internalised Wg is subsequently re-released into the extracellular space and hence presented to more distant cells (Bejsovec, 1995 and Moline et al., 1999). Of course, internalisation of Wg could also constitute the first step towards degradation in lysosomes (van den Heuvel et al., 1989). An alternative to the planar transcytosis model is that Wg diffuses in the extracellular space, interacting with membrane-associated glycoproteins (Hacker et al., 1997; Binari et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999 and Baeg et al., 2001).

Irrespective of the transport mechanism, one would expect that if the Wg protein were artificially tethered to the membrane of secreting cells and hence prevented from being released into the extra-cellular space, its range would be reduced. The experiments described in this chapter were conducted to test this prediction.
Membrane-tethered Wingless Rescues a wingless Mutation

In the ventral abdominal epidermis of Drosophila embryos, Wingless signalling specifies bald cuticle (Fig. 2.1c). The range of action can be appraised in cuticle preparations from larvae carrying a wg-lacZ reporter gene. Such preparations show that bald cuticle is made by cells located as much as 4 cell diameters away from the wg transcription domain (Fig. 2.1a). As expected for a secreted product, Wg protein is distributed more widely than wg mRNA. Transcription of wg is in single cell-wide stripes while the protein is detected over 3-4 cell diameters (Fig. 2.1b).

To test whether Wg needs to be released from wg transcribing cells to act at a distance, I expressed membrane-tethered Wg (Wg[teth]) as a UAS construct (UAS-nrt-flu-wingless, Zecca et al., 1996) in a wg null mutant with a wg-GAL4 driver. Surprisingly, wg mutants rescued by Wg[teth] can hardly be distinguished from wild type embryos (Fig. 2.1c-e). In particular, the bands of naked cuticle are as wide as in the wild type, suggesting that Wg when tethered to the membrane of transcribing cells can still act as far as 4 cell diameters away from its source of synthesis.
**Figure 2.1: Membrane-tethered Wingless acts at a distance.**

a) Larva carrying *wg-GAL4* and *UAS-lacZ* stained with X-Gal (dark blue) and DAPI (light blue). Individual denticles appear black. The *wg-GAL4* transgene used in this experiment comprises 5kb of *wg* regulatory sequences upstream of a minimal promoter and *GAL4* coding sequences (Jacques Pradel, personal communication). Up to 4 nuclei can be seen between the lacZ-positive, *wg*-expressing cells and the nearest denticle at the anterior. b) Composite triple staining for *wg* RNA (blue), Wg protein (green) and En (red) at stage 11. This composite was assembled from two separate wild type embryos: one double stained with an anti-En antibody and a *wg* mRNA probe and another embryo double stained with anti-En and anti-Wg antibodies. For each embryo, a dual colour picture of the 5th abdominal segment was acquired with a confocal microscope at a given magnification. The two pictures were subsequently merged and aligned using *en*-expressing cells as a reference. This is a projection of several focal planes and therefore includes most detectable Wingless-containing vesicles. Such vesicles are seen up 3-4 cell diameters anterior to the domain of *wg* transcription, which is in a single cell wide stripe adjoining the *en* domain. c) Cuticle pattern of a wild type first instar larva. d) Cuticle pattern of a *wingless<sup>CM4</sup>* larva carrying *wg-GAL4* and *UAS-nrt-flu-Wingless*. 'Rescued' embryos can be recognised from wild type because they die before the second instar. Some rescued embryos do not hatch and have cuticle defects probably because of delayed expression from *wg-GAL4* (rescue by *wg-GAL4 UAS-wingless* is also not fully penetrant). Note that the relatively normal denticle pattern of rescued embryos implies that a Wg gradient is not required for the generation of denticle diversity. e) Cuticle pattern of a *wingless<sup>CM4</sup>* larva. f) Transcription of *wg* in a wild type stage 11 embryo as detected with an RNA probe. g) Transcription of *GALA* in a *wingless-GAL4* stage 11 embryo as detected with a *GALA* RNA probe. The scale bar is 44 μm in a), 3.5 μm in b), 25 μm in c-e) and 16 μm in f-g).
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(a) 6
(b) 4
(c) 3
(d) 2
(e) 1
(f) 5
(g) 7
Two trivial explanations could account for the rescue. One is that the membrane tether of Wg[teth] might be leaky. Tethering Wg to the cell membrane was achieved by fusing it to the transmembrane protein Neurotactin (Zecca et al., 1996). Rescue could be explained if this fusion protein were cleaved, thus releasing active Wg into the extracellular space. However, there is no indication of cleavage from western blots (Zecca et al., 1996). Moreover, two functional assays confirm that Wg[teth] remains attached to expressing cells. First, clones of cells expressing Wg[teth] in wing imaginal discs activate Wg target genes only in adjoining cells (Zecca et al., 1996). Second, when this fusion protein is expressed in embryos with the en-GAL4 driver, it specifies naked cuticle only in cells adjoining the transcription domain (Sanson et al., 1999). Thus, in this embryonic assay, as in imaginal discs Wg[teth] acts only on adjoining cells as designed.

A second possible explanation for the rescue of wg mutants by Wg[teth] is that the wg-GAL4 driver could be expressed in a wider area than the domain of endogenous wg expression. I stained wg-GAL4 embryos with a GAL4 RNA probe and found its distribution to be identical to that of wg mRNA in wild type embryos (Fig. 2.1f-g). Therefore, broader-than-expected expression of wg-GAL4 does not account for the wide range of action of Wg[teth]. Overexpression itself is unlikely to extend the range of Wg[teth] because whatever the level of expression, it should remain confined to expressing cells. Therefore another explanation has to be sought for the ability of
The Parasegment Boundary Imposes Directionality to Cell Spreading

During normal development, *en*-expressing cells do not cross into the anterior compartment, where *wg* is expressed (Vincent and Lawrence, 1994). However, *wg*-expressing cells and their progeny may be free to roam in the anterior direction. This suggests an alternative explanation for the 'long range action' of Wg[teth]: it could be carried anteriorward by moving cells and their progeny. To test the feasibility of such a mechanism, I tracked the progeny of single cells marked at the time when *wg* expression commences. Single cells were marked by activating a photoactivatable lineage tracer with the UV laser beam of a confocal microscope (Fig 2.2a). In order to have a spatial landmark at later developmental times this experiment was performed with embryos expressing Green Fluorescent Protein (GFP) in the posterior compartment (*en-GAL4 UAS-nlsGFP*). The progeny of marked cells was identified in live embryos at late stage 11 (after 3 mitoses) and mapped relative to the domain of *en* expression. I find that although no clones crossed the parasegment boundary, those located just in front of the *en* domain span several cell diameters (up to 5) in the antero-posterior direction. One example is shown in Fig. 2.2b. Four other clones located within the posterior region of
**Figure 2.2:** Cell clones colonise a wide area within the range of Wingless.

**a)** The final photoactivatable lineage tracer reagent consisted of a dextran backbone, nuclear localisation peptides and caged rhodamine. **b)** The lineage tracer was injected prior to cellularisation and embryos were left to develop to stage 5 at room temperature. For photoactivation, the embryos were mounted in a custom-built chamber and viewed under the confocal microscope. The UV laser of the confocal microscope (excitation wavelengths at 361 nm and 365 nm) was used for uncaging. Embryos were then cultured under oil and examined at late stage 11. This is a live, late stage 11 embryo bearing the progeny of a single cell marked at the cellular blastoderm (red) and expressing nuclear GFP (green) under the control of en-GAL4. **c)** The diagram outlines the presumed behaviour of cells during this period of development. A wg-expressing cell gives rise to 8 daughters that spread anteriorly. The scale bar is 8 μm.
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a) 

b) 

c)
the anterior compartment were obtained. They were similar in aspect to the one shown in Fig. 2b and spanned 4-5 cells along the anterior-posterior axis revealing the extent of clonal spread along that axis. Since the parasegment boundary is a clonal boundary, it imposes directionality to this spread resulting in the net movement of wg-expressing cells towards the anterior. Importantly, clonal spread covers a broad area of the ectoderm and can account for the range of Wg[teth] in the rescue experiment.

If Wg is carried by moving cells and their progeny, a stable non-secreted protein should also be transported towards the anterior. This prediction was tested using UAS-nuc-lacZ, which encodes a nuclear targeted β-Galactosidase (nls-β-Gal). nls-β-Gal was found to be relatively stable (Fig. 2.3a). In the ventral ectoderm, expression of wg (and of GAL4 in wg-GAL4) continues throughout development. However, in the lateral ectoderm (marked by a bracket in Fig. 2.3a) expression terminates around 5 hrs AEL (Fig 2.1g) and stability can therefore be assessed in these cells. In wg-GAL4 UAS-nuc-lacZ embryos, nls-β-Gal protein persists in these lateral cells until 8 hrs AEL (stage 12), approximately 3 hrs after GAL4 transcription has ended there (Fig 2.3a).

I then asked whether this stable, non-secreted protein is carried forward in the ventral epidermis. Indeed, in embryos carrying wg-GAL4 and UAS-nuc-lacZ, β-Gal is detected in 3-4 cells wide stripes in front of the parasegment boundary (Fig. 2.3b).
Figure 2.3: Spread of a non-secreted protein within the range of Wingless.

a) Lateral view of a stage 12 embryo (8 hrs AEL) carrying wg-GAL4 and UAS-nuc-lacZ. As outlined in the text, this view allows an estimate of the stability of the product encoded by UAS-nuc-lacZ. As can be seen by anti-β-Gal staining, in wg-GAL4 UAS-nuc-lacZ embryos, β-Gal protein persists in these lateral cells until 8 hrs AEL (stage 12), approximately 3 hrs after GAL4 transcription has ended there. b) Late stage 11 embryo of the same genotype. In this ventral view, the β-Gal stripes are clearly wider than the domain of GAL4 expression (compare also with Fig. 2.1g). c) Hatching larva of the same genotype as in panels a) and b) Stripes of β-Gal activity are about three cells wide and lie in the middle of the naked domains. Note that nuclear β-Gal encoded by this transgene is more stable than cytoplasmic β-Gal made by the embryo shown in Fig. 3.1a) as assayed in the lateral region (not shown). The narrowing of β-Gal stripes near the ventral midline mirrors that of Wg staining in the wild type. Although this narrowing is consistently seen, its functional significance, if any, is unclear. d) Expression of UAS-nrt-flu-wingless driven by wg-GAL4 in a late stage 11 embryo. Stripes containing Wg[teth] (detected with anti-HA.11 antibody) are 3-4 cell wide, approximately the same as wild type Wg, shown in the next panel. e) Wild type stage 11 embryo stained with an anti-Wg antibody. Wg-containing vesicles are detected in stripes 3-4 cells wide. f) Diagram outlining how wg-expressing cells of a stage 6 embryo come to colonise a large area of the naked domains in the larval epidermis. The scale bar is 30 μm in a-b), 50 μm in c) and 10 μm in d-e).
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(a) Image showing striped pattern
(b) Close-up of striped pattern
(c) Image with blue and black patterns
(d) High magnification of patterned region
(e) Another view of patterned region
(f) Diagram illustrating signal spread
This is substantially wider than the GAL4 RNA stripes. In hatched larvae of the same genotype, β-Gal activity is detected within similarly wide bands of cells occupying the middle of the naked regions (Fig. 2.3c). This suggests that β-Gal made by wg-expressing cells is retained even when cells move away from the source of Hh and therefore shut off the wg promoter. Since cells can only move towards the anterior of the parasegment boundary, β-Gal appears to spread in the anterior direction. Note that such spread could not occur through cellular extensions such as cytonemes (Ramirez-Weber and Kornberg, 1999) since the β-Gal product is nuclear in this experiment. Thus, a non-secreted protein can spread by being passed on to the progeny of expressing cells and this explains why Wg[teth] acts at a distance in the embryonic epidermis.

As expected then, when driven by wg-GAL4, Wg[teth] is detected in stripes that are similar in width to the stripes of Wg protein in wild type embryos (3-4 cells wide; Fig. 2.3d-e). This suggests that, like nuclear β-Galactosidase, Wg[teth] driven by wg-GAL4 is retained by cells as they spread anteriorward and this accounts for its range of action (Fig. 2.3f). I now address whether wild type Wg protein is also carried forward by cell inheritance during normal development.
Wingless Co-localises with Secretory Vesicles

As I have shown, the Wg-containing vesicles found at the anterior of the transcription domain are within cells that descended from wg-expressing cells. These vesicles are therefore not necessarily endocytic. They could equally be secretory, i.e., contain unsecreted protein inherited from past expression. To label the secretory pathway, I used flies expressing GFP fused to the signal peptide of Wingless (UAS-GFPsecr; gift from C. Alexandre). When this fusion is expressed with the en-GAL4 driver, fluorescence is detected in bright intracellular dots within the en domain as well as weakly throughout the perivitelline space (Fig. 2.4a). The punctuate fluorescence in expressing cells most probably represents GFP transiting through the secretory pathway and thus identifies secretory vesicles. In live embryos that express GFPsecr under the control of wg-GAL4, vesicular staining is detected in 3-4 cells wide stripes at stage 11 (Fig. 2.4b). Thus secretory vesicles are present several cell diameters beyond the domain of expression. These embryos were fixed and stained with anti-Wg antibody. Although much GFP fluorescence is lost upon fixation, extensive colocalisation between Wg protein and the remaining GFP signal can be detected, even outside the wg expression domain (Fig. 2.4c-e). This suggests that many Wg-containing vesicles are secretory as opposed to endocytic (although endocytic exist as well, see Chapter 4).
Fig. 2.4: Many Wingless-containing vesicles are 'secretory'.

a) Live stage 11 embryo carrying en-GAL4 and UAS-GFPsecr. Punctuate staining seen in expressing cells most likely marks secretory vesicles. b) Live stage 11 embryo carrying wg-GAL4 and UAS-GFPsecr. For this experiment, I used a different wg-GAL4 transgene to that of Figs. 2.1 and 2.3. I used an enhancer trap that expresses GAL4 more strongly than the earlier construct with 5 kb of regulatory sequence. Note the presence of vesicular staining in a 4 cells wide domain. c-e) Co-localisation (yellow in e) of Wg (red; single channel in c) and GFPsecr (green; single channel in d) in wg-GAL4 UAS-GFPsecr. Images were acquired on a confocal microscope. To minimise cross-talk between the green and the red channel, they were acquired sequentially, i.e. the laser line to excite the green fluorophore (λ=488 nm) was switched off before the red image was acquired (excitation at λ=568 nm). Arrows point to co-localised vesicles outside the wg expression domain. There are Wg-containing vesicles that do not contain GFP fluorescence. They may represent endocytic vesicles or, alternatively, GFP is below the detection level in these vesicles (substantial GFP fluorescence is lost upon fixation and subsequent antibody staining). The scale bar is 8 μm in a-b) and 12 μm in c-e).
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The presence of Wg-containing vesicles at the anterior of the wg transcription domain is often taken as evidence for transport from cell to cell. Our result shows that this assumption must be revised. It also shows that, during normal development, cell spreading contributes significantly to the anterior movement of endogenous Wingless protein.

Conclusion

I have shown that, as cells proliferate and spread, they can retain Wg and thus affect target cells some distance away from the site of wg transcription. It is important to note that Wingless can also spread independently of cell movement (Sanson et al., 1999). It remains to be seen whether this occurs by restricted diffusion or active transport. I have been able to uncouple the two mechanisms of Wg movement and thus have shown that either is sufficient to ensure a normal range of action. Presumably, both contribute during wild type development. Interestingly, the parasegment boundary only allows cells to carry Wg towards the anterior and this adds to other mechanisms ensuring an asymmetric range of Wg in Drosophila embryos (Sanson et al., 1999). Without this border, cells carrying Wg could wander towards the posterior and disrupt segment polarity.
CHAPTER 3: A BRIEF ANALYSIS OF CELL MOVEMENT

Introduction

In chapter two I have shown that the progeny of wg expressing cells can deliver the signal at a distance. This novel mode for Wg transport emphasises the importance of cell movement and cell lineage for correct segmental patterning. However, epidermal cell movement has only been studied scantily in *Drosophila* embryos. Irvine and Wieschaus used time-lapse video microscopy to analyse cell movement during the first 45 min of germband extension (Irvine and Wieschaus, 1994). In their study Irvine and Wieschaus demonstrate extensive cell intercalation during germband extension. No study of cell movement in living embryos during germband retraction is available to date. The aim of my work described in this chapter was to establish methods that would allow detailed studies of epidermal cell movement in living *Drosophila* embryos. Once developed, I choose to apply these methods as a proof of concept to study epidermal cell movement during germband retraction mainly because this is the time when Wg specifies epidermal cell fates.
The Use of Various GFP Constructs for Cell Tracking in Live Embryos

There are two different approaches to mark a cell fluorescently: one can either label the cell outline with a membrane-bound fluorescent construct or one can label the cell interior (or a subset of it, e.g. the nucleus). In my first attempt I marked the plasma membrane of all cells. I used flies transgenic for Armadillo-GFP (Arm-GFP), kindly provided by C. Alexandre (NIMR, London). arm-GFP contains a DNA fragment encoding a N-terminal fusion of GFP to Arm downstream of the arm promoter. Arm-GFP seems to be most stable when associated with the cell membrane, only weak cytoplasmic Arm-GFP fluorescence was detected. Therefore, when imaged under a fluorescence microscope, the outline of cells expressing Arm-GFP is highlighted (Fig. 3.1a). In collaboration with A. Dempster (Dept. Electronic Systems, University of Westminster, London) a computer programme was designed that was able to segment fluorescently labelled cells (Ortiz De Solorzano et al., 2001) and to track such segmented cells. This programme was able to follow single cells expressing arm-GFP. Cells were manually picked when the fluorescence time-lapse movie was played back on a computer. However we were unable to design an algorithm that could reliably keep track of cells when they undergo mitosis (Fig 3.1b-e). I therefore abandoned the approach of labelling cell membranes.
Fig 3.1: Labelling cell membranes in live embryos to track cell movement.

a) Image of the ventral epidermis taken from a fluorescence time-lapse movie (movie 3.1) of a live stage 11 embryo expressing Arm-GFP, anterior is to the left. b-e) Close-ups taken from the same movie as in a), d) and e) are consecutive frames. A custom-written computer programme has outlined one cell (in red) and is tracking this cell successfully through the movie. However, when the tracked cell divides, the programme loses track of it (d-e). Cells appear to become bigger just before they divide (c-d). The scale bar is 30 μm in a) and 10 μm in b-e), the time is in minutes.
I then decided to label the cell nucleus. First I used flies transgenic for UAS-GFPnls. UAS-GFPnls contains a DNA fragment encoding the nuclear localisation signal of SV40 large antigen fused to GFP (Neufeld et al., 1998). However during early metaphase, when the nuclear envelope breaks down, GFP spills into the cytoplasm, the fluorescent signal becomes much weaker and resolution is lost (data not shown). I therefore decided to use a Histone-GFP fusion protein to label cell nuclei. I used flies carrying a modified genomic fragment which encodes the Drosophila melanogaster variant H2A.F/Z class histone (His2AvD) fused to GFP (His2AvD-GFP, Clarkson and Saint, 1999). I acquired fluorescence time-lapse movies of the ventral epidermis of live embryos carrying His2AvD-GFP (Movie 3.2). These movies show that His2AvD-GFP remains on chromosomes throughout all phases of mitosis and daughter cells can be identified unambiguously (Fig 3.2, Movie 3.3).

**Time-lapse Data Analysis using XVTRACK**
After acquiring fluorescence time-lapse movies of the ventral epidermis of live embryos carrying His2AvD-GFP (Movie 3.4) I processed individual frames with the image processing and analysis programme NIH Image 1.62 (Rasband and Bright, 1995).
Fig 3.2: Mitosis in the ventral epidermis during late embryonic stage 11.

a-f) Images taken from a fluorescence time-lapse movie (Movie 3.3) of a live embryo expressing His2AvD-GFP, ventral view of the epidermis, anterior is to the left. The nucleus in the upper right corner (red arrow) undergoes mitosis. a) The nucleus is already in prophase at the beginning of the movie. b) Chromosomes align to form the metaphase plate. c-e) Chromosomes segregate in anaphase. f) Chromosomes reach the poles in telophase. After that cytokinesis occurs. The scale bar is 5 μm and the time is in seconds.
Fig 3.3: Automated tracking of fluorescently labelled cells.

a) Starting frame taken from a fluorescence time-lapse movie of a live late stage 11 embryo expressing His2AvD-GFP, ventral view of the epidermis, anterior is to the left. XVTRACK acquires cell targets from the starting frame of a time series. Each target is marked with a red cross. To determine what is a target, i.e. a cell to track, the programme uses several criteria such as cell shape and brightness. XVTRACK then tracks all targets throughout the time-lapse series continuously calculating parameters such as target position and velocity. 

b) Example of 4 cells that have been tracked throughout the movie (45min). Their trajectories are overlaid onto the starting frame (same as in panel a) of the movie.

c) Vector representation of the trajectories of all tracked targets. The vector points from the starting position of each cell to its final position. Sister cells are easily recognisable by virtue of their common starting point. It is obvious that there is still mitotic activity throughout the epidermis during germ band retraction.

d-f) Example of a cell that undergoes mitosis while tracked (blue arrows). XVTRACK generates a new target for one of the daughter cells and logs the position where it appeared first. Both nuclei are tracked. The scale bar is 20 μm in a-c) and 5 μm in d-f), time is in seconds.
To test the feasibility of automating the tracking of cells, I analysed the data visually and with a 2-D cell tracking computer programme (XVTRACK; J. Solomon and S. Speicher, Computational Biology Centre, California Institute of Technology, Pasadena). XVTRACK acquires cell targets from an initial image of the time series and uses several criteria, including cell shape and brightness, to track cells throughout the time-lapse series (Nguyen Ngoc et al., 1997 and Fig 3.3a). In most cases the majority of cells could be tracked throughout the entire time-lapse sequence with the programme continuously calculating parameters such as cell position and velocity (Fig 3.3b-c). Any targets that are lost or suddenly appear along the way are indexed by the programme. Some of these were cells moving in or out of focus. Sometimes new targets appeared when the GFP fluorescence pattern changed suddenly. In such cases one nuclei was marked with more than one target. Unlike the computer programme that tracked the outline of cells (described above), XVTRACK is capable of following cells marked with His2AvD-GFP throughout mitosis (Fig 3.3d-f). Using fluorescence time-lapse movies of live embryos carrying His2AvD-GFP it is possible to mark any number of cells and follow them and their progeny visually or with the help of XVTRACK throughout the movie. One can also group cells into distinct domains and analyse the spread of such domains across the epidermis and their movement relative to each other (Fig 3.4).
Fig. 3.4: Analysis of cell movement during germband retraction.

a) Starting frame taken from a fluorescence time-lapse movie of a live late stage 11 embryo expressing His2AvD-GFP, ventral view of the epidermis, anterior is to the left. All fluorescent nuclei were tracked using XVTRACK, but only a subset of the targets ("virtual clone") is displayed (10 red nuclei). b) Finishing frame of the movie. The clone of initially marked cells has grown to 14 cells and is wider than it was in the beginning. c) Same frame as in panel a), this time two different groups of cells have been selected (10 red and 10 green nuclei). d) Same frame as in panel b). The clone of cells marked in green has not grown whereas the clone of red cells has grown to 14 cells and is wider than it was in the beginning. The two different groups of cells did not intermingle during the course of the analysis. The scale bar is 20 μm, time is in minutes.
Fig 3.5: Mitotic activity of wg-expressing cells during germband retraction.

a) Starting frame taken from a fluorescence time-lapse movie of a live late stage 11 embryo expressing His-YFP under the control of wg-GAL4, ventral view of the epidermis, anterior is to the left. All fluorescent nuclei were tracked using XVTRACK, but only a subset of the targets is displayed (6 nuclei, all shown in different colours). b) Finishing frame of the movie. All of the initially marked cells have divided once during the course of the movie. Cell division and rearrangement caused some cells to change their neighbours. c) Close-up of several fluorescent nuclei shortly after anaphase. The orientation of the mitotic spindle is depicted with coloured arrows connecting sister cells. The angle of the spindle with the AP axis of the embryo is approximately 23° (green arrow) and 110° (red arrow). d) Same image as in c, showing fluorescent nuclei shortly after anaphase, which has been filtered with the “emboss” tool (Adobe Photoshop) to make it easier to visually distinguish individual nuclei outlines. e) Plot of the orientation of cell divisions in the epidermis. The arrow indicates the mean value and the coloured area shows the standard deviation, 30° intervals are shown on the circular plot. Scale bar is 30micron (a-b) or 15micron (c-d).
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(a) 0 min

(b) 64.7 min

(c)

(d)

(e) Pie chart showing:
- 101 ± 19
- 23 ± 15
Chapter 3: A Brief Analysis of Cell Movement

Mitosis in the Embryonic Epidermis during Germband Retraction

It is thought that by the onset of the germband shortening mitotic activity has ceased in the ventral epidermis, with the exception of neuroblasts that undergo additional rounds of mitosis (Hartenstein and Campos-Ortega, 1985 and Foe, 1989; reviewed in Foe et al., 1993). But the fluorescence time-lapse movies of the ventral epidermis that I acquired showed a surprising amount of mitotic activity during germband retraction. I therefore decided to investigate this further in a subset of epidermal cells. I acquired fluorescence time-lapse movies of the ventral epidermis of live, late stage 11 embryos expressing UAS-

\[ \text{H2B-mYFP} \] (Bellaiche et al., 2001) under the control of \[ \text{wg-GAL4} \]. H2B-mYFP encodes human histone H2B fused to the red-shifted GFP variant mYFP (Haseloff, 1999). An analysis of these movies shows that many of the tracked \[ \text{wg-GAL4} \] expressing cells undergo mitosis during the course of image acquisition (typically 1-1.5 hrs, Fig 3.5a-b, Movie 3.5). I also measured the orientation of the mitotic spindle during cell division (Fig. 3.5c-d). These measurements show that epidermal cells do not divide randomly, but that there are two preferred orientations of the mitotic spindle: parallel and perpendicular to the AP axis (Fig. 3.5e). This suggests that there are mechanisms to control spindle orientation. Observations of spindle alignments prior to cell division support this hypothesis (Movie 3.5).
Conclusion

The analysis of cell movement during later stages of embryonic development was hampered by the lack of suitable imaging and analysis techniques. In earlier studies of epidermal cell movement epi-illumination and time-lapse video microscopy was used and the trajectories of a limited number of cells were reconstructed by hand (Irvine and Wieschaus, 1994). Labelling cell nuclei with fluorescent histones allows to automatically track the movement of any number of cells and makes a detailed computational analysis of many trajectories possible.

I applied the developed imaging and analysis tools to the study of epidermal cell movement during germband retraction. The long-term nature of my observations allows me to catch transient events and show that there is a surprising amount of cell division during germband retraction. It has been thought that at this stage epidermal cells do not divide anymore, with the exception of neuroblasts (Foe et al., 1993). The orientation of the observed cell divisions fell with similar frequency into one of two classes: either parallel or perpendicular to the AP axis. This suggests that there are mechanisms in place that control spindle orientation. The function of such a control mechanism could be to orchestrate mitotic activity and cell movement. My findings also challenge the prevailing assumption that cells do not change neighbours during germband retraction (Martinez Arias, 1993).
CHAPTER 4: WINGLESS TRAFFICKING IN LIVE EMBRYOS

Introduction

In chapter 2 I have shown that as cells proliferate and spread, they retain the Wg protein in secretory vesicles and that this can account for the spread of the signal within the embryonic epidermis of Drosophila. However, this may represent a specialisation of this fast developing system. The mechanism of transcytosis, involving endocytosis and release in the extracellular space, appears to drive the spread of the TGF-β homologue Dpp along the wing imaginal epithelium (Entchev et al., 2000). Planar transcytosis had previously been suggested to transport Wg along the embryonic epithelium of Drosophila (Moline et al., 1999). However, clathrin-mediated endocytosis is dispensable for Wg transport in wing imaginal discs suggesting that transcytosis is not required in this tissue (Strigini and Cohen, 2000). Whether Wg transcytosis operates in Drosophila embryos is still unclear. An alternative model for both discs and embryos is that diffusion occurs in the extracellular space in association with cell surface molecules such as HSPGs (Hacker et al., 1997; Binari et al., 1997 and Haerry et al., 1997). Whatever the mechanism of transport, Wg trafficking is probably under tissue-specific control since the range and distribution of Wg differs between embryos and discs. In discs, Wg spreads rapidly and over relatively long distances (10-12 cell diameters; Strigini and Cohen, 2000 and
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Cadigan et al., 1998) while in embryos, the action of Wg is confined to 3-4 cell diameters at most. In order to understand the reason for this difference and explore the cell biological basis of Wg transport, I made transgenic Drosophila embryos that express biologically active GFP-Wg. In this chapter, I focus on the spread of Wg towards the anterior of each stripe of wg expression. Towards the posterior, the range is restricted by specific degradation of the signal (L. Dubois, in press).

**GFP-Wingless is Active**

GFP was inserted at the amino terminus of Wg, just downstream of the signal peptide. To first assess the activity of GFP-Wg I asked whether it could replace endogenous Wg during embryonic development. I expressed UAS-GFP-wg in a wg null mutant with wg-GAL4. In most embryos, the mutant phenotype is rescued to a wild type pattern (Fig. 4.1a) showing that GFP-Wg is active. However, this does not necessarily mean that GFP-Wg spreads normally along the epithelium because, as mentioned above, the signal can be delivered by the progeny of expressing cells. Indeed, as I have shown in chapter 2, a membrane-tethered form of Wg (Wg[teth]) also rescues a wg null mutant in this assay (Pfeiffer et al., 2000). To eliminate the contribution of cell movement, UAS-GFP-wg was expressed on the posterior side of the parasegment boundary (with en-GAL4) in a wg mutant.
Figure 4.1: GFP-Wingless rescues wingless mutants.

(a) Cuticle pattern of a \textit{wg}^{CX4} larva carrying \textit{wg}-GAL4 and \textit{UAS-GFP-wg}, the dentine pattern is rescued to a wild type phenotype  (b) Cuticle pattern of a \textit{wg}^{CX4} larva carrying \textit{en-GAL4} and \textit{UAS-GFP-wg}, the dentine pattern is indistinguishable from that of a \textit{wg}^{CX4} larva carrying \textit{en-GAL4} and \textit{UAS-wg}. Naked cuticle at the anterior of the \textit{en} expression domain implies that GFP-Wg is secreted and spreads across the segment.  (c) Expression of \textit{ser} (black) in a wild type embryo at stage 12. Wg (\textit{wg} mRNA in red) represses \textit{ser} over 2-3 cell diameters.  (d) Expression of \textit{ser} and \textit{wg} in a \textit{wg}^{CX4} embryo carrying \textit{en-GAL4} and \textit{UAS-GFP-wg} at stage 12, secreted GFP-Wg represses serrate over 2-3 cell diameters, the number of \textit{en} cells is increased due to GFP-Wg expression.  (e) Schematic representation of \textit{wg} expression in a wild type embryo. The dotted line represents movement of \textit{wg} expressing cells and their progeny, the solid lines represent the spread of Wg independent of cell movement.  (f) Schematic representation of \textit{wg} expression in a \textit{wg}^{CX4} embryo carrying \textit{en-GAL4} and \textit{UAS-GFP-wg}. The parasegment border (PS) prevents movement of \textit{wg} expressing cells towards the anterior. The scale bar is 50 \textmu m in (a-b) and 11 \textmu m in (c-d).
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- **e)**
  - $ser$ expressing cell
  - $wg$ expressing cell

- **f)**
  - $en$ expressing cell
  - $wg$ and $en$ expressing cell
Since the parasegment boundary is a clonal boundary, it prevents the anteriorward spread of *en*-expressing cells and any action of Wg towards the anterior in this assay must follow from the spread of the signal itself (Fig. 4.1e-f). The signal can spread independently of cell movement since Wg expressed in the *en* domain acts over several cell diameters towards the anterior (in *wg*; *en-GAL4 UAS-wg*) while, by comparison, membrane-tethered Wingless (in *wg*; *en-GAL4 UAS-wg[teth]*) acts only on adjacent cells (Sanson et al., 1999). Using the same assay, I found GFP-Wg to behave like wild type Wg. In embryos whose sole source of Wg is GFP-Wg expressed in the *en* domain (*wg*; *en-GAL4 UAS-GFP-wg*), large expanses of naked cuticle (an indication of Wg signalling) form and a near normal cuticle pattern develops (Fig 4.1b). Because the cuticle pattern is a rather late readout of Wg signalling, I also used a more immediate assay, the embryonic expression of *ser*, a gene that is repressed by Wg signalling. The posterior edge of each *ser* stripe marks the anterior limit of the range of Wg at stage 11-12 (Alexandre et al., 1999; Fig 4.1c, e-f). As shown in Fig 4.1d, GFP-Wg driven with *en-GAL4* represses *ser* across several cell diameters at the anterior of the *en-GAL4* expressing cells. On the basis of the two assays described above, I conclude that GFP-Wg spreads along the embryonic epidermis like the wild type protein.
**Extracellular Distribution of Wingless**

To assess the spread of Wg in the extracellular space independently of cell inheritance, I expressed GFP-Wg in the *en* domain and imaged live embryos (Fig 4.2a-a"). As expected, expressing cells are very bright. However, little or no fluorescence is detectable outside the expression domain (Fig 4.2a). For comparison, secreted GFP (GFP$^{secr}$) was expressed either with *en-GAL4* or *wg-GAL4*. In both cases, GFP fluorescence fills the perivitelline space, highlighting grooves and cell boundaries (Fig 4.2b). Thus, GFP itself diffuses readily in the extracellular space while Wg appears to be specifically retained by expressing cells. The bright signal from expressing cells arises in part from GFP-Wg transiting through the secretory pathway and removal of this contribution might allow a better view of extracellular Wg across the embryonic epidermis. Unfortunately, I was unable to distinguish unambiguously extracellular from intracellular fluorescence with either traditional widefield or single-photon confocal microscopy. And Total Internal Reflection Fluorescence Microscopy (TIRF) could not be used because of the thickness of the vitelline membrane (data not shown).

Therefore, Sara Ricardo (a member of Dr. Vincent's laboratory at NIMR) adapted to the embryo a procedure designed to detect only extracellular Wg in fixed imaginal discs (Strigini and Cohen, 2000).
Figure 4.2: GFP-Wingless is retained by expressing cells.

(a-a") Co-localisation (arrowheads in (a")) of GFP-Wg (a) and endocytosed TR-Dextran (a') in intracellular vesicles. The panels show the ventral epidermis of a live wg^{CX4} embryo carrying en-GAL4 UAS-GFP-wg at stage 11. No obvious GFP-Wg fluorescence can be seen outside the expression domain, however GFP-Wg must be present because fluorescent endocytic vesicles (arrowheads) can be detected. (b) Live stage 11 wg-GAL4 UAS-GFP^{sect} embryo. GFP^{sect} is secreted from the expressing cells and diffuses readily across the epidermis. Note the different fluorescence pattern in (c) and (b) demonstrating the retention of GFP-Wg within its expression domain. (c, d) Extracellular Wg (red) and intracellular En (green) in a wg^{CX4} carrying en-GAL4 and UAS-GFP-wg (c) and a wild type (d) embryo at stage 11. Embryos stained for extracellular Wg and for intracellular En were kindly provided by S. Ricardo. In the rescue situation (c) no cell movement towards the anterior occurs and GFP-Wg is mainly found at the membrane of expressing cells. In the wild type situation (d) extracellular Wg is found over the entire range of Wingless action. All the cells within that range could have inherited Wg (Pfeiffer et al., 2000).

See page 67 for remaining figure legend.
Figure 4.2 (continued): GFP-Wingless is retained by expressing cells
(e-e") Co-localisation (arrowheads in (e")) of GFP-Wg (e) and endocytosed TR-Dextran (e') in intracellular vesicles. The panels show the ventral epidermis of a wg-GAL4 UAS-GFP-wg live embryo at stage 11. The inset in (e) shows the number of GFP-Wg vesicles (present in a 128x128 pixels ROI over 300 frames acquired at 0.2 frames per second) and their intensity within (light green) and at the posterior (dark green) of the expression domain. The histogram for the vesicles within the expression domain is shifted to the right with respect to the histogram of the posterior vesicles because the fluorescence intensity is higher in the expression domain. In addition, the area under the histogram is bigger within the expression domain because there are more vesicles there than at the posterior. This distribution mirrors the asymmetry of Wingless action. The inset in panel (e') shows the number of Rho-Dx vesicles and their intensity that were counted in the same ROI as in inset (e). There is no difference in the two distributions showing that generic endocytic vesicles form uniformly across the epidermis. The scale bar is 10 μm for all panels except (c, d) where it is 11.5 μm.
As it can be seen in Fig. 4.2c, all detectable extracellular Wg is confined to the surface of expressing cells even though some Wg must be present in anterior non-expressing cells (over 2-3 cell diameters) because they respond to Wg (they repress ser and make naked cuticle). This contrasts with the situation in imaginal discs where the same staining procedure reveals extracellular Wg over up to 12 cell diameters. I conclude that in the embryonic epidermis, most detectable extracellular Wg is associated with the surface of cells that secrete it.

Using the same staining procedure, one finds that, in wild type embryos, extracellular Wg is detectable in 3-4 cell-wide stripes corresponding roughly to the range of Wingless action (Fig 4.2d, Fig 4.1e). In particular, Wg is seen at the surface of cells that transcribe \( wg \) (adjacent to the \( en \) domain) and of cells located 2-3 cell diameters at the anterior. Since, as shown in the previous experiment, extracellular Wg is only detectable at the surface of cells that secrete it (Fig 4.2c), surface staining at the anterior of the domain of transcription most likely arises from delayed release of persisting secretory vesicles in the progeny of expressing cells (Pfeiffer et al., 2000). This means that most of the cells that respond to Wg also secrete it and therefore, the distinction between sending and receiving cells is blurred.

Even though no extracellular Wg can be detected in recognisable non-expressing cells in \textit{en-GAL4 UAS-GFP-wg} embryos (at the anterior of the parasegment border; Fig
4.2c), a few Wg-containing vesicles do form there (Fig. 4.2a) confirming that these cells were reached by Wg. Since these vesicles are in non-expressing cells, they are likely to be endocytic. This was confirmed by using 10kD tetramethylrhodamine dextran (Rho-Dx, Molecular Probes) as a marker for endocytosis. Upon injection in the perivitelline space, Rho-Dx is readily taken up by epidermal cells (Fig 4.2 a'). As shown in Fig 4.2a'', GFP-Wg vesicles seen in non-expressing cells also contain Rho-Dx confirming their endocytic nature.

There is one difficulty in interpreting the results obtained with the adapted extracellular staining protocol. This protocol should identify extracellular Wg only on the apical surface of the epidermis because only the apical side is accessible to anti-Wg in unfixed embryos. Therefore some extracellular Wg might be missed. However, I assume that most of the extracellular Wg in the embryonic epidermis is located apically for two reasons. Firstly, in fixed wild type embryos most of the Wg protein detected is enriched in apical vesicles (Simmonds et al., 2001). Secondly, GFP-Wg is also enriched in the apical cytoplasm of GFP-wg expressing cells (Fig 4.3). My interpretation is, that Wg is secreted and endocytosed mostly apically and therefore most of the extracellular Wg should be on the apical surface. When GFP-Wg is degraded, its fluorescence signal will most likely vanish. Most late endosomes and lysosomes are found baso-laterally (L. Dubois, personal communication) and this might account for the absence of fluorescence
So far, I can conclude that expressing cells retain most secreted Wg, releasing only a trickle and that receiving cells internalise Wg quickly. In imaginal discs, extracellular as well as vesicular Wg is detected up to 12 cell diameters away from the source (Strigini and Cohen, 2000). Maybe in this system, Wg is less strongly tethered to the surface of expressing cells and is allowed to spread away as a result. One obvious class of molecule that could tether Wingless at the surface of expressing cells are HSPGs (Reichsman et al., 1996 and Baeg et al., 2001) and differences in the regulation of these molecules might explain the differences between discs and embryos.

Two Populations of Wingless Vesicles
As suggested above, Wg is readily internalised by receiving cells. In the wild type many vesicles are seen at the anterior of the parasegment boundary but there, the distinction between signalling and receiving cells is blurred. Indeed, in this domain, many vesicles are known to be secretory, even in cells that no longer transcribe \textit{wg} (Pfeiffer et al., 2000). Therefore, one expects that, at the anterior of the parasegment border, secretory and endocytic Wg-containing vesicles coexist. To assess the relative importance of these two populations, I expressed GFP-Wg under the control of \textit{wg-GAL4}. As with \textit{en-GAL4}, most of the fluorescence is confined to expressing cells (or their progeny) confirming that
the Wg signal remains tightly associated with expressing cells (Fig 4.2e). Many intracellular vesicles are seen and Rho-Dx was used to recognise endocytic vesicles. The perivitelline space of wg-GAL4 UAS-GFP-wg embryos was injected with Rho-Dx around stage 9 and embryos were subsequently imaged live, around stage 11-12 (Fig 4.2e-e”).

GFP-Wg-positive vesicles were identified as endocytic (endoGFP-Wg) by co-localisation with intracellular Rho-Dx. Fig. 4.2e” shows that many vesicles (about 54%) contain endoGFP-Wg. This number probably represents a lower estimate because some endoGFP-Wg vesicles could have formed before Rho-Dx injection. Therefore, wg-expressing cells endocytose Wg at a high rate, perhaps reflecting the availability of Wg at the cell surface. In contrast, when GFP-Wg originates from the en domain thus allowing non-expressing cells to be unambiguously recognised, very few endocytic vesicles are present outside the expression domain (arrowheads in Fig 4.2a-a”). I conclude that, although much intracellular Wg is endocytic in wild type embryos, endocytosis of Wg is mostly confined to secreting cells, which have plenty of Wg available at their surface.

The Fate of Endocytosed Wingless

I now address what happens to Wg after it has been endocytosed. Is Wg trafficking specifically regulated after endocytosis? To answer this question, I investigated the behaviour of GFP-Wg vesicles in live embryos.
Figure 4.3: Intracellular Wingless Distribution.

(a) Image of the ventral epidermis of a live wg-GAL4 UAS-GFP-wg embryo at stage 11. This image represents a projection of a 10 μm deep volume into the epidermis at a step-size of 0.5 μm. (b) Computer reconstruction of a sagittal section so that location of the GFP-Wg vesicle with respect to the apical/basal axis can be seen. Most vesicles are found in the apical cytoplasm of GFP-wg expressing cells. The scale bar is 6 μm.
I tracked the movement of fluorescent vesicles in two dimensions in stage 11-12 embryos expressing GFP-Wg with wg-GAL4. This is the stage when Wg activity specifies epidermal cell fate (Bejsovec and Martinez Arias, 1991). It is also the stage when epidermal cells undergo extensive rearrangements during germ band retraction (see Chapter 3) which complicates the analysis of vesicle movement. I therefore developed a computational tool to adjust for this offset movement (see chapter 6, Fig 4.4 and Movie 4.2). After removing the offset movement, the mean square displacement (MSD) was calculated for increasing time intervals \( \Delta t \). A MSD vs. \( \Delta t \) plot was used as a quantitative characteristic of vesicle motion and also to classify various types of movement.

Three classes of movement were recognised: random walks, directed diffusion, and diffusion in a cage (Fig 4.5d-g). The MSD was calculated for endoGFP-Wg vesicles (containing both GFP-Wg and Rho-Dx), generic endocytic vesicles (only Rho-Dx positive) and secretory vesicles (fluorescent vesicles in wg-GAL4 UAS-GFP\textsuperscript{secr} embryos) which generated distributions of diffusion coefficients for all classes of vesicles (Fig 4.5h-j).

Looking at the relative contribution of the different modes of motion of a given class of vesicles, I found differences between endoGFP-Wg and generic endocytic vesicles (Table 4.1).
Figure 4.4: Removing offset movement caused by germband retraction.

(a) Image of the ventral epidermis of a live \textit{wg-GAL4 UAS-GFP-wg} embryo at stage 11.
(b) Image from panel (a) after a FFT low-pass filter (see chapter 6). This fluorescent cloud was tracked to calculate the rate of germband retraction. The inset shows a plot of the x and y position of the centre of mass of the tracked fluorescent cloud as a function of time. The x and y components of the rate of germband retraction ($v_x$ and $v_y$ respectively) were calculated from the slopes of these plots. In this example $v_x = -0.0128$ \textmu m/s and $v_y = -0.0004$ \textmu m/s. (c-d) Trajectory of a tracked vesicle before (c) and after (d) the offset movement was removed. (e-f) MSD vs. $\Delta t$ plot of the tracked vesicle from panels (c) and (d) before (e) and after (f) the offset movement was removed. Before removing the offset movement caused by germband retraction the vesicle seemingly undergoes directed diffusion (e). But the directed component of the motion is caused by cell movement. When the offset cell movement is removed, it becomes apparent that the vesicle undergoes diffusion in a cage (f). The scale bar is 10 \textmu m in (a-b).
For instance while 43% of endoGFP-Wg vesicles undergo directed diffusion, only 36% of generic endocytic vesicles do so. This may seem like a small difference but one should bear in mind that, in this experiment, endogenous, untagged Wg was present. Therefore, generic endocytic vesicles may have included Wingless-containing vesicles, thus reducing the apparent difference between the two populations. This difference becomes more obvious when one compares the relative contributions of simple and directed diffusion. Only 6% more endocytic vesicles undergo directed rather than simple diffusion whereas in the case of endoGFP-Wg vesicles this proportion amounts to 17%. Increased directed diffusion suggests that, once internalised, Wg-containing vesicles are preferentially captured, possibly by the cytoskeleton.

I also found quantitative differences between the different modes of motion (Table 4.2). The diffusion coefficient of endoGFP-Wg vesicles undergoing directed diffusion is on average lower than that of generic endocytic vesicles ($D=1.8 \times 10^{-3} \, \mu m^2/s$ v. $D=2.8 \times 10^{-3} \, \mu m^2/s$ respectively, $P<0.01$). This means that endoGFP-Wg vesicles deviate less from their track (possibly fall off less frequently from their cytoskeletal anchor). In addition to directed diffusion, I also observed trajectories best explained by diffusion in a cage. A common interpretation of such behaviour is that the motion of the vesicles is restricted by a tether to the plasma membrane (Steyer and Almers, 1999).
Figure 4.5: Analysis of endocytic GFP-Wingless vesicle movement.

(a-c) Example of time-lapse fluorescence microscopy imaging of endocytic GFP-Wg vesicles in a live stage 11 wg CX4 embryo carrying en-GAL4 UAS-GFP-wg. Rho-Dx was injected into the perivitelline space 2hrs prior to imaging. Images were acquired at 0.2 dual colour frames/second. The vesicle was positively identified as endocytic by co-localisation with endocytosed Rho-Dx (red channel not shown). The scale bar is 2 μm and time is in seconds. (d-g) Examples of modes of motion observed for GFP-Wg vesicles. Shown is a fit of the MSD as a function of the time interval Δt as derived from the vesicle trajectory (see chapter 6). (d) Simple diffusion with a diffusion coefficient D was fitted with: $MSD(Δt) = 4DΔt$, here $D = 1.7 \times 10^{-3} \, \text{μm}^2/\text{s}$. (e) Directed diffusion at velocity v and a diffusion coefficient $D$ was fitted with $MSD(Δt) = 4DΔt + v^2Δt^2$, here $D = 2.4 \times 10^{-3} \, \text{μm}^2/\text{s}$ and $v = 1.1 \times 10^{-2} \, \text{μm}/\text{s}$. (f) Diffusion in an immobile cage and (g) diffusion in a mobile cage both fitted with $MSD(Δt) = R^2[1 - a_i \exp(-4a_2 D Δt / R^2)] + 4D_{\text{cage}}Δt$ where R is the radius of the cage, $D_{\text{cage}}$ is the diffusion coefficient of the cage, D is the diffusion coefficient of the vesicle inside the cage, and $a_i = 0.99$, $a_2 = 0.85$ are two constants. In (f) $R = 824 \, \text{nm}$, $D = 3.5 \times 10^{-3} \, \text{μm}^2/\text{s}$ and $D_{\text{cage}} = 10^{-13} \, \text{μm}^2/\text{s}$ and in (g) $R = 523 \, \text{nm}$, $D = 3.4 \times 10^{-3} \, \text{μm}^2/\text{s}$ and $D_{\text{cage}} = 3 \times 10^{-4} \, \text{μm}^2/\text{s}$. (h-j) Distribution of diffusion coefficients for generic endocytic (h), endoGFP-Wg (i) and secretory (j) vesicles. Data was compiled from the observation of 50 generic endocytic vesicles, 51 endoGFP-Wg and 88 secretory vesicles.
Chapter 4: Wingless Trafficking in Live Embryos
**Table 4.1: Relative contribution of the different modes of motion.**

This table shows the relative contribution of the different modes of motion (simple and directed diffusion and diffusion in a mobile and immobile cage) of generic endocytic, endoGFP-Wg and secretory vesicles.
### Table

<table>
<thead>
<tr>
<th>Vesicle class</th>
<th>% Simple</th>
<th>% Directed</th>
<th>% CagedImm</th>
<th>% CagedMob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generic endocytic</td>
<td>30</td>
<td>36</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>EndoGFP-Wingless</td>
<td>27</td>
<td>43</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>Secretory</td>
<td>30</td>
<td>38</td>
<td>19</td>
<td>13</td>
</tr>
</tbody>
</table>
Table 4.2: Parameter of all observed modes of motion.

This table shows the mean value of parameters calculated from MSD v. Δt plots of generic endocytic, endoGFP-Wg and secretory vesicles. For simple diffusion the mean diffusion coefficient is shown, for directed diffusion the mean diffusion coefficient and the mean velocity of the vesicle is shown, for diffusion in a cage the mean diffusion coefficient of the vesicle and the cage as well as the radius of the cage is shown.
<table>
<thead>
<tr>
<th>Vesicle class</th>
<th>Simple</th>
<th>Directed</th>
<th>Caged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generic endocytic</td>
<td>$2.53 \times 10^{-3}$</td>
<td>$2.79 \times 10^{-3}$</td>
<td>$1.28 \times 10^{-2}$</td>
</tr>
<tr>
<td>EndoGFP-Wingless</td>
<td>$2.51 \times 10^{-3}$</td>
<td>$1.84 \times 10^{-3}$</td>
<td>$1.19 \times 10^{-2}$</td>
</tr>
<tr>
<td>Secretory</td>
<td>$1.92 \times 10^{-3}$</td>
<td>$2.11 \times 10^{-3}$</td>
<td>$2.11 \times 10^{-2}$</td>
</tr>
</tbody>
</table>
For endoGFP-Wg vesicles undergoing diffusion in a cage (either mobile or immobile) the size of the cage was on average significantly smaller than that of generic endocytic vesicles diffusing in a cage (476 nm vs. 630 nm, P<0.05). Overall then, this analysis shows that endoGFP-Wg vesicles are handled differently from generic endocytic vesicles suggesting that endocytic trafficking of Wg is under distinct control.

**Recycling of Endocytosed Wingless**

Endocytosed material can either be sent to lysosomes for degradation or recycled to the plasma membrane. Often, ligand-receptor complexes dissociate in the endocytic pathway with the ligand being forwarded to lysosomes and the receptor being recycled. I specifically asked whether endoGFP-Wg is ever recycled. This was done in live *wg−* embryos expressing GFP-Wg with *en-GAL4*. As argued above, vesicles found at the anterior of the expression domain are necessarily endocytic and no Rho-Dx injection is required. Fluorescence time-lapse microscopy shows that such vesicles can return to the cell surface (Fig 4.6 and Movie 4.3), providing the first direct observation of ligand recycling in a living embryo. In epidermal cells lysosomes are found baso-laterally (L. Dubois, personal communication). It is therefore unlikely that the disappearance of the vesicle at the apical surface is due to degradation of its content.

This observation could be taken as supporting the transcytosis hypothesis. However, one
should note that in this experimental situation, very few endoGFP-Wg vesicles are present in non-expressing cells even though Wg transport does occur over 2-3 cell diameters (Fig 4.2a-a"). This is not a peculiarity of GFP-Wg because, in *wg* mutant embryos that express wild type Wg under the control of *en-GAL4*, immuno-fluorescence reveals very few intracellular Wg-containing vesicles in non-expressing cells (data not shown). It could be that a very small number of endocytic vesicles are sufficient to carry Wg over this distance.

However recycling of endocytic Wg may serve another purpose. Since increased ligand recycling has been documented to enhance the potency of a signal in tissue culture cells (Fallon et al., 2000), an alternative is that cells at the anterior of the parasegment boundary (which require sustained Wg signalling) recycle Wg to ensure maximal signalling.

**Specific Covalent Labelling of Extracellular Wingless**

The analysis of the dynamics of extracellular Wg was hampered by the strong intracellular signal from expressing cells and the very weak extracellular GFP-Wg fluorescence outside the expression domain. I decided to specifically label extracellular Wg in living embryos. I made flies transgenic for a Wg protein that was tagged with an \(\alpha\)-helical tetracysteine-containing peptide and a HA tag (*UAS-4Cys-HA-Wg*).
Figure 4.6: Recycling of endocytosed GFP-Wingless.

(a-d) 4D time-lapse fluorescence microscopy imaging of a live stage 11 wg CX4 embryo carrying en-GAL4 UAS-GFP-wg. I imaged a 6 µm deep volume into the epidermis at a step-size of 0.5 µm at 1.4 frames/second. The top image shows the section of the volume were the tracked vesicle (blue arrow in (a)) was in focus. The lower image shows a computer reconstruction of a sagittal section so that position of the tracked vesicle with respect to the apical/basal axis can be seen. The tracked vesicle disappears between panel (c) and (d), which show consecutive volumes, most likely due to secretion of its contents. It is very unlikely that the vesicle left the volume at the basal side. This would have required the vesicle to move with a velocity exceeding any speed I have seen in my experiments (in the order of 10^{-2} µm/s). In epidermal cells lysosomes are found basolaterally (L. Dubois, personal communication). It is therefore unlikely that the disappearance of the vesicle at the apical surface is due to degradation of its content. In the sagittal reconstruction it is obvious that the vesicle is outside the en cells and therefore must be endocytic. (e) 3D trajectory of the tracked vesicle. The axes are in µm with the starting position of the vesicle set to z=0 µm. The vesicle leaves the volume at z= 2µm. Note that the z resolution is less than the x-y resolution. The scale bar is 10 µm.
The α-helical tetracysteine-containing peptide serves as a receptor domain for a trivalent arsenic ligand called FLASH (Fig 4.7a and Griffin et al., 2000; Griffin et al., 1998). The original FLASH reagent is membrane permeable. However, S. Adams (University of California San Diego, USA) kindly provided a sulfonated FLASH derivative (sFLASH) which is membrane impermeable (Fig 4.7b). FLASH, and its derivative sFLASH, has relatively few binding sites in wild type embryos but binds to the tetracysteine motif with nanomolar or even lower dissociation constant. The ligand is initially non-fluorescent but becomes strongly fluorescent upon binding to the tetracysteine motif (Fig 4.7c).

The idea was to “stain” embryos transgenic for 4Cys-HA-Wg by injecting sFLASH into the perivitelline space. Since sFLASH is membrane impermeable, only extracellular 4Cys-HA-Wg would be fluorescently labelled. To test the membrane impermeability of sFLASH and the specificity of the sFLASH-tetracysteine peptide interaction in live Drosophila embryos I made two additional constructs. One encoded the transmembrane domain of CD2 fused to the α-helical tetracysteine-containing peptide on the intracellular side (negative control) and the positive control contained the tetracysteine motif on the extracellular side.

Unfortunately the 4Cys-HA-Wg protein turned out to be biologically inactive, judging from its ability to induce naked cuticle. Anti-HA-antibody stainings showed that the
protein itself was expressed (data not shown). Since I was unable to genetically engineer a biologically active 4Cys-Wg protein I abandoned the sFLASH approach.

**Conclusion**

I have shown that Wingless is retained at the surface of expressing cells thus leading to limited availability in the extracellular space of non-expressing cells. Retention is probably essential to ensure a short range of action within the embryonic epidermis. In the wild type, inheritance of Wg in proliferating cells ensures that plenty of Wg is available with the normal domain of action. If the source of Wg is shifted posteriorly to the other side of the parasegment boundary thus restricting the availability of Wg in the normal expression domain, recycling of endocytosed Wg ensures that signalling is sustained and possibly amplified. In the wild type, internalisation occurs mostly within expressing cells or their progeny, where surface-associated Wg is abundant. Therefore, within the anterior domain of Wingless action, there is no distinction between cells sending the signal and cell receiving it and, in this domain, secretory and endocytic Wg coexist. Towards the posterior, there is no contribution from cell proliferation because the parasegment boundary prevents the progeny of expressing cells from spreading posteriorly. In addition, at the posterior, endocytosed Wg is rapidly degraded (L. Dubois, *in press*) thus further reducing Wg signalling.
Figure 4.7: Specific covalent labelling of a tetracysteine-containing peptide.

(a) The biarsenical ligand 4',5'-bis(1,3,2-dithioarsolan-2yl)fluorescein, which is also called FLASH, was prepared in a single step by transmetallation of commercially available fluorescein mercuric acetate (Griffin et al., 1998). (b) Sulfonated FLASH (sFLASH) kindly provided by S. Adams (Howard Hughes Medical Institute and University of California San Diego, USA). (c) Proposed structure of the complex formed when sFLASH binds to an α-helical tetracysteine-containing peptide. Although the structure is drawn with i and i+4 thiols bridged by one arsenic and the i+1 and i+5 thiols bridged by the other, one cannot rule out the isomeric complex in which one arsenic links the i and i+1 thiols while the other links the i+4 and i+5 thiols. Redrawn from Griffin et al., 1998.
Chapter 4: Wingless Trafficking in Live Embryos

a) 

b) 

1 + 5 + 5

SH SH

SH COO-

3OS
CHAPTER 5: DISCUSSION

Wg -Expressing Cells Spread the Signal

The Wnt/Wg family of secreted proteins are important in many different patterning events during development. It has been demonstrated that these proteins can act at a distance from their site of synthesis and it is generally assumed that this occurs via diffusion, although it has been argued that active transport is important. I have investigated the mechanism underlying long range Wg activity in the embryo and discovered a novel mechanism for Wg transport: Wg function at a distance is mediated by the movement of wg expressing cells. In addition to being a novel mechanism for Wg transport, my findings also demonstrate the importance of cell lineage in embryonic development because it shows that the manner in which the anterior compartment of a segment is populated effects the distribution of Wg and therefore patterning. So far cell lineage has not been considered to play a major role in embryonic development.

These findings raise several questions that are worth clarifying in future experiments. One question, which is difficult to address, is how much of the patterning effects of Wg in the wild type situation are due to Wg diffusion (or some other transport mechanism) versus cell delivery of Wg. Since I have shown in several independent experiments that there is enough mitotic activity and cell movement to account for the range of Wg in
embryos, this questions leads ultimately to the issue of the stability of wild type Wg protein. One obvious approach would be to tag Wg with a fluorescent timer (Terskikh et al., 2000) and express this fusion protein under wg-GAL4 control. However, it is difficult to infer the stability of the wild type protein from the stability of a fusion protein and therefore it might not be that satisfying. Instead, endogenous protein needs to be labelled in a manner that allows pulse-chase experiments. But the labelling procedure must not interfere with the structure of the protein (or its activity) and this point would need addressing in very carefully controlled experiments.

A second issue raised concerns the nature of the vesicles that unsecreted Wg can be found in. Are these vesicles specialised ‘storage compartments’? A more biochemical approach seems appropriate here. One could use the fluorescence signal of ectopically expressed GFP-Wg for purification of those vesicles (Fluorescence Activated Cell Sorting, FACS, could be used for this). It is then possible to characterise the protein composition of these vesicles, preferably with mass spec and HPLC, and compare the protein composition to that of other vesicle populations. This raises another question immediately. Is the release of Wg from these vesicles somehow regulated? It is interesting to note in this context that naked cuticle (nkd), an inducible antagonist of Wg signalling, has significant similarity to the high affinity Ca^{2+}-binding EF hand of the
recoverin family of myristoyl switch proteins (Zeng et al., 2000). Ca\(^{2+}\) plays a central role in the function of many different cell types, e.g. as the trigger for neurotransmitter release in neurons. This is mediated by Ca\(^{2+}\)-binding proteins such as recoverin and calmodulin (see Burgoyne and Weiss, 2001 for a recent review). It is tempting to speculate that the release of Wg might be regulated in a similar fashion. One possible experimental approach to address this question is to photoactivate caged Ca\(^{2+}\) in embryos that express GFP-Wg and monitor the secretion of GFP-Wg.

Another question raised is whether movement of \(wg\) expressing cells contributes significantly to the distribution of the protein in other tissues. It is quite possible that this transport mechanism plays a role in places close to the \(wg\) expressing cells. Clones of wild type cells in the wing are often oriented along the dorsal-ventral axis. In principle they could very well carry inherited Wg protein. However, Wg seems to move rapidly across wing imaginal discs. After a temporary block to secretion, Wg can be detected over many cell diameters after only 30 min. Cell proliferation and movement cannot account for such rapid Wg movement (Strigini and Cohen, 2000).
A Brief Analysis of Cell Movement

Because the findings described in chapter 2 highlight the importance of cell lineage for segmental patterning, I set out to establish a system for the detailed study of epidermal cell movement in living embryos that could be used for future studies in the laboratory of Dr J-P. Vincent. Although cell movement had been studied before in embryos, the techniques utilised in those studies were limited: the precise position of the imaged cells with respect to the segment boundary was unknown and only a few cells could be tracked manually over a short period of time. Whereas the methods established in chapter 3 allowed me to follow many cell trajectories in living embryos and can potentially be used to perform very detailed examinations of time-lapse data. I conducted experiments to validate my experimental approach. These experiments indicated a degree of mitotic activity that had not been described before for stage 11/12 embryos. I also found regularity in the orientation of mitosis during germband retraction: cells divide either parallel or perpendicular to the AP axis.

Morphogenesis in general involves changes in the shapes of populations of cells and encompasses a complex set of behaviours such as cell division, rearrangements and signalling events which have to be co-ordinated with each other. The embryonic epidermis of Drosophila is an attractive model system for the study of morphogenesis.
because it displays all these behaviours, yet it is comparatively easy to perturb and to investigate. The ability to apply the powerful genetic and molecular techniques available in *Drosophila* combined with live imaging and sophisticated computational techniques to the process of morphogenesis should provide important insights into this fundamental process in general. The complexity of the process lends itself naturally to a 'systems biology' approach. An important prerequisite for such an approach however is a detailed description of the mophogenetic processes. Whereas rapid progress has been made with respect to cataloguing mRNA expression patterns and cell-cell signalling events, a comprehensive description of cell rearrangements and divisions is lacking. A key first step was taken in chapter 3 when a method to derive such data sets was established.

The subsequent stages of a 'systems biology' analysis will involve devising an abstract computational model for morphogenesis that can be tested against the observational data and finally proposing a mechanistic biological model. A systematic, computer-oriented heuristic for uncovering complex patterned behaviour in dynamic data sets can be applied to the data derived from the time lapse movies to build the computer model (Valdes-Perez and Perez, 1994).
Wingless Trafficking in Live Embryos

In chapter 2 I have shown that the Wg protein can be delivered at a distance by cells that were originally wg-expressing cells. This mechanism for Wg transport is clearly sufficient for long range Wg signalling since it rescues a wg mutant to a wild type pattern. However, there must be other mechanisms for Wg transport in place, because Wg can still act at a distance in the absence of cell movement. The two alternative mechanisms that have been discussed in the past are diffusion in the extracellular space and transcytosis. To gain further insight into the transport of Wg I followed the movement of GFP-Wg in live embryos. There are three main conclusions that can be drawn from the results of the studies described in chapter 4. First, GFP-Wg remains tightly associated with the cells that make it suggesting that it does not diffuse freely. Second, the fate of endocytosed GFP-Wg appears to be under distinct control and third, in anterior cells that receive GFP-Wg it is rapidly endocytosed and can be recycled back to the surface. Although my studies do not resolve the issue of diffusion versus transcytosis, which would be very difficult anyway, they provide information about Wg movement at a level of detail that is unprecedented.

One problem with studies of GFP tagged proteins in general is, that one can always argue that the absence of a fluorescence signal might reflect limits in the sensitivity of the
detection method, rather than the absence of the fusion protein. The apparent distribution of GFP-Wg at the anterior of its expression domain highlights these problems. No fluorescence signal can be detected. Yet GFP-Wg must be present because it can be detected when it is concentrated in intracellular vesicles. Obviously it would be very useful to be able to detect extracellular GFP-Wg. This might be achieved by a combination of a more sensitive detection system and a fluorescent tag with a higher quantum yield. This would open the door for investigations of the dynamics of extracellular GFP-Wg movement by techniques such as ‘Fluorescence Recovery After Photobleaching’ (FRAP) measurements. It would be useful in this context to develop a method to relate GFP-Wg fluorescence intensity to the number of molecules present. This would make it possible to estimate the number of molecules present at a given time and to test the various models of morphogen movements (Kerszberg, 1999).

However, the observation that GFP-Wg is retained at the surface of expressing cells is unambiguous. This immediately raises the question of how Wg is tethered to the surface of expressing cells. One obvious possibility is that HSPGs might be involved in the retention of Wg, since interactions of HSPGs and Wg have already been demonstrated (Baeg and Perrimon, 2000). Therefore it would be very informative to examine the distribution of GFP-Wg in embryos with impaired proteoglycan biosynthesis.
The observation that endocytosed Wg can be recycled back to the surface of Wg receiving cells raises some interesting questions. First of all, what mechanisms are involved in the recycling of Wg? And how is the decision to recycle Wg, as opposed to degrade it, made by cells? Interestingly, the range of Wg towards the posterior is restricted by specific degradation of the signal (L. Dubois, in press). It is therefore possible that the range of Wg is controlled by a cellular ‘switch’ that controls this decision of whether to recycle or to degrade internalised Wg.

Recycling of endocytic Wg may also serve a purpose in Wg signalling. Since increased ligand recycling has been documented to enhance the potency of a signal in tissue culture cells (Fallon et al., 2000), it is possible that cells at the anterior of the parasegment boundary (which require sustained Wg signalling) recycle Wg to ensure maximal signalling. The recent finding that LRPs play a role in the Wg pathway (Wehrli et al., 2000) supports this hypothesis. Because it has been suggested that LRPs play a role in the recycling of Fz to the cell surface (Bejsovec, 2000), and recycling of the receptor is formally equivalent to recycling of the ligand (both are part of the same signalling complex).

At this point it is useful to re-visit the model of transcytosis and some of the evidence it is based on, especially the finding that certain mutations in the Wg protein seem to interfere with its transport, but not its signalling capabilities (Dierick and Bejsovec,
The distribution of these mutant proteins is restricted although when over-produced they can still signal. In the light of all the issues raised in the previous paragraph it seems misleading to distinguish between 'transport' and 'signalling'. Because if the mutant protein is not recycled to the surface but is degraded instead, this would certainly restrict its range, and the lack of recycling would at the same time lower signalling efficiency (which is what all the biological read-outs really are about). Over-expression of the mutant protein would of course elicit a response but only because of increased ligand concentration. It would be interesting to investigate whether these mutant Wg proteins are recycled. The only feasible way to do this is to tag them with GFP and observe their trafficking just like I did with wild type Wg.
DNA manipulations

Restriction enzymes from New England Biolabs (NEB) were used in the recommended buffer and digests were carried out at 37°C. The Klenow fragment of DNA Polymerase I (Promega) was used to create blunt ends. Digests were incubated with 1 unit Klenow per μg DNA and 33 μM of each dNTP (Pharmacia) for 15 min at 37°C, and heat inactivated at 75°C for 10 min. To de-phosphorylate DNA fragments Calf Intestine Alkaline Phosphatase (Roche) was used in the buffer supplied, incubated with DNA for 45 min at 37°C, and heat inactivated at 75°C for 10 min. T4 DNA Ligase (NEB) was used for the ligation of restriction fragments. Ligations were carried out with the buffer supplied, for 4hrs (or overnight) at 16°C. 1 μM T4 DNA Ligase was used for cohesive end ligation, or 50 μM for blunt end ligation.

Agarose Gel Electrophoresis was performed in TBE buffer with a concentration of 1μg/ml ethidium bromide (Sigma) in the gel, a percentage of Agarose (BioRad) suitable for the range of DNA fragment sizes to be separated was used. Both restriction digests and DNA fragments isolated from Agarose gels were purified using QIAquick Gel Extraction Kit (QIAGEN).
**Bacterial Transformation and preparation of Plasmid DNA**

For transformation of competent cells with ligation DNA approximately 10 ng DNA was added to 50 µl subcloning efficiency competent cells. The mixture was left on ice for 30 min and then heat shocked at 42°C for 90 s. It was then placed on ice for a further 2 min before adding 950 µl SOC and incubating at 37°C for 45 min. The cells were spun down and 800 µl of supernatant removed. The remaining 200 µl was used to re-suspend the cells which were then plated on LB plates containing ampicillin (Sigma). After plates were incubated at 37°C overnight colonies were picked.

Potential positive clones were tested directly by PCR or grown in a pre-culture at 37°C for at least 4 hrs in 1.5 ml LB + ampicillin. For direct PCR testing of colonies a 25 µl reaction mixture containing 1 µM each primer oligonucleotide (Oswell), 200 µM each dNTP and 1 unit Taq DNA polymerase was prepared in PCR reaction buffer supplied with the Expand High Fidelity PCR Kit (Roche). Colonies were removed from the plates and mixed directly into the PCR mix. Conditions used for the PCR amplification were as follows: denature 94°C, 1 min; anneal 55°C, 1 min; extend 72°C, 1-1.5 min; cycle 25 times. From pre-culture not tested directly by PCR plasmid DNA was isolated using the QIAGEN Plasmid Mini Kit. Positive clones were grown overnight in 25 ml LB + ampicillin and plasmid DNA isolated using QIAGEN Plasmid Midi Kit. Purified plasmid
DNA was then used for microinjections.

**Transforming flies by microinjection**

P-element vectors carrying the wild type $w^+$ eye marker gene and the transforming DNA were used for transformation by microinjection. Embryos from a $yw$ homozygous stock were collected for 30 min and aged for a further 15 min. They were dechorionated in bleach (Hays Chemical Distribution) for 2 min, individually oriented and glued on microscope slides. The embryos were dried for 3 min on silica gel (BDH Laboratory Supplies) and then covered with Voltalef 10S mineral oil (ATO, France). DNA at a concentration of 500 ng/ml in 1/10 PBS was injected with 150 ng/ml turbo vector (Rubin and Spradling, 1982) into the posterior end of the embryos. Upon hatching the larvae were transferred into a fresh yeast corn-meal tube and grown at 25°C. These G0 flies were crossed to $yw$ flies and the G1 progeny screened for the presence of $w^+$ (red eye colour). Single G1 transformants were then crossed to $yw$ and single G2 males selected from each cross. These males were again crossed to $yw$ and a homozygous stock generated which contained a single insertion. Males from this stock were used to map the insertion and obtain balanced stocks where required.
Cloning of UAS constructs

**UAS-Wingless-GFP:** First a GFP PCR fragment was generated using the following oligonucleotides (Oswell):

Forward: 5’GATCGCTAGCGGCCCTGTGGGGCAGTAAAGGAGAAGAA3’

Reverse: 5’GATCACTAGTGCCCCACAGGCTTTTGTATAGTTCATCCATGCCC3’

These oligonucleotides contain BgII sites (in bold) that are compatible with the unique PflmI site present in Wingless at position aa38. The forward primer is also flanked by a NheI site on its 5’ end. The PCR product was then cut with NheI and SpeI and subcloned in the NheI site of pTrcHisB vector (Invitrogen) to create pTrcHisB-GFP. Positive clones were selected under a fluorescence microscope. Positive clones were then cut with BgII. The BgII fragment was inside the NheI and SpeI sites in the PCR fragment. Individual BgII fragments were subsequently cloned in the PflmI site of Wingless to generate KS-Wingless-GFP. The joints between Wingless and GFP are as follows:

Trp(38)Gly[Ser(2)...Lys(238)]Ser Leu TrpTrp(39). The amino acid sequence from GFP is bracketed and the extra amino acids created by the fusion are underlined. The amino acid positions of the respective proteins are indicated in parentheses.

In order to check the GFP-Wingless fusion, different inserts coding for the fusion molecule were cut with EcoRV (position aa2 in Wg and BgIII position aa163) and ligated
with pTrcHisB prepared as a Neol filled in site and BglII vector. Again, positive clones were selected under the fluorescence microscope. To generate pUAS-GFP-Wingless the GFP-Wingless fragment was obtained by cutting KS-GFP-Wingless (gift from C. Alexandre) with NotI and Asp718 and subsequently cloned in pUAS.

UAS-4Cys-HA-Wingless: The KS-Wingless plasmid was cut with PflmI (a unique site present in Wingless, see above). Two complementary phosphorylated oligonucleotides were synthesised (Genosys) containing a tetracysteine motif (AlaGluAlaAlaAlaArgGluAlaCysCysArgGluCysCysAlaArgAla, small underlined letters in forward primer sequence) and a HA tag (TyrProTyrAspValProAspTyrAla, capital underlined letters in forward primer sequence) flanked by PflmI ends.

Forward:
5' GTGGgccgaggccgccgcccgcgaggcctgctgccgcgagtgctgcgcccgcgcc TACCCCTACGATG TGCCCGATTACGCCTCCATGTG

Reverse:
5' ATGGAGGCGTAATCGGGCACATCGTAGGGGTAGGCGCGGGCGCAGCACTC GCGGCAGCAGCCTCGCCGGGCGGCGGCAGCAGCTC

These oligonucleotides were hybridised by mixing equal amounts (25 µl, 1 µg/ml) with 50 µl TE, placing at 100°C for 5 min and allowing to cool to room temperature overnight.
The annealed, double stranded oligonucleotide was then cloned in the PflmI site of Wingless to generate KS-4Cys-HA-Wingless. The joints between Wingless and the tetracysteine motif are as follows:

\[ \text{Trp}(38)\text{Trp}[\text{Ala}(2)\ldots\text{Ala}(27)]\text{Ser Met Trp Trp}(39) \]  

The amino acid sequence from the oligonucleotide is bracketed and the extra amino acids created by the fusion are underlined. The respective amino acid positions are indicated in parentheses. The insertion of the tetracysteine motif was checked by direct PCR from picked colonies. To generate pUAS-4Cys-HA-Wingless the 4Cys-HA-Wingless fragment was obtained by cutting KS-4Cys-HA-Wingless with NotI and Asp718 and subsequently cloned into pUAS.

UAS-CD2-HA-4Cys\text{\textsuperscript{intra}}: The vector pTrcCD2-GFP (gift from L. Dubois) containing the signal peptide and transmembrane domain of CD2 was cut with NotI which is a unique site downstream of the transmembrane domain of CD2. Two complementary phosphorylated oligonucleotides were synthesised (Genosys) containing a HA tag (TyrProTyrAspValProAspTyrAla, capital underlined letters in forward primer sequence), a tetracysteine motif (AlaGluAlaAlaAlaArgGluAlaCysCysArgGluCysCysAlaArgAla, small underlined letters in forward primer sequence) flanked by NotI ends and a stop codon at the 3’ end.
Forward:
5'GGCCGCTTACCCCTACGATGTCGCCGATTACGCCgccgaggccgccgcccgcgaggcctgccgccgagtgctgcgcccgcgccTGAGC
cgccgagtgtgcgccgcccgccctTGAGC

Reverse:
5'GGCCGCTCAGGGCGCGGCGAGCAGCAGCGCGCAGCAGGCCCTCGCGGGGC
CGGCCCTCGGCACGTGCTAGGGGTAGGC

These oligonucleotides were hybridised as described above. The annealed, double stranded oligonucleotide was then cloned in the NotI site of pTrcCD2-GFP to generate pTrcCD2-HA-4Cys\textsuperscript{intra}. The orientation of the insert was checked by direct PCR. To generate pUAS-CD2-HA-4Cys\textsuperscript{intra} the CD2-HA-4Cys\textsuperscript{intra} fragment was obtained by cutting pTrcCD2-HA-4Cys\textsuperscript{intra} with EcoRI and NotI and subsequently cloned into pUAS. UAS-CD2-4Cys-HA\textsuperscript{extra}. The vector pTrcCD2-GFP was cut with BsmI, which is a unique site between the signal peptide and the transmembrane domain of CD2. Two complementary phosphorylated oligonucleotides were synthesised (Genosys) containing a tetracysteine motif (AlaGluAlaAlaAlaArgGluAlaCysCysArgGluCysCysAlaArgAla, small underlined letters in forward primer sequence) and a HA tag (TyrProTyrAspValProAspTyrAla, capital underlined letters in forward primer sequence)
flanked by BsmI ends.

Forward:

5’gccgaggccgccgccgccccgccaggtgtgccgagtgctgcgccgccgcgccTACCCCTACGATGTGCCC
GATTACGCCACGAATGCC

Reverse:

5’CATTCGTGGCGTAATCGGCCACATCGTAGGGGTAGCGCGCCGCGCGCGACTCGCGGGCGGCCTCGGCGG
CGCGGCAGCAGGCCTCGCGGGCGGCGCCGCTCGCGGG

These oligonucleotides were hybridised as described above. The annealed, double-stranded oligonucleotide was then cloned in the BsmI site of pTrcCD2-GFP to generate pTrcCD2-4Cys-HA\textsuperscript{extra}. The insertion was checked by direct PCR. To generate pUAS-CD2-4Cys-HA\textsuperscript{extra} the CD2-4Cys-HA\textsuperscript{extr} fragment was obtained by cutting pTrcCD2-4Cys-HA\textsuperscript{extr} with EcoRI and NotI and subsequently cloned into pUAS.

**Whole-mount antibody staining**

Staged embryo collections were dechorionated and fixed for 30 min at the interface of a heptane 7.5% formaldehyde in PBS fix. The aqueous phase of the fix was removed and the embryo devitellinised by adding equal amounts of methanol and shaking vigorously. Embryos were then re-hydrated, washed for 30 min in PTX, blocked for 30 min in PTX-
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NGS and incubated with the primary antibody overnight at 4°C. After washing thoroughly with PTX the embryos were blocked for 30 min in PTX-NGS and then incubated with secondary antibody for 2 hrs at room temperature and again washed thoroughly. When fluorescent secondary antibodies (Alexa conjugates Alexa 488 and Alexa 594, Molecular Probes) were used, single embryos were directly mounted in VectaShield (Vector Labs), oriented on the slide and the coverslip was sealed with nail varnish (Boots). When HRP-coupled secondary antibodies were used (Vector Labs), the staining was visualised with a DAB substrate (Roche) before mounting. Antibodies used were rabbit anti-β-Galactosidase (Cappel), rabbit anti-Engrailed (gift from C. H. Girdham and P. O'Farrell, UCSF), mouse anti-HA.11 (BAbCO), mouse anti-Wingless (4D4, gift from S. Cohen, EMBL) and mouse anti-Armadillo (DSHB). Alexa 594-Phalloidin conjugate was bought from Molecular Probes.

**Whole-mount *in situ* hybridisation**

Embryos were fixed as described above. Re-hydrated embryos were rinsed in 1:1, PBT:hybridisation buffer, pre-hybridised in hybridisation buffer for 1 hrs at 58°C and incubated with heat-denatured probe (kindly provided by C. Alexandre, NIMR) overnight at 58°C. Embryos were then washed in hybridisation buffer, in 1:1, PBT:hybridisation buffer and PBT for 20 min each at 58°C and then washed with PBT 5x 20 min at room temperature. After that embryos were incubated with antibody (1:5000, alkaline
phosphatase-coupled anti-Digoxigenin FAB or anti-fluorescein FAB fragments, Roche) for 1 hr at room temperature and washed overnight at 4°C. NBT/BCIP (Gibco) and FastRed (Roche) were used for probe detection. For double in situ antibodies were applied sequentially and the enzyme was inactivated with 100 mM glycine-HCl, pH2.2 for 30 min at room temperature before the second antibody incubation.

**Detection of β-Galactosidase activity in cuticle preparations**

Prehatching larvae were injected with 8% glutaraldehyde in PBS and subsequently transferred to PBS. The fix was allowed to act for about 5 min. The anterior and posterior ends were then cut away with a piece of razor blade, the remaining trunks were transferred to X-Gal staining solution and stained for several hours at 37°C. After staining larvae were cut in half longitudinally. The ventral halves were mounted in Hoyer’s after the CNS and most of the internal tissue was removed. Some of the preparations were stained with Hoechst (Sigma, bis-benzimide, 10 μg/ml in H₂O/0.1% Tween-20) to visualise the nuclei and mounted in Glycergel (DAKO).

**Synthesis of photoactivatable lineage tracer**

The photoactivatable lineage tracer has three components; a dextran backbone, a nuclear localisation peptide and a photoactivatable caged rhodamine. The caged rhodamine was linked to dextran to prevent intercellular diffusion. Nuclear localisation peptides were
coupled to the dextran to target the lineage tracer to the nucleus to allow activation in the syncytial blastoderm and enhance resolution of the resulting clones.

Caged-rhodamine N-hydroxy succinimidyl ester (Molecular Probes) was mixed with 70kD amino dextran (approximately 36 amino groups per dextran molecule, Molecular Probes) in 50mg/ml anhydrous DMSO together with a small amount of triethylamine as a proton acceptor. The ratio of N-hydroxy succinimidyl ester to dextran was 10 mole ester/mole dextran. After 30 min at room temperature the remaining amino groups on the dextran were reacted with iodacetic acid-N-hydroxy succinimidyl ester (Sigma, 50 mole ester/mole dextran) to allow conjugation of the nuclear localisation peptide. After a further 30 min the reaction was dialysed against 100 mM Hepes, pH7, to remove excess iodacetic acid-N-hydroxy succinimidyl ester. The nuclear localisation peptide (CGYGVSRSKPRPG, gift of D. Chelsky, UCSF) was coupled to the dextran via the thiol group on the N-terminal cysteine. The peptide, in water, was added to the iodoacetyl-caged rhodamine dextran (approximately 10 peptide molecules per dextran molecule) and allowed to react overnight at room temperature. Remaining unreacted iodacetate groups were reduced with 5% β-mercaptoethanol (Sigma). All synthesis steps were performed in the dark.

**Cell lineage analysis using the photoactivatable lineage tracer**

A 1 mg/ml solution of the photoactivatable lineage tracer was injected prior to
cellularisation and embryos were left to develop to stage 5 at room temperature. For photoactivation, the embryos were mounted in a custom-built chamber (described in Girdham and O'Farrell, 1994) and viewed under a confocal microscope. The UV laser of the confocal microscope (excitation wavelengths at 361 nm and 365 nm) was used for photoactivation. Embryos were then cultured under oil and examined at late stage 11 under the confocal microscope as described below.

**Fluorescence Microscopy**

Confocal microscopy was done on a Leica TCS-4D confocal microscope equipped with an argon/krypton laser providing excitation wavelengths at 488 nm and 568 nm. Images were collected using a x63 oil-immersion Plan APO objective (NA 1.32) with x2 electronic zoom. For live recordings embryos were cultured in a custom-made chamber. Embryos were imaged on a DeltaVision (AppliedPrecision) widefield microscope based on an Olympus IX70 inverted microscope. An Olympus x63, 1.35 NA U Plan APO oil objective was used for high magnification imaging. DeltaVision software was used to control the stage and to select the appropriate excitation (FITC 490/20 nm, Rhodamine 555/28 nm) and emission filters (FITC 528/38 nm, Rhodamine 617/73 nm). Emitted light was recorded on a Micromax (Roper Scientific) liquid cooled CCD camera typically with 2x2 pixel binning. Typical time-lapse movies consisted of 150 to 300 frames of 512x512
pixel images acquired at 0.2 frames/second with a spatial resolution of 0.1352 μm/pixel.

Image analysis

Movies were analysed using Optimas software (Media Cybernetics L. P., Silver Spring, USA). Customised macros were written in the C++ based language ALI (Analytical Language for Images) and run under Optimas. To analyse vesicle movement, the offset cell movement caused by germband retraction was removed (see below). In short, the movement of the centre of mass of the expression domain was tracked and the x and y components of the rate of germband retraction was calculated. Movies in the reference frame of the embryo were then generated for both red and green channels. From the original 512x512 pixels image a 128x128 pixels regions of interest (ROI) was selected. For each frame in the original movie this ROIs was cut, and saved as a new image and then moved in the next frame with the same velocity as the rate of germband retraction. The vesicles were automatically detected by thresholding the images and tracked in two-dimensions from the filtered 128x128 pixels movie for a maximum number of frames (N frames, separated by the time interval δτ=5 s) using a custom-written ALI macro.

From the two-dimensional co-ordinates x(t), y(t) of the vesicle, the mean squared displacement (MSD) travelled by the vesicle during a time interval Δt=nδτ was calculated according to:
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\[ MSD(\Delta t = n\delta t) = \frac{1}{N - n} \sum_{j=1}^{N-n} \left[ (x(j\delta t + n\delta t) - x(j\delta t))^2 + (y(j\delta t + n\delta t) - y(j\delta t))^2 \right]. \]

The analysis of the plot of the MSD as a function of the time interval \( \Delta t \) allowed the definition of three classes of motion: simple diffusion, directed motion superimposed on diffusion, diffusion in a mobile or immobile cage. The following equations to fit the MSD data were used (Steyer and Almers, 1999 and Oheim and Stuhmer, 2000): simple diffusion with a diffusion coefficient \( D \) was fitted with: \( MSD(\Delta t) = 4D\Delta t \), directed diffusion at velocity \( v \) and diffusion coefficient \( D \) was fitted with: \( MSD(\Delta t) = 4D\Delta t + v^2\Delta t^2 \), diffusion in a mobile or immobile cage was fitted with: \( MSD(\Delta t) = R^2[1 - a_1 \exp(-4a_2D\Delta t / R^2)] + 4D_{\text{cage}}\Delta t \) where \( R \) is the radius of the cage, \( D_{\text{cage}} \) is the diffusion coefficient of the cage, \( D \) is the diffusion coefficient of the vesicle inside the cage, and \( a_1 = 0.99, a_2 = 0.85 \) are two constants. The cage was considered to be immobile when the fit parameter \( D_{\text{cage}} \) was less than \( 10^{-6} \mu m^2/s \). This was the limit of resolution of the microscope as determined by tracking 0.1 \( \mu m \) fluorescent beads adsorbed to a glass slide and imaged under the same experimental conditions as when movies of live embryos were acquired.

**Removing the offset movement caused by germband retraction**

During the 20 min acquisition time, germband retraction causes an offset component to the movement of tracked vesicles. To remove this offset motion the images in the green
channel were processed by a Fast Fourier Transformation (FFT) low-pass filter, and smoothed by a 3x3 pixels Trimmed Mean filter and four consecutive 7x7 Median filters. The resulting images consisted of a fluorescent cloud indicating the position of the GFP-Wingless expression domain. The centre of mass of the expression domain was tracked using a custom written 2D tracking macro. The x and y components of the rate of germband retraction was calculated from the slope of the x and y co-ordinates of the centre of mass as a function of time. In most cases, this rate was constant and the data were well described by a single slope. In few cases however, the rate of germband retraction changed significantly during the acquisition and two distinct slopes were used to fit the data. Movies in the reference frame of the embryo were then generated for both red and green channels, inside and outside the expression domain. From the original 512x512 pixels image a 128x128 pixels region of interest (ROI) was selected. For each frame in the original movie, this ROI was cut and saved as a new image, and then moved in the next frame with the same velocity as the rate of germband retraction. The vesicles were tracked in these new movies.

**Detecting and counting vesicles**

The raw 128x128 pixels images were filtered to enhance the visibility of vesicles. First a low-pass FFT filter was applied to remove the non-uniform background, then a 3x3 pixels trimmed mean filter was applied to further smooth the image. Since the fluorescence
intensity in the red channel was in most cases larger than in the green channel the
brightness and contrast of the look-up table (LUT) was adjusted to achieve similar
intensity levels in both channels. The vesicles were automatically detected by
thresholding the images: bright areas detected above the threshold were scored as vesicles
if their size was larger than 4x4 pixels. For the histograms the number of vesicles that
could be detected using a fixed threshold in each frame of the movie was accumulated
throughout the whole movie. Since the same vesicle could be scored several times during
the whole sequence, this number represents the convolution of the spatial density of the
vesicles with their lifetime and not the actual number of distinct vesicles detected in the
movie. For both red and green channels spatial maps showing the locations of the
vesicles during the entire movie were also generated by adding the binarised and
thresholded images frame by frame. Superimposing the red and the green channels
allows to easily visualise vesicles that were visible either in one channel only or in both
channels.

Quantifying the fluorescence intensity

The image processing described above is necessary to remove the background and to
detect the vesicles but it also modifies the raw grey values in a non-linear manner. This
made it impossible to use the filtered 128x128 pixels images to quantify fluorescence
intensity data. To quantify fluorescence intensity a 5x5 pixels ROI was centred on the
centre of mass of the vesicle after it was detected in the filtered image. The fluorescence intensity was then averaged over the same ROI in the corresponding raw 128x128 pixels image.

Buffers and solutions

Laboratory chemicals were obtained from Sigma, Glibco-BRL or Roche unless otherwise stated.

Hoyer's (in 50 ml): 30 g gum arabic, 200 g chloral hydrate, 20 g glycerol

hybridisation buffer: 50% formamide, 5xSSC, 100 μg/ml yeast RNA, 0.1% Tween 20, 50 μg/ml Heparin, pH6.5 adjusted with citric acid

LB (per litre): 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl

PBS: 0.13 M NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄

PTX: 1xPBS, 0.1% Triton X-100, 0.1% BSA

PTX-NGS: 1xPTX, 5% Normal Goat Serum

SOC (per litre): 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl, 3.6 g glucose

SSC: 150 mM NaCl, 15 mM tri-sodium citrate

TBE: 90 mM Tris-borate, 2 mM EDTA

TE: 10 mM Tris-HCl, pH8, 1 mM EDTA

X-gal staining solution: 0.2 M Na₂HPO₄, 0.2 M NaH₂PO₄, 5 M NaCl, 1 M MgCl₂, 50mM K₃[Fe(CN)₆], 50 mM K₄[Fe(CN)₆], 0.2% X-gal (added fresh before staining)
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Signalling at a distance: Transport of Wingless in the embryonic epidermis of Drosophila

Sven Pfeiffer and Jean-Paul Vincent*

Secreted signalling molecules affect the behavior of cells at a distance. Here we discuss how the Wnt family member Wingless reaches distant cells within the embryonic epidermis of Drosophila. We consider three possible mechanisms: free diffusion, restricted diffusion and active transport. We argue that free diffusion is unlikely to occur. However, a variant of restricted diffusion may account for Wingless transport. It may be that Wingless is carried from one side of a cell to the other by a drifting transmembrane protein such as a specific receptor or a glycosaminoglycan. Transfer from cell-to-cell would involve release from the donor cell and recapture in an adjacent cell. Alternatively, Wingless might be transported by a mechanism akin to transcytosis. This would involve the packaging of Wingless in specialized vesicles at one end of a cell, active transport across the cell, vesicle fusion and Wingless release on the other side. We describe the evidence in favor and against these two alternatives.

Key words: Drosophila / embryogenesis / morphogen / transcytosis / Wingless

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a mechanism involving exo- and endocytosis, which we will call planar transcytosis. Note that the term transcytosis was initially used to refer to transport along the baso–apical axis while here, transcytosis would occur within the plane of the epithelium.  

**Receptor-restricted diffusion**

In original models of long-range signalling by morphogens a localised morphogen-synthesizing source is assumed and, from there, the morphogen diffuses freely away. Depending on the chosen boundary conditions, a steady-state concentration gradient with desired properties would ensue. We see several problems with free diffusion in epithelia. Foremost, it is not immediately obvious how size invariant positional information can be obtained (this is a general problem, not necessarily restricted to epithelia). In addition, the Wingless protein appears to stick to cell membranes (see later) and it is therefore very unlikely to diffuse freely along the epithelium. Even uncharged large solutes are unlikely to diffuse freely as suggested by biophysical measurements of diffusion coefficients in epithelia: Xia et al. found restriction to diffusion of fluorescein dextran in intercellular spaces of epithelial cell monolayers whereas no restriction was found for small solutes.

The spread of Wingless could be regulated by the presence of interacting molecules whose localized expression would then cause an anisotropic diffusion coefficient leading to an asymmetric distribution of Wingless. Kerszberg and Wolpert studied theoretically how the binding of a diffusing morphogen to its membrane-bound receptor influences its distribution (Kerszberg, this issue). Their study uses the TGFβ activin as an example. They first consider a simple model whereby activin exists in either of two states: freely diffusing in the extra-cellular space or bound to its high affinity receptor. In such a system, they find that a narrow front of unbound activin would advance, leaving behind stable receptor–activin complexes. In other words, under the assumption that activin diffuses freely except when captured by an immobile high affinity receptor, no graded distribution could form as previously suggested. Kerszberg and Wolpert propose an alternative way whereby a graded distribution would arise in the presence of a high affinity receptor. In their model, receptor molecules would transport bound morphogen. The key assumption they make is that there is a difference
in binding affinities for different oligomeric receptor states. The morphogen would bind to a receptor monomer, leading to heterodimerisation of that receptor. If the heterodimer has lower affinity for the morphogen, the ligand would be forced to hop onto an unoccupied receptor if spatially permitted. An activin molecule could therefore move along cell membranes bound to the heterodimer and could be transferred between cells by hopping from a heterodimer to a monomer. If the lifetime of the heterodimer is sufficiently long, morphogen diffusion becomes directed, because already visited receptors cannot be occupied.

It is not clear yet whether activin is transported by its receptor in the manner envisaged by Kerszberg and Wolpert. However, there is now good experimental evidence that a receptor can alter the range of its ligand. This was shown for the Patched (Ptc) receptor and its ligand Hh. Absence of Ptc allows Hh to act at a longer distance, presumably because it is allowed to spread further (normally, cells expressing ptc would soak up oncoming hh). Since the hh pathway positively regulates the transcription of ptc, Hh appears to prevent its own action at a distance. In the case of Wingless, the interplay between receptor and ligand leads to a reverse situation. *Drosophila* Frizzled 2 (DFz2) is a receptor for Wingless. In wing imaginal disks, DFz2 appears to stabilize the Wingless protein thereby allowing it to be transported further. Thus, contrary to Hh, the range of Wingless is lengthened in the presence of its receptor. One should point out here that these findings are based on overexpression experiments and that they should be confirmed by studying the behavior of Wingless protein in clones lacking DFz2 function. It is also important to note that these experiments do not address whether the signalling Wingless receptor is part of the transport machinery or whether there are different Wingless receptor species for signalling and transport.

**GAG-restricted diffusion**

Another set of molecules that interact with Wingless in the extracellular space are glycosaminoglycans (GAGs). GAGs are unbranched, highly negatively charged polysaccharides consisting of repeating units (sometimes up to 100) of disaccharides (reviewed in ref 32). These sugar chains are attached to a core protein forming a proteoglycan. Proteoglycans are abundant on cell surfaces, creating a high charge density. Bernfield estimated that there are $10^6$ molecules of just one type of core protein (Syndecan-
on mammary epithelial cells. One class of glycosaminoglycans, glucosaminoglycans (GuAGs) such as heparin and heparan sulfate has been implicated in Wingless signalling/transport. Heparin binds Wingless as well as other Wnts and this binding is responsible for the tight association of Wingless to the surface of Wingless-expressing cells in cell culture. In vivo evidence for the role of GAGs in Wingless function comes from flies mutant for a gene encoding UDP-glucose dehydrogenase (UDP-GlcDH). The mutation has been named sugarless, kiwi or suppennkasper. UDP-GlcDH is required for the biosynthesis of glucuronic acid, a precursor of all GAGs and flies lacking UDP-GlcDH are therefore not expected to have any GAG. This has been confirmed by immunoblotting embryo extract with anti-heparan antibodies. Flies mutant for UDP-GlcDH die in late embryogenesis and show a segment polarity phenotype reminiscent of wingless-. Two models have recently been discussed for the role of proteoglycans in Wingless function. One is that GuAGs sequester Wingless thereby regulating the overall protein distribution and local concentration. In sugarless mutant embryos, the domain of engrailed expression is temporarily widened suggesting a longer range of action for Wingless. However, engrailed expression subsequently decays suggesting insufficient signalling possibly due to dilution of the signal. A second model is that GuAGs function as coreceptors. In tissue culture, wingless activity can be inhibited by treating cells with GAG lyases or with sodium perchlorate, a treatment known to suppress sulfation of GAGs. Adding back heparin to chlorate-treated cells restores Wingless activity to control levels implying a role for GAGs in Wingless signal transduction. However, embryos lacking UDP-GlcDH respond to additional Wingless showing that GuAGs are not absolutely required for the response to Wingless. In any case, these findings do not allow a clear distinction between a signalling and/or transporting role for GuAGs. One could imagine that GuAGs transport Wingless much like the activin receptor transports activin in the Kerszberg and Wolpert model (Figure 3a). Wingless molecules would hop from one tripartite complex (Wingless-Frizzled-GuAG, where Frizzled represents the signalling receptor) to another unoccupied GuAG. With the available data, one cannot confirm or infirm this possibility. However, a body of work suggests the existence of an alternative transport mechanism akin to transcytosis.

Figure 3. Models of transport. (a) Transport by a transmembrane protein which could be either a receptor, a glycosaminoglycan, or a complex of both. Diffusion of the transmembrane protein provides lateral movement along the A/P axis and transfer from cell-to-cell involves release from the donor cell and recapture in an adjacent cell. (b) Simplified model of transepithelial transcytosis (adapted from ref 53). Immunoglobulin A (■) is picked up at the basal surface and delivered to the apical side as a complex with a portion of its receptor. (c) Simplified model of planar transcytosis as envisioned by Bejsovec and Wieschaus. Net movement of Wingless (●) occurs in a plane that is parallel to the epithelium. Specialized transport vesicles would carry wingless from one side of a cell to the other.

Vesicle-mediated transport

The first lead came from immuno-EM localization of the Wingless protein in embryos. Initially Wingless was found in various intracellular bodies: small membrane-bound vesicles and multivesiculate bodies (MVBs), as well as in the extracellular space. Van den Heuvel et al. argued that the MVBs were endocytic because their occasional presence was detected in engrailed positive cells. Using immunogold, rather
than silver intensification of horseradish peroxidase substrate, Gonzalez et al obtained more precise localization of the Wingless protein. In early stage 10 embryos, Wingless is in the Golgi complex and the endoplasmic reticulum of wingless expressing cells. In addition Wingless is found in MVBs and small vesicles and also at the membrane of large intracellular vesicles. These large vesicles, by their morphology and location, could correspond to endosomes or export vesicles. On this basis, Gonzalez et al suggested that Wingless might be directly passed from cell-to-cell through an active process linked to vesicular traffic. Of course, the presence of vesicles does not necessarily imply that they transport Wingless. Wingless could equally reach distant cells by an extracellular route, bind to a receptor, and finally be internalized. Such receptor–ligand internalization is common and well studied for the Epidermal Growth Factor Receptor (EGFR).\textsuperscript{41,42} In fact, proper signalling by the EGF receptor is dependent on its internalization.\textsuperscript{43}

Evidence for the involvement of endocytosis in Wingless transport came from studies of shibire (shi) mutant embryos. shi encodes a dynamin\textsuperscript{44} which is required for clathrin-mediated endocytosis.\textsuperscript{45} In shi mutant embryos, Wingless no longer seems to act at a distance, at least with the limited assay available.\textsuperscript{46} Assaying Wingless action is limited because of the pleiotropy of the shi mutation and hence the inability to keep mutant embryos healthy for very long. Currently, the only practical immediate assay for Wingless signalling is the stabilization of Arm protein. In wildtype embryos, Wingless is expressed in single-cell-wide stripes and this leads to the stabilization of Arm over up to three to four cell diameters on either side of these stripes.\textsuperscript{14,47} In shi mutants, Arm is stabilized only at the site of Wingless expression and in flanking cells, suggesting that Wingless is not transported.\textsuperscript{48} The stabilization of Arm in shi mutant cells indicates that they are still able to respond to Wingless. One caveat however, is that the shi mutation could conceivably compromise the response to Wingless in a more general way and only cells receiving high levels of Wingless would show detectable Arm stabilization.\textsuperscript{49} Also one cannot exclude the possibility that the shi gene product could be involved in secretion/import in addition to endocytosis.\textsuperscript{49} Thus evidence from shi mutants is not definitive.

Recent work suggests that transport and signalling are separably mutable in the Wingless protein.\textsuperscript{50} Bejsov\v{c} and Wieschaus identified a mutant wingless allele, wingless\textsuperscript{CE7} whose protein product is distributed indistinguishably from the wild-type protein during early stages (stage 8–10), but is inactive then (in terms of stabilization of Arm and maintenance of engrailed expression). Thus, transport can occur without signalling and since wingless\textsuperscript{CE7} encodes a protein truncated at residue 367, the C-terminal region (368–468) appears dispensable for transport. Recently, Dierick and Bejsov\v{c} identified three mutants which accumulate the Wingless protein in expressing cells but not in neighboring cells, suggesting a transport defect. These mutant forms of Wingless can activate the Wingless pathway in overexpression assays (this is particularly clear for one mutant, wingless\textsuperscript{NE2} which is a single point mutation, disrupting a conserved cysteine at position 247) and therefore, on the face of it, signalling can occur in the absence of transport. However, NE2 mutants suffer from extensive loss of engrailed expression. This is surprising since engrailed maintenance is a short-range function of Wingless and would thus be expected to continue even in the absence of transport as long as Wingless is secreted. One could argue that since ectopic expression of wingless\textsuperscript{NE2} induces naked cuticle, the mutant protein must be active and hence secreted. However, it is also conceivable that the Wingless\textsuperscript{NE2} protein accumulates inside expressing cells where it interacts with the receptor (maybe even within the secretory pathway) and activates the pathway intracellularly only. Thus autocrine signalling may not involve any protein export and one cannot exclude the possibility that the Wingless\textsuperscript{NE2} protein suffers from an export (not transport) defect. Nevertheless, Dierick and Bejsov\v{c} conclude from their observations that signalling can occur in the absence of transport and that different receptors might exist for transport and signalling. Apparently consistent with this suggestion is the recent finding that embryos lacking both DFz1 and DFz2, two known signalling receptors, can still transport Wingless away from its site of synthesis.\textsuperscript{52} However, one weakness in the argument here, is that yet another signalling receptor (DFz2?) may exist. In fact, fi fj2 double mutant embryos do show residual signalling activity.\textsuperscript{52,53}

Transepithelial transcytosis as a model

So far we have outlined the evidence that Wingless transport may involve receptor-mediated uptake and subsequent re-secretion. We referred to this mechanism as planar transcytosis by analogy to transepithelial transcytosis, which also involves a cycle of endo-
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The progeny of wingless-expressing cells deliver the signal at a distance in Drosophila embryos
Sven Pfeiffer*, Cyrille Alexandre*, Manuel Calleja† and Jean-Paul Vincent*

Pattern formation in developing animals requires that cells exchange signals mediated by secreted proteins. How these signals spread is still unclear. It is generally assumed that they reach their target site either by diffusion or active transport (reviewed in [1,2]). Here, we report an alternative mode of transport for Wingless (Wg), a member of the Wnt family of signaling molecules. In embryos of the fruit fly Drosophila, the wingless (wg) gene is transcribed in narrow stripes of cells abutting the source of Hedgehog protein. We found that these cells or their progeny are free to roam forwards over a distance of up to four cell diameters. The cells leaving the expression domain retain inherited Wg protein in secretory vesicles, however, and carry it forwards over a distance of up to four cell diameters. Experiments using a membrane-tethered form of Wg showed that this mechanism is sufficient to account for the normal range of Wg. Nevertheless, evidence exists that Wg can also reach distant target cells independently of protein inheritance, possibly by restricted diffusion. We suggest that both transport mechanisms operate in wild-type embryos.

Membrane-tethered Wg rescues a wg mutation
Irrespective of the transport mechanism, one would expect that, if the Wg protein were artificially tethered to the membrane of secreting cells and, hence, prevented from being released into the extracellular space, its range would be reduced and the area of bald cuticle would narrow. We tested this by expressing membrane-tethered Wg (UAS-Nrt–flu–Wg [8]) in a wg null mutant with a wg–GAL4 driver. To our surprise, wg mutants rescued by membrane-tethered Wg could hardly be distinguished from wild-type embryos (Figure 1c,d). In particular, the bands of naked cuticle were as wide as in the wild type, suggesting that membrane-tethered Wg can act as far as four cell diameters away.

Two trivial explanations could account for the rescue. One is that membrane-tethered Wg might be leaky. Tethering Wg to the cell membrane was achieved by fusing it to the transmembrane protein Neurotactin (Nrt) [8]. Rescue could be explained if this fusion protein were cleaved, releasing active Wg into the extracellular space. There is, however, no indication of cleavage from western blots [8]. Moreover, two functional assays confirm that Nrt–flu–Wg remains attached to expressing cells. First, clones of cells expressing Nrt–flu–Wg in wing imaginal discs activate Wg target genes only in adjoining cells [8]. Second, when this fusion protein is expressed in embryos with the engrailed (en)–GAL4 driver, it specifies naked cuticle only in cells adjoining the expression domain [9]. Thus, Nrt–flu–Wg acts only on adjoining cells as designed. A second possible explanation for the rescue of wg mutants by Nrt–flu–Wg is that the wg–GAL4 driver could be expressed in a wider area than the domain of endogenous wg expression. We
stained *wg-GAL4* embryos with a *GAL4* RNA probe and found its distribution to be identical to that of *wg* mRNA in wild-type embryos (Figure 1e,f). Therefore, broader-than-expected expression of *wg-GAL4* does not account for the wide range of action of Nrt-flu-Wg. Another explanation for the ability of membrane-tethered Wg to reach distant cells must be sought.

### The parasegment boundary imposes directionality to cell spreading

During normal development, *en*-expressing cells do not cross into the anterior compartment where *wg* is expressed [10]. However, *wg*-expressing cells and their progeny may be free to roam in the anterior direction. This suggests an alternative explanation for the 'long-range action' of Nrt-flu-Wg: it could be carried anteriorwards by moving cells and their progeny (Figure 2; diagram). To test the feasibility of such a mechanism, we tracked the progeny of single cells marked at the time when *wg* expression commences. Single cells were marked by photoactivating caged rhodamine with the ultraviolet (UV) laser beam of a confocal microscope. To have a spatial landmark at later developmental times, this experiment was performed with embryos expressing the green fluorescent protein (GFP) in the posterior compartment (*en-GAL4 UAS-nlsGFP*). The progeny of marked cells were identified in live embryos at late stage 11 (after three mitoses) and mapped relative to the domain of *en* expression. We found that, although no clones crossed the parasegment boundary, those located just in front of the *en* domain spanned several cell diameters (up to five) in the anteroposterior direction. One example is shown in Figure 2. Four other clones located within the posterior region of the anterior compartment were obtained. They were similar in aspect to the one shown in Figure 2 and spanned 4–5 cells along the anteroposterior axis, revealing the extent of clonal spread along the anteroposterior axis. As the parasegment boundary is a clonal boundary, it imposes directionality to this spread, resulting in the net movement of *wg*-expressing cells towards the anterior. Importantly, clonal spread covered a broad area of the ectoderm and can account for the range of Nrt-flu-Wg in our rescue experiment.

If, as we propose, Wg is carried by moving cells and their progeny, a stable non-secreted protein should also be transported towards the anterior. This prediction was tested using a Gal4-responsive transgene encoding nuclear-targeted β-galactosidase (*β-gal*), which we found to be relatively stable (see legend to Figure 3). We determined whether this product was carried forwards in the ventral epidermis. Indeed, in embryos carrying *wg-GAL4* and *UAS-nuc-lacZ*, *β-gal* was detected in stripes 3–4 cells wide in front of the parasegment boundary (Figure 3b). This is substantially wider than the *GAL4* RNA stripes. In hatched larvae of the same genotype, *β-gal* activity was detected within similarly wide bands of cells occupying the middle of the bald regions (Figure 3c). We suggest that *β-gal* made by *wg*-expressing cells is retained even when cells move away from the source of Hedgehog and therefore shut off the *wg* promoter. As cells could move only towards the anterior of the parasegment boundary, *β-gal* appears to spread in the anterior direction. Note that such spreading could not have occurred through cellular extensions such as cytonemes [11] as the *β-gal* product was nuclear in this experiment. Thus, a non-secreted protein can spread by being passed on to the progeny of expressing cells. As expected then, when driven by *wg-GAL4*, Nrt-flu-Wg was detected in stripes that were...
Embryos expressing GFP under the control of tory pathway and thus identities secretory vesicles. In live domain as well as weakly throughout the perivitelline space vesicular staining was detected in stripes was detected in bright intracellular dots within the Wg colocalizes with secretory vesicles contain unsecreted protein inherited from past expression. most probably represents GFP transiting through the secretory vesicles descended from z-expressing cells. These vesicles are anterior of the transcription domain are within cells that wards by cell inheritance during normal development. As we have shown, the Wg-containing vesicles found at the spread anteriorly.

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**Wg colocalizes with secretory vesicles**

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These embryos were fixed and stained with anti-Wg antibody. Although much GFP fluorescence was lost upon fixation, we did detect extensive colocalization between Wg and the remaining GFP signal even outside the Wg expression domain (Figure 4c–e). This suggests that many Wg-containing vesicles are secretory as opposed to endocytic (although endocytic vesicles may exist as well). The presence of Wg-containing vesicles at the anterior of the Wg expression domain is often taken as evidence for transport from cell to cell. Our result shows that this assumption must be revised. It also shows that, during normal development, cell spreading contributes significantly to the anterior movement of endogenous Wg protein.

In conclusion, we have found that, as cells proliferate and spread, they can retain the Wg signal and thus affect target cells some distance away from the site of Wg transcription. It is important to note that Wg can also spread independently of cell movement [9], possibly by restricted diffusion. We have been able to uncouple the two mechanisms of Wg movement and, thus, have shown that either is sufficient to ensure a normal range of action. Presumably, both contribute during wild-type development although we cannot yet assess their relative importance. Interestingly, the parasegment boundary allows cells to carry Wg only towards the anterior and this adds to other mechanisms ensuring an asymmetric range of Wg in Drosophila embryos [9]. Without this border, cells carrying Wg could wander towards the posterior and disrupt segment polarity.

Supplementary material
Supplementary material including additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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