

# The endocytic pathway and formation of the Wingless morphogen gradient

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Controlling the spread of morphogens is crucial for pattern formation during development. In the *Drosophila* wing disc, Wingless secreted at the dorsal-ventral compartment boundary forms a concentration gradient in receiving tissue, where it activates short- and long-range target genes. The glypican Dally-like promotes Wingless spreading by unknown mechanisms, while Dynamin-dependent endocytosis is thought to restrict Wingless spread. We have utilized short-term expression of dominant negative Rab proteins to examine the polarity of endocytic trafficking of Wingless and its receptors and to determine the relative contributions of endocytosis, degradation and recycling to the establishment of the Wingless gradient. Our results show that Wingless is internalized via two spatially distinct routes: one on the apical, and one on the basal, side of the disc. Both restrict the spread of Wingless, with little contribution from subsequent degradation or recycling. As previously shown for Frizzled receptors, depleting Arrow does not prevent Wingless from entering endosomes. We find that both Frizzled and Arrow are internalized mainly from the apical membrane. Thus, the basal Wingless internalization route must be independent of these proteins. We find that Dally-like is not required for Wingless spread when endocytosis is blocked, and propose that Dally-like promotes the spread of Wingless by directing it to lateral membranes, where its endocytosis is less efficient. Thus, subcellular localization of Wingless along the apical-basal axis of receiving cells may be instrumental in shaping the Wingless gradient.

**KEY WORDS:** Rab5, Rab7, Rab11, Imaginal disc, Polarized endocytosis, *Drosophila*

## INTRODUCTION

Morphogens are signaling proteins secreted by restricted groups of cells and forming a graded distribution in surrounding tissue (Neumann and Cohen, 1997). This concentration gradient activates different genes as a function of distance from the source, specifying cell identities in developing tissues. Gradient formation requires both a source of morphogen and a sink. Possible sinks include degradation in lysosomes of receiving tissue, degradation by extracellular proteases or release from tissue.

The endocytic pathway in receiving tissue is an important regulator of morphogen gradients, although it can have opposing effects on the spread of different morphogens. The TGF $\beta$  homolog Decapentaplegic (Dpp) depends on the functions of Dynamin (Shibire – FlyBase) to move over long distances through the wing disc epithelium of *Drosophila* (Entchev et al., 2000) (but see also Belenkaya et al., 2004). Dynamin is a GTPase mediating scission of endocytic vesicles (Sever, 2002); thus, Dpp molecules are proposed to be actively passed through cells by planar transcytosis (i.e. successive rounds of internalization and recycling to the surface). By contrast, internalization restricts the range of FGF-8 movement in zebrafish – a process called restrictive clearance (Scholpp and Brand, 2004).

Morphogens of the Wingless (Wg) and Hedgehog (Hh) families harbor covalently attached lipid moieties (Pepinsky et al., 1998; Porter et al., 1996; Willert et al., 2003; Zhai et al., 2004). Although lipid confers high membrane affinity on these proteins, they can escape cell membranes by binding to Lipoproteins. In the *Drosophila* wing disc, Wg and Hh require Lipoproteins to signal over long distances (Panáková et al., 2005). Neither Wg nor Hh

require Dynamin activity to spread through receiving disc tissue (Strigini and Cohen, 2000; Torroja et al., 2004); therefore, they probably move extracellularly rather than by planar transcytosis in imaginal discs. Loss of Dynamin function does perturb the range of Wg signaling in the embryonic ectoderm, however (Bejsovec and Wieschaus, 1995), suggesting that different transport mechanisms operate in different tissues.

Several observations suggest that the endocytic pathway helps shape the Wg gradient. In imaginal discs, Wg accumulates on the cell surface of Dynamin mutant clones (Strigini and Cohen, 2000), suggesting that normal cells internalize and degrade Wg. In embryos, increased Wg lysosomal degradation appears to shorten its signaling range posteriorly (Dubois et al., 2001). Furthermore, recycling Wg back to the extracellular space was proposed to replenish the pool available for movement (Pfeiffer et al., 2002). Nevertheless, the respective contributions of internalization, degradation and recycling in controlling Wg spreading have not been systematically examined.

The receptors that restrict the spread of Wg remain unidentified. Removing proteins that mediate Wg internalization and degradation should elevate Wg concentration on cell surfaces. Conversely, overexpressing such proteins might cause excess internalization and deplete extracellular Wg. However, ectopic Frizzled 2 (Fz2) overexpression actually stabilizes extracellular Wg (Cadigan et al., 1998; Baeg et al., 2004), suggesting that this receptor facilitates the spread of Wg. While Wg accumulates on the surface of cells missing both Frizzled receptors Fz1 and Fz2, it is nevertheless still internalized; thus, the role of Fz receptors appears complex (Baeg et al., 2004). The LDL receptor family protein Arrow is thought to act as a Wg co-receptor; its role in Wg trafficking is also unclear. Wg accumulates extracellularly on *arrow* mutant clones, but this may be an indirect effect; loss of Wg signaling increases the transcription of *dally-like* (Han et al., 2005), of which the protein product stabilizes Wg at the cell surface (see below). It is not yet known whether Wg internalization depends on Arrow.

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Heparan sulfate proteoglycans (HSPGs) play a crucial, if little understood, role in promoting Wg spreading. Wg does not accumulate in tissue that cannot synthesize heparan sulfate (Baeg et al., 2004), and heparinase treatment in vitro causes Wg to disappear from both producing and receiving tissue (Greco et al., 2001). This mainly reflects the activity of the HSPG Dally-like (Dlp), which is required for long-range Wg movement and signaling (Franch-Marro et al., 2005; Han et al., 2005; Kirkpatrick et al., 2004; Kreuger et al., 2004). Dlp overexpression in imaginal discs causes massive accumulation of extracellular Wg (Baeg et al., 2001; Han et al., 2005). Thus, Dlp is thought to promote the interaction of Wg with the cell surface (Baeg et al., 2001; Baeg et al., 2004; Franch-Marro et al., 2005). Cleavage of the Dlp gpi anchor by Notum has also been proposed as a mechanism to promote long-range Wg movement (Kreuger et al., 2004). The possibility that Dlp might influence the endocytic trafficking of Wg has not yet been examined.

## MATERIALS AND METHODS

### Inducible UAS constructs

pUHR was constructed by inserting an FRT>*HcRed*>FRT cassette (*HcRed* and SV40 terminator were PCR-amplified from pHcRed1-N1 [Clontech] with 5' and 3' primers both incorporating a wild-type FRT sequence) between the UAS and the multiple cloning site (MCS) of pUAST (Brand and Perrimon, 1993). When the cassette is present, Gal4 transcribes *HcRed* and any gene cloned in the MCS is silent. Heat-shock-mediated induction of Flipase excises the FRT cassette and triggers expression of the gene in the MCS.

We generated pUHR derivatives containing the following cDNAs: *GFP-Dlp* (GFP inserted at a.a. 221); *rab5SN* subcloned from pUAST-Rab5SN (Entchev et al., 2000); *rab7TN*; *rab11SN*; *rab4SN* (donated by M. González-Gaitán).

Larvae containing pUHR constructs, a GAL4 driver and hs-Flipase were heated for 1 hour 30 minutes to 37.2°C (causing cassette excision in virtually 100% of the cells) and dissected at indicated times.

### Inducible tissue-specific RNAi

A 406-bp PCR product generated from the *dlp* coding sequence was cloned as an inverted repeat separated by an intron into pUAST. Transgenic flies were crossed with *apterous*-Gal4 also expressing ubiquitous Gal80<sup>ts</sup>, which represses GAL4 at the permissive temperature of 18°C (McGuire et al., 2003). Dlp dropped to undetectable levels within 36 hours of shift to 29°C. Experiments were performed 40 hours after temperature shift. To induce Dlp RNAi and Rab5SN sequentially, we first inactivated Gal80 (resulting in *dlp* RNAi) and 2 days later heat-shocked larvae to excise the FRT cassette in pUHR-Rab5SN.

For *rab7* RNAi, inverted repeats separated by an intron were cloned into pUHR. For *rab11* RNAi, inverted repeats were cloned without spacer intron into pUHR. For *arrow* RNAi, we inserted a 575-bp *arrow* cDNA fragment into pFRIFE. pFRIFE is a pUAST derivative in which inverted repeats can be inserted in one step by Gateway-based recombination (Invitrogen) on either side of an FRT-HcRed-FRT cassette containing a transcription terminator. The FRT cassette separating the two repeats provides inverted repeat stabilization during *Escherichia coli* growth, and RNAi inducibility in flies by heat-shock dependent cassette excision, which generates an inverted repeat without spacer.

### FP-Rab constructs

To label Rab5, Rab11, Rab4 and Rab7 endosomes, we fused CFP and YFP (Venus) to the N-terminus of Rabs, cloned them into pCasper4 under control of the ubiquitous *tubulin* promoter and generated transgenic flies. Fluorescence intensity indicates that these constructs drive much weaker expression than the GAL4/UAS system.

### Dextran uptake

Wing imaginal discs were dissected in M3 medium, transferred to medium containing 5 mg/ml Alexa 488-labeled Dextran 10,000 (Molecular Probes) and incubated for 10-15 minutes at 29°C. Discs were washed for 10 minutes in ice-cold PBS and fixed in PBS + 4% formaldehyde.

### Immunofluorescence

Antibodies used were mouse anti-Wg (Strigini and Cohen, 2000), mouse anti-Dlp (Lum et al., 2003), rabbit anti-Dfizzled2 (Strigini and Cohen, 2000), rat anti-DE-Cadherin2 (Oda et al., 1994), rabbit anti-Caspase (Cell Signalling Technology), recognizing *Drosophila* Drice and Dcp1 (Yu et al., 2002), rat anti-EGF Receptor (Jékely and Rørth, 2003). To generate Arrow antibodies, guinea pigs were immunized with a fragment of the protein corresponding to amino acids 1222-1450. Antibody specificity was confirmed by staining imaginal discs containing *arrow*<sup>-</sup> clones. Antibody stainings were performed as described (Strigini and Cohen, 2000), except that for extracellular protein staining, discs were incubated <3 minutes at room temperature with the Wg antibody before incubation on ice. Confocal images were acquired at 100× magnification on a Zeiss Axiovert 200 microscope.

### PIPLC treatment

To release gpi-linked proteins, wing imaginal discs dissected from third instar larvae were transferred to Grace's insect medium containing 10 U/ml of PIPLC enzyme (Molecular Probes) and incubated at 25°C with mild agitation for indicated times.

### Image analysis

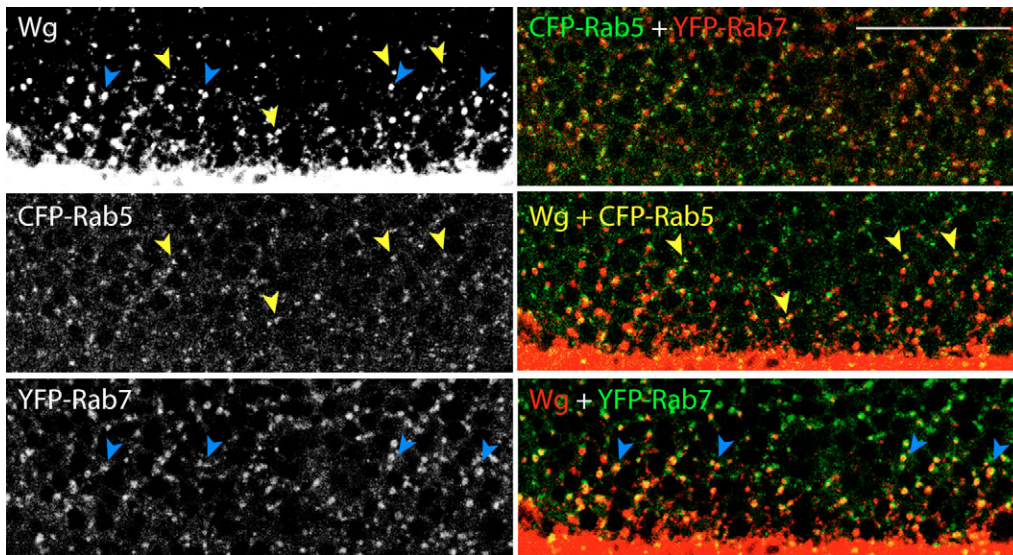
To quantify Wg staining intensity in Fig. S3 (see supplementary material) and Dlp staining intensity in Fig. S6 (see supplementary material), pixel intensity along different lines centered on the dorsal-ventral boundary were quantified using the Plot Profile function of ImageJ and values at each point were averaged.

To quantify co-localization between Wg, CFP-Rab5 and YFP-Rab7 in receiving tissue, we used threshold and despeckle functions of Image J to eliminate cytoplasmic signals from FP-Rab proteins. For Wg, this treatment virtually eliminated the dispersed staining outlining cell boundaries. Wg-expressing cells were excluded. We defined a particle as a signal of at least three contiguous pixels and counted particles automatically using the Analyze particles function of Image J. Co-localization was assessed for single confocal sections using the RGB-colocalization plugin and defined as an overlap of at least three pixels. Similar methods were used to quantify co-localization between membrane proteins and Rab5 or 7 endosomes, but the threshold was set higher to reduce signal from the plasma membrane.

## RESULTS

### Wingless is found in Rab5 and Rab7 endosomes

The Rab5 GTPase regulates endocytic vesicle formation and early endosome fusion (Stenmark et al., 1994; Zerial and McBride, 2001). As Rab5 is replaced by Rab7, these early endosomes acquire degradative capacity and mature into lysosomes (Bucci et al., 2000; Rink et al., 2005). We used transgenic flies ubiquitously expressing moderate levels of CFP-Rab5 and YFP-Rab7 to localize these endosomes in discs. Endosomes positive for Rab5 only were most abundant in the apical- and basal-most regions of disc cells. The fraction of endosomes containing only Rab7 increased in central regions (see Fig. S1A in the supplementary material), suggesting endosomes move centrally as they mature. Consistent with the model that Rab5 is gradually replaced by Rab7, we observed significant co-localization between these two markers (see Fig. S1 in the supplementary material). To test whether Wg was present in these compartments, we examined co-localization between Wg, CFP-Rab5 and YFP-Rab7 in receiving tissue. Between 43 and 58% of Wg spots co-localized with Rab5 and/or Rab7 in the apical half of disc cells (Fig. 1). Basally, between 25 and 29% was found in these compartments (see Fig. S1B in the supplementary material). Thus a significant fraction of Wg was found in Rab5- and Rab7-positive endosomes. Non-co-localizing Wg may be extracellular, or in a different endocytic compartment.



**Fig. 1. Wingless is found in Rab5 and Rab7 endosomes.** Wing imaginal discs ubiquitously expressing moderate levels of CFP-Rab5 and YFP-Rab7 stained with antibodies against Wg. Panels depict the different channels from a single confocal section 4  $\mu\text{m}$  below the apical surface (including a small part of the Wg expression domain, at bottom of panel). Yellow arrowheads indicate co-localization between Wg and CFP-Rab5, blue arrowheads co-localization between Wg and YFP-Rab7. Scale bar: 20  $\mu\text{m}$ .

### Rab5-dependent endocytosis restricts Wingless spread

Previous studies using a temperature-sensitive *dynamain* mutant to block endocytosis indicated that Wg spreads extracellularly in the wing disc (Strigini and Cohen, 2000). As Dynamin also functions at the Golgi apparatus and Rab11 endosomes (Jones et al., 1998; Pelissier et al., 2003; Sever, 2002; van Dam and Stoorvogel, 2002), we specifically perturbed endocytosis by expressing the dominant negative Rab5SN (Entchev et al., 2000; Stenmark et al., 1994) in different subsets of the wing disc. When Rab5SN expression was induced during the third larval instar (see Materials and methods; see also experimental scheme in Fig. S2A in the supplementary material), endocytosis was inhibited within 2 hours, and cells in the wing pouch began to undergo apoptosis after 6-8 hours (see Fig. S2 in the supplementary material). We therefore limited our observations to discs that had expressed Rab5SN for 2-6 hours.

Wg is expressed in a stripe of cells straddling the dorsal-ventral boundary and forms a symmetrical gradient in dorsal and ventral compartments. To ask how Rab5-dependent endocytosis affects Wg distribution, we expressed Rab5SN in the dorsal compartment of third instar discs and compared the shape of the Wg gradient in dorsal and ventral compartments (Fig. 2). One hour after inducing Rab5SN in dorsal cells, no change in Wg distribution was evident (not shown). By 2-4 hours (shortly after the block in Rab5-dependent internalization), Wg puncta disappeared but Wg protein levels were elevated in dorsal tissue nearest the Wg-expressing cells (Fig. 2A). By 5 hours, large amounts of Wg had invaded the entire dorsal wing pouch (Fig. 2B). Elevated Wg levels were not caused by induction of ectopic Wg expression (see Fig. S2H in the supplementary material). The spatial progression of Wg accumulation with time suggests that intervening tissue no longer acts as a sink for Wg when endocytosis is disrupted, and that Rab5-dependent internalization normally limits the range over which Wg spreads.

Although Dynamin is needed for Wg secretion from producing cells (Strigini and Cohen, 2000), Rab5-dependent endocytosis is dispensable; targeted Rab5SN expression in the entire *wg* expression domain had no impact on Wg release even after long-term Rab5SN expression (up to 23 hours; data not shown).

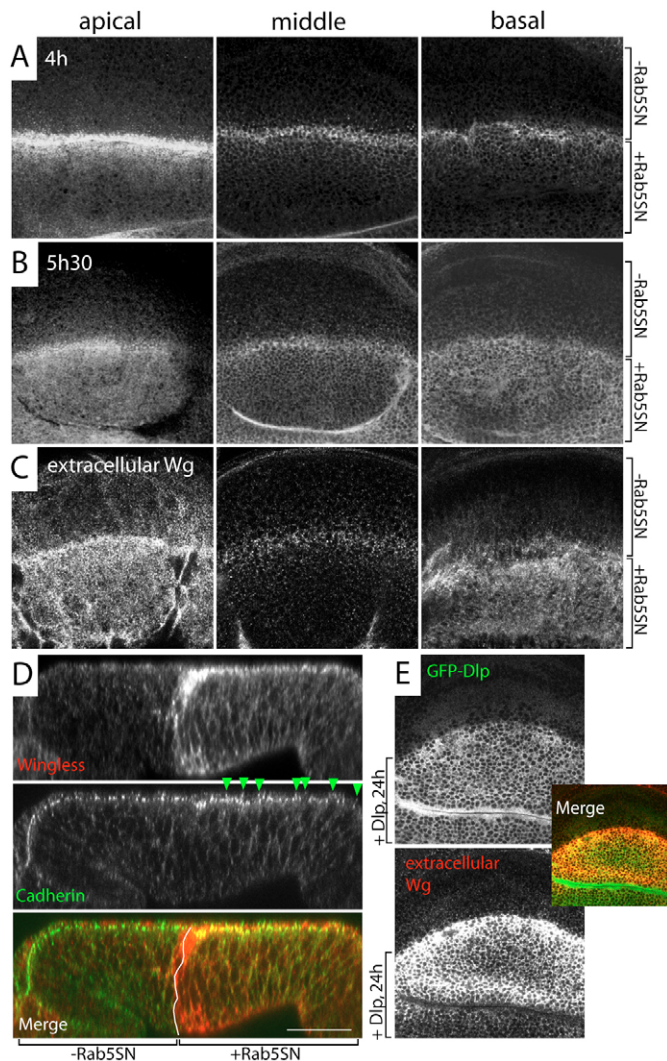
To identify sites of Wg internalization, we examined the subcellular localization of Wg in Rab5SN-expressing cells. Wg was strongly enriched apical to adherens junctions, and at the very basal side of the epithelium (Fig. 2A-D; quantified in Fig. S3 in the supplementary material), especially when discs were stained specifically for extracellular Wg (Fig. 2C). Polarized accumulation is not an artifact of reduced antibody access to lateral cell membranes, because lateral extracellular Wg accumulation was detected upon Dlp overexpression (Fig. 2E). This suggests that Wg is internalized from apical and basal (but not lateral) surfaces and that internalization normally limits its spread.

### Rab7-dependent degradation does not modulate the extracellular spread of Wingless

To investigate whether the balance of Wg recycling versus degradation affected levels of extracellular Wingless, we perturbed these pathways. In vertebrate cells, expression of dominant negative Rab7 disperses the lysosomal compartment and inhibits degradation of endocytosed proteins (Bucci et al., 2000). In *Drosophila* disc cells, Rab7TN expression dispersed the punctate distribution of YFP-Rab7, leaving CFP-Rab5-labeled endosomes undisturbed (see Fig. S4A,B in the supplementary material) and caused accumulation of Hedgehog and Patched (Eugster and Eaton, unpublished), suggesting that lysosomal degradation is inhibited. Furthermore, YFP-Rab11-labeled recycling endosomes were more abundant in Rab7TN-expressing cells, suggesting that inhibiting the degradative pathway increased trafficking to recycling endosomes (see Fig. S4E in the supplementary material).

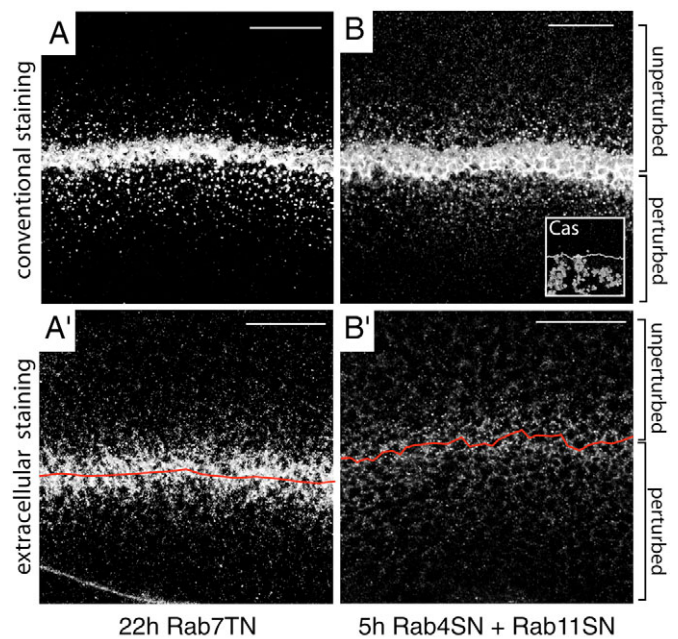
Wg-positive endosomes in Rab7TN-expressing tissue were increased in size and number, and were present at a greater distance from the source than in non-expressing control tissue (Fig. 3A). The range of extracellular Wg distribution was unchanged, however (Fig. 3A'). Thus, the increased range of detection of Wg in Rab7TN-expressing tissue is not due to an increase in the distance over which extracellular Wg spreads, but rather to enhanced perdurance of internalized Wg protein. Slowing degradation does not increase the pool of Wg available for extracellular movement.

Wings of adult flies that expressed Rab7TN were slightly upcurved and showed frequent vein deltas near the margin, but no notching or bristle defects indicative of gain or loss of Wg signaling (not shown). RNAi directed against Rab7 reduced RNA levels and



**Fig. 2. Wingless spreads further when Rab5-dependent internalization is blocked.** (A–C) Wg staining in single confocal sections of discs that expressed Rab5SN for 4 hours (A) or 5 hours 30 minutes (B, C) in the dorsal compartment. Apical, middle and basal sections are shown. Discs in A, B were stained using a protocol detecting both intra- and extracellular Wg; C shows extracellular Wg. (D) Single xz section perpendicular to the dorsal/ventral boundary of a disc 5 hours 30 minutes after the onset of Rab5SN expression. Dorsal Rab5SN-expressing compartment is to the right. The dorsal-ventral compartment boundary is indicated by a white line. Cadherin (green) marks apical junctions. Elevated Wg (red) accumulates apically at the level of and above junctions. Green arrows indicate junctions within the overlying peripodial membrane. (E) Single confocal section through the middle (8  $\mu\text{m}$  below apical surface) of a wing disc that overexpressed GFP-Dlp for 24 hours in the dorsal compartment (indicated by brackets). The section passes through lateral cell membranes. Top: endogenous GFP-Dlp fluorescence. Bottom: extracellular Wg. GFP-Dlp overexpression elevates Wg on the lateral surface. Scale bar: 20  $\mu\text{m}$ .

produced identical results (see Fig. S4F in the supplementary material). The surprisingly moderate effects of both Rab7 dominant negative expression and RNAi may reflect residual activity of Rab7; alternatively, non-Rab7-dependent degradative pathways may exist in *Drosophila*.



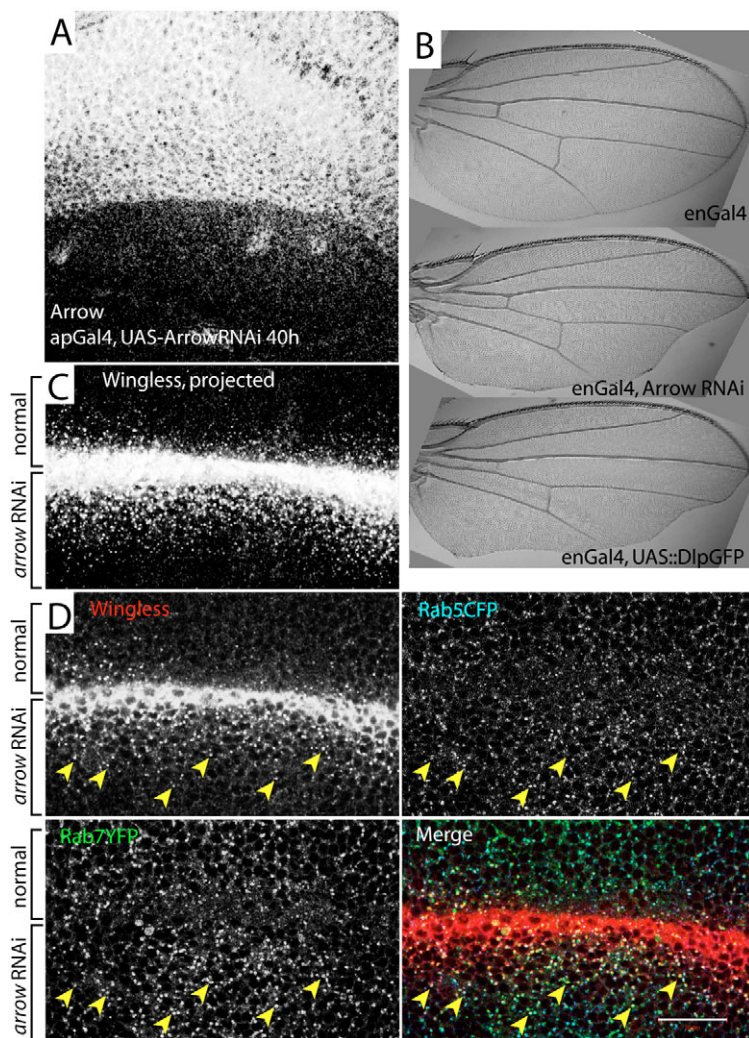
**Fig. 3. Inhibiting the degradative or recycling pathways does not affect extracellular Wingless distribution.** (A, B) Wg conventional staining of wing discs expressing (A) Rab7TN, (B) Rab4SN + Rab11SN (inset shows nuclei positive for activated Caspase). Compare dorsal expressing and ventral non-expressing compartments. (A', B') Wg extracellular staining of the same genotypes. The dorsal-ventral boundary is indicated by a red line. Wg extracellular distribution is not altered by any of these treatments, but note intracellular Wg accumulation in A. Scale bars 20  $\mu\text{m}$ .

### Canonical recycling pathways do not promote Wingless spread

To examine whether recycling played a role in Wg gradient formation, we expressed the dominant negative proteins Rab11SN and Rab4SN. Rab11 is required to transport both endocytosed proteins and some de-novo synthesized proteins through recycling endosomes to the plasma membrane (Ang et al., 2004; Lock and Stow, 2005; Maxfield and McGraw, 2004; Satoh et al., 2005). Vertebrate Rab4 directs rapid recycling from early endosomes to the plasma membrane and is essential for recycling certain receptors (de Renzis et al., 2002; Lazzarino et al., 1998; Seachrist et al., 2000; van der Sluijs et al., 1992).

Inducing Rab11SN expression in imaginal discs dispersed wild-type YFP-Rab11 within 4 hours (see Fig. S5A in the supplementary material) and inhibited recycling of Delta (Emery et al., 2005), Cadherin (Shotgun – FlyBase) (Classen et al., 2005) and the EGF receptor (see Fig. S5C in the supplementary material). Furthermore, Rab7 endosomes are enlarged by Rab11SN expression, suggesting that inhibiting recycling may increase trafficking to Rab7 endosomes (see Fig. S5B in the supplementary material). Rab4SN expression prevented endosomal localization of wild-type YFP-Rab4 (see Fig. S5D in the supplementary material), but did not affect the morphology of other endosomes (not shown). Expression of Rab11SN, but not Rab4SN, activated apoptosis within 5 hours (Fig. 3B inset and not shown).

Expression of either dominant negative alone, or both Rab11SN and Rab4SN together, did not affect Wg distribution (intra- or extracellular) within 5 hours (Fig. 3B, B' and not shown). Similarly, RNAi directed against Rab11 reduced Rab11 RNA levels (see Fig.



**Fig. 4. Arrow knockdown causes intracellular Wg accumulation and loss of Wg signaling.** (A) Anti-Arrow staining of wing disc with *arrow* RNAi induced in the dorsal compartment. Dorsal cells lack Arrow, except in four small clones that failed to excise the HcRed cassette and initiate RNAi (imaged using higher gain than Fig. 5C to maximize residual Arrow detection). (B) Top panel shows a control wing. Middle panel shows a wing from a fly that emerged 6 days after *arrow* RNAi induction in the posterior compartment. Lower panel shows a wing from a fly that emerged 6 days after induction of GFP-Dlp overexpression in the posterior compartment. (C) Projection of 12 confocal sections 1  $\mu\text{m}$  apart from a disc that expressed *arrow* RNAi in the dorsal compartment for 48 hours, stained for Wg. (D) Single confocal section of the disc shown in (C) showing Wg, CFPRab5 and YFP Rab7. Yellow arrowheads show examples of co-localization between Wg and endosomes. Scale bar: 20  $\mu\text{m}$ .

S5E in the supplementary material), but did not alter Wg distribution (see Fig. S5F,F' in the supplementary material). Thus, it seems unlikely that either Rab11- or Rab4-dependent recycling pathways contributes to the spread of Wg in imaginal discs, although as yet unknown recycling pathways might exist. These data show that Wg gradient formation is controlled by apical and basal internalization, with little contribution from canonical degradative or recycling pathways.

#### Arrow is not required for Wingless endocytosis

Although it accumulates extracellularly, Wg is still also found in endosomes in *Fz1-Fz2* double mutant clones, suggesting that Wg internalization can occur via other receptors (Baeg et al., 2004). Whether Arrow (a Lipoprotein receptor family member required for Wg signaling) internalizes Wg is unknown. To investigate its role in Wg trafficking, we reduced Arrow protein levels by RNAi in the dorsal compartment. Arrow protein became undetectable 2 days after RNAi induction (Fig. 4A), and emerging adults had phenotypes suggesting loss of Wg signaling (Fig. 4B). Wg-positive Rab5 and Rab7 endosomes were more abundant ( $2.9 \pm 0.6$ -fold,  $n=3$ ) over a longer range in Arrow RNAi tissue (Fig. 4C,D). These endosomes were also located more apically than those of wild-type cells (see Fig. S6A in the supplementary material). Thus Arrow is not required for Wg internalization, but appears to modulate its trafficking. As

Wg is also detected in endosomes over a longer range when Rab7 activity is reduced (Fig. 3A), loss of Arrow may reduce the rate of Wg degradation after endocytosis.

Like *arrow* mutant clones (Han et al., 2005), tissue undergoing *arrow* RNAi accumulated some extracellular Wg (data not shown); however, this may reflect increased stabilization caused by elevated Dlp protein levels near the dorsal-ventral boundary (see Fig. S6B in the supplementary material), consistent with previous observations (Han et al., 2005).

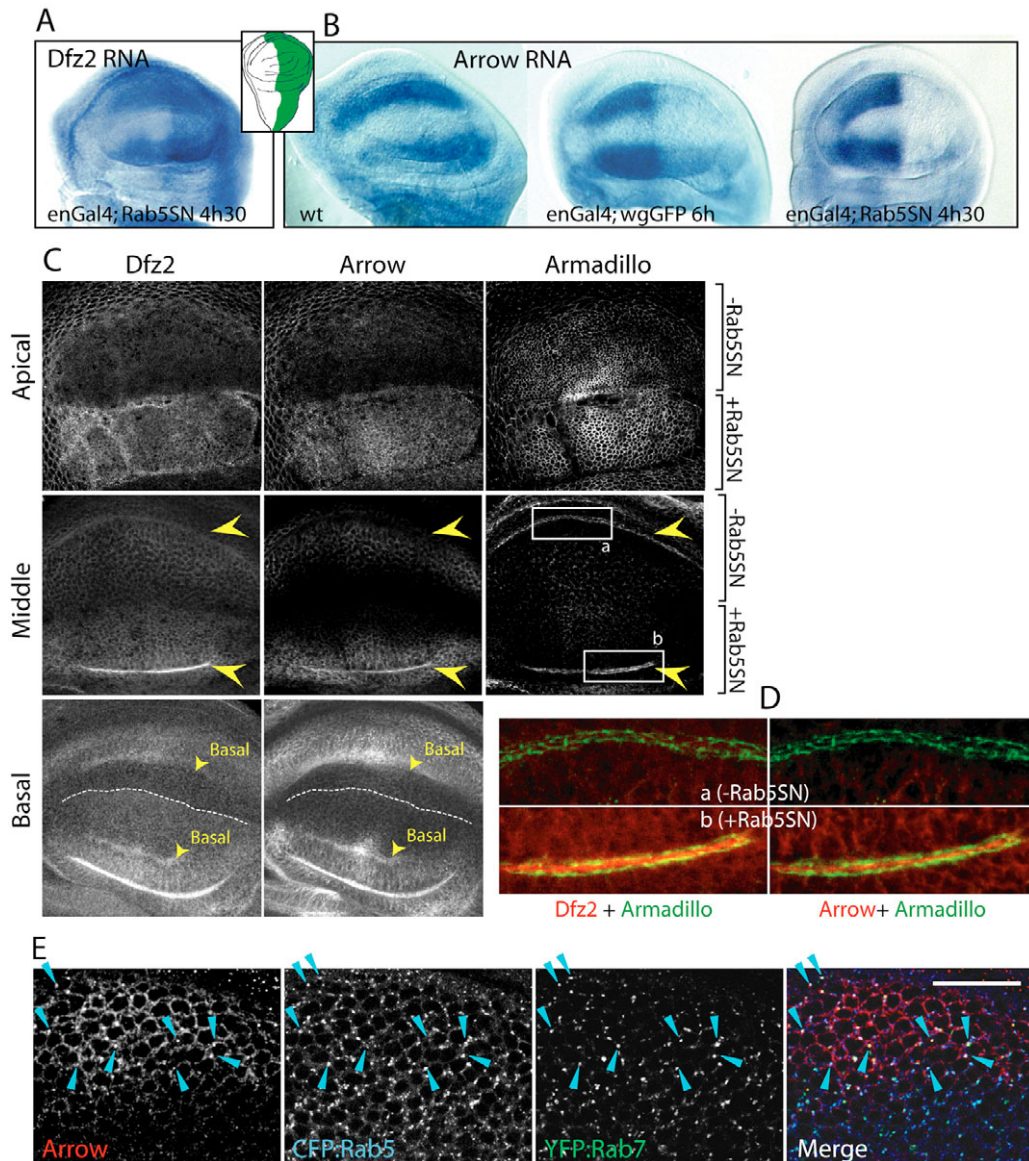
#### Fz2 and Arrow are internalized apically by Rab5-dependent endocytosis

Our results suggest that Wg is internalized from both the apical and basal disc surfaces. We expected that receptors mediating Wg internalization should co-accumulate with Wg on these surfaces when endocytosis was blocked. We therefore examined the subcellular localization of Fz1, Fz2 and Arrow after Rab5SN induction. In the steady state, Fz2 is found predominantly on the basal-lateral side of wing epithelial cells (Wu et al., 2004) (Fig. 5C, non-Rab5SN-expressing region). However, after 5 hours of Rab5SN expression, Fz2 accumulated dramatically on the apical side of the epithelium (Fig. 5C,D and Fig. S6C in the supplementary material) and was only slightly elevated on basal and lateral membranes (Fig. 5C). These data suggest that, despite its steady state basal-lateral

localization, Fz2 is delivered to the apical side of the cell but does not accumulate there because of rapid internalization. By contrast, Fz1-GFP localization did not change obviously under these conditions (not shown). As Fz1 and 2 function redundantly, it is possible that Fz1 internalization might be more apparent in the absence of Fz2. Unexpectedly, some Fz2 accumulation may reflect an increase in synthesis, rather than a decrease in endocytosis, because *Fz2* mRNA levels rose in response to Rab5SN expression (Fig. 5A). However, the apical shift in Fz2 subcellular distribution

when endocytosis was blocked indicates that it is normally internalized from the apical surface. As Fz2 is internalized slowly, if at all, from the basal surface, it is unlikely to mediate Wg endocytosis there.

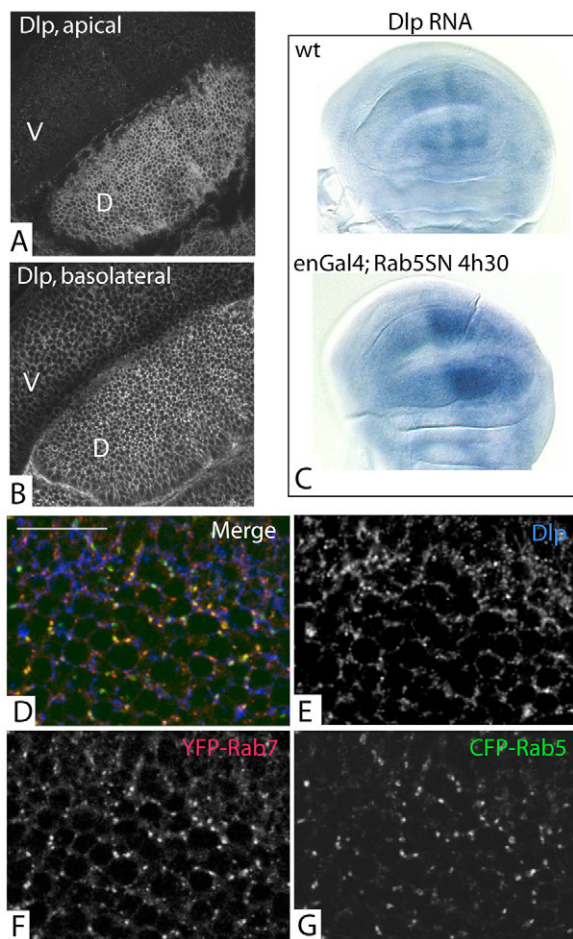
Like Fz2, endogenous Arrow protein levels rise with distance from the dorsal-ventral boundary (Fig. 5C, middle). This reflects its transcription (Fig. 5B, left), which, like that of *Fz2* (Cadigan et al., 1998), is lowered by Wg signaling (Fig. 5B, middle). Arrow protein is mainly basal-lateral in the steady state (Fig. 5C, non-



**Fig. 5. Rab5SN expression alters subcellular localization and mRNA levels of Fz2 and Arrow.** (A) In-situ hybridization to *Fz2* mRNA in a disc after 4 hours 30 minutes Rab5SN expression in the posterior compartment. Inset shows Rab5SN expression domain (green). (B) In-situ hybridizations detecting *arrow* mRNA in discs of different genotypes. Left: wild type. Middle: disc expressing GFP-Wg in the posterior compartment for 6 hours. Right: disc expressing Rab5SN in the posterior compartment for 4 hours 30 minutes. (C) Discs expressing Rab5SN in the dorsal compartment for 5 hours 30 minutes stained for Arrow, Fz2 and Armadillo to mark apical junctions. Apical, middle sections and the basal region are shown. Note the accumulation of Fz2 and Arrow above the junctions. Large arrows in middle sections indicate the apical cell surface. In the basal region, the wing pouch curves so that the apical-basal axes of the epithelial cells at the edges of the wing pouch lie parallel to the focal plane. Thus, a complete longitudinal section of these cells is visible. Small arrows point to their basal sides. (D) Larger magnification of the areas boxed in C. Armadillo staining reveals the apical junctions of two rows of epithelial cells facing each other. Fz2 and Arrow accumulate above cellular junctions in Rab5SN-expressing tissue. (E) Single confocal section of a wing imaginal disc expressing YFP-Rab7, CFP-Rab5 and stained for Arrow. Arrowheads indicate co-localization between Arrow and Rab5/7 endosomes. Quantifying ten confocal sections corresponding to the apical-most 10  $\mu\text{m}$  of the disc shown indicates that 49% of the brightest Arrow spots co-localize with either YFP-Rab7 or CFP-Rab5. Scale bar: 20  $\mu\text{m}$ .

Rab5SN-expressing cells) and is also present in both Rab5 and Rab7 endosomes (Fig. 5E). In response to Rab5SN expression, punctate Arrow staining disappeared and Arrow accumulated at the apical membrane, co-localizing with Fz2 (Fig. 5C,D and Fig. S6C). Thus Arrow, like Fz2, is internalized apically and is unlikely to mediate basal Wg endocytosis. While these proteins may internalize Wg from the apical surface, an Arrow and Fz2-independent Wg internalization pathway must operate at least on the basal surface.

We wondered whether *arrow* mRNA levels, like those of *Fz2*, might rise in response to Rab5SN expression. Surprisingly, the opposite was true. *Arrow* mRNA plunged to undetectable levels by 4 hours after Rab5SN induction (Fig. 5B, right). Arrow protein, however, was not reduced within this time.



**Fig. 6. Rab5SN expression alters subcellular localization and mRNA levels of Dlp.** (A,B) Wing disc after 5 hours 30 minutes Rab5SN expression in dorsal compartment, stained for Dlp. Dlp accumulates more strongly apically (A) than basal-laterally (B) in response to Rab5SN expression. (C) In-situ hybridization to *dlp* mRNA in a wild-type disc (top) and a disc after 4 hours 30 minutes Rab5SN expression in the posterior compartment (to the right). (D-G) Single confocal section of a disc stained for endogenous Dlp and expressing the indicated FP-Rab proteins. Quantifying ten confocal sections corresponding to the apical-most 10  $\mu\text{m}$  of this disc indicates that 19% of the brightest Dlp spots co-localize with either YFP-Rab7 or CFP-Rab5. D, dorsal compartment; V, ventral compartment.

### Dlp accumulates upon Rab5SN expression

Dlp assists extracellular Wg spreading in discs (Baeg et al., 2001; Franch-Marro et al., 2005; Giraldez et al., 2002; Han et al., 2005) and localizes predominantly to the basal-lateral membrane in the steady state (Baeg et al., 2004) (Fig. 6A,B). As early as 4 hours after inducing Rab5SN expression in the dorsal compartment, endogenous Dlp dramatically accumulated around the apical-lateral region of expressing cells and more modestly on the basal-lateral membrane (Fig. 6A,B and Fig. S6D). *dlp* mRNA levels rose within 4 hours 30 minutes of initiating Rab5SN expression (Fig. 6C), suggesting that Dlp protein accumulates in part because of increased synthesis. However, the strong apical shift in Dlp subcellular localization suggests that Rab5-dependent internalization normally prevents apical-lateral accumulation of Dlp.

We were surprised that Rab5SN expression trapped Dlp at the cell membrane, because gpi-linked proteins are thought to be internalized by a Rab5-independent mechanism (Sabharanjak et al., 2002). To investigate whether Dlp, like Wg and Arrow, was internalized into Rab5 endosomes, we examined co-localization between endogenous Dlp, YFP-Rab7 and CFP-Rab5. Unlike Wg or Arrow, the Dlp signal only rarely overlaps with that of CFP-Rab5 or YFP-Rab7 (Fig. 6D-G). Therefore, apical-lateral enrichment of Dlp in Rab5SN-expressing tissue may occur by an indirect mechanism. For example, Dlp may be recruited there by other proteins accumulating when Rab5 endocytosis is blocked. Alternatively, Dlp may move swiftly through these compartments, failing to accumulate to easily detectable levels.

### Wingless is not recruited by elevated Dlp during the endocytosis block

Because Dlp overexpression elevates Wg levels at the plasma membrane (Baeg et al., 2001; Franch-Marro et al., 2005; Giraldez et al., 2002; Han et al., 2005), we wondered whether Wg accumulation on Rab5SN-expressing cells was an indirect effect of increased Dlp. To see whether Wg was bound to these cells via Dlp, we treated discs that had expressed Rab5SN for 5 hours 30 minutes with Phosphatidylinositol-Phospholipase C (PI-PLC) to release gpi-linked proteins. One hour of PI-PLC treatment entirely removed even the high levels of Dlp present on Rab5SN-expressing cells (Fig. 7C). Wg, however, was not released by this treatment (Fig. 7D). Therefore, Wg is not trapped at the surface of Rab5SN-expressing cells by binding to Dlp or any other gpi-anchored protein. As long as endocytosis is prevented, Wg does not require glypicans to accumulate extracellularly on disc epithelial cells.

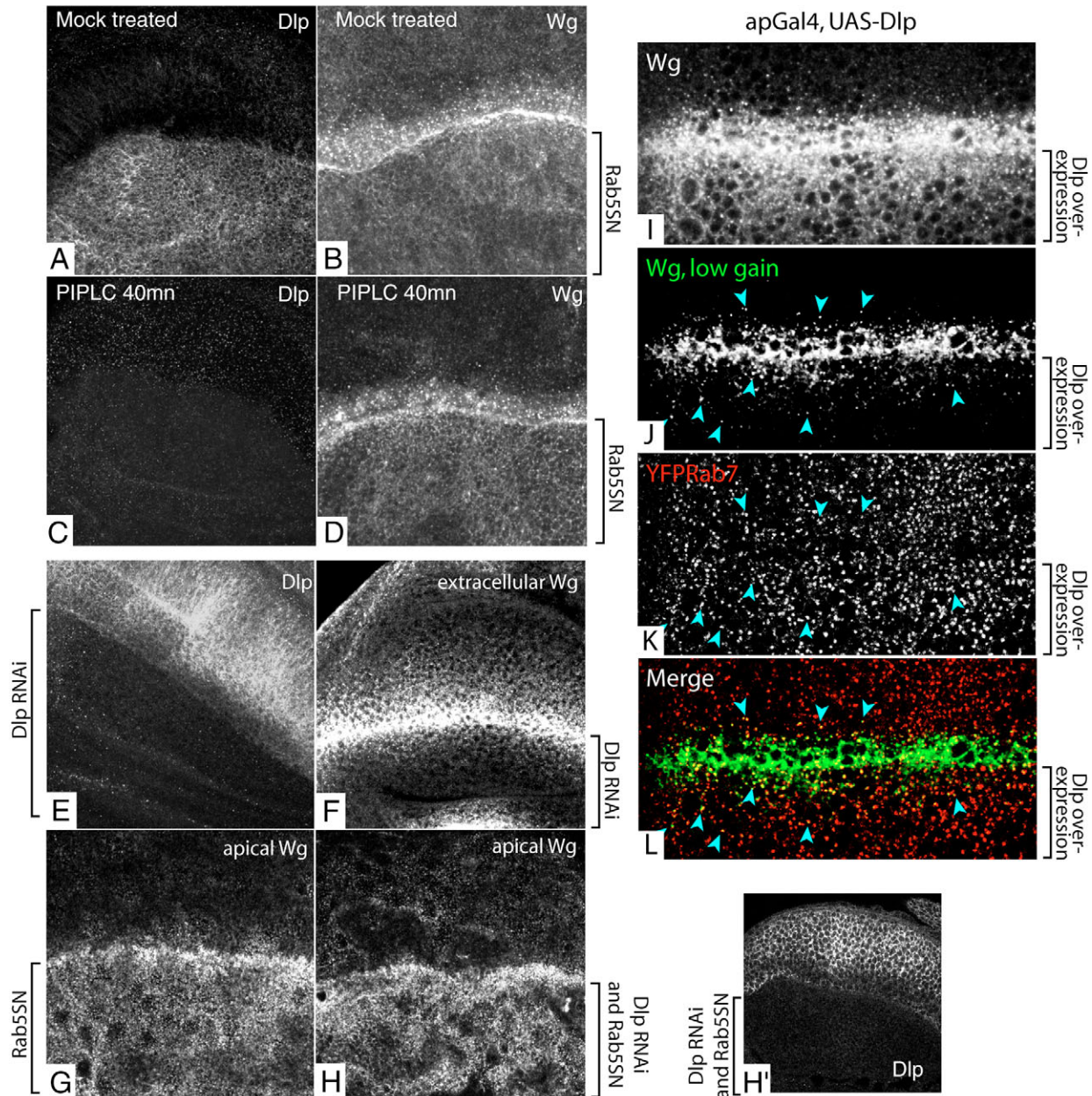
### Wingless spreads independently of Dlp during the endocytosis block

Although Dlp is not needed to maintain Wg on the surface of Rab5SN-expressing cells, it might be required earlier to promote Wg spread across Rab5SN-expressing tissue. To address this possibility, we used RNAi to deplete Dlp from the dorsal compartment before Rab5-dependent endocytosis was blocked (see Materials and methods). In discs that did not express Rab5SN, inducing *dlp*-specific RNAi rendered Dlp protein undetectable within 2 days, and reduced the range of extracellular Wg (Fig. 7E,F), consistent with the effect of *dlp* null clones (Franch-Marro et al., 2005; Han et al., 2005). *dlp* RNAi even abolished Dlp detection in Rab5SN-expressing cells (Fig. 7H'), which normally have much higher levels of Dlp on their surface (Fig. 6A). Despite the absence of detectable Dlp, blocking Rab5-dependent internalization still caused accumulation of extracellular Wg throughout the wing pouch within 5 hours 30 minutes (Fig.

7G,H). These data show that Dlp is not needed for the spread of Wg if Rab5-dependent internalization is prevented. Other HSPGs might then ensure the cell-to-cell transfer of Wg.

We wondered whether Dlp might normally promote the spreading of extracellular Wg by antagonizing Wg internalization. Dlp overexpression strongly elevates the level of extracellular Wg,

especially on lateral membranes (Fig. 2E). To ask whether accumulated Wg was accessible to the endocytic pathway, we compared co-localization of Wg with endosomal markers in Dlp-overexpressing versus normal tissue (Fig. 7I-L). Although Dlp overexpression increased the range over which Wg-positive endosomes were found, their frequency was not significantly higher



**Fig. 7. Removal of Dlp by PI-PLC treatment or by RNAi does not affect Wg accumulation on Rab5SN-expressing cells.** (A-D) Imaginal discs after 5 hours 30 minutes Rab5SN expression in the dorsal compartment (dorsal Rab5SN-expressing tissue indicated by brackets). Images are projections of 1- $\mu$ m-spaced confocal sections over 20  $\mu$ m. (A) Disc mock-incubated for 1 hour, stained for Dlp. (B) Disc mock-incubated for 1 hour, stained for Wg. (C) Disc incubated for 1 hour with PI-PLC, stained for Dlp. (D) Disc incubated for 1 hour with PI-PLC, stained for Wg. Wg is not released with Dlp. (E,F) Discs subjected to Dlp RNAi for 40 hours in the dorsal compartment (indicated by brackets) stained for Dlp (E) or extracellular Wg (F). Dlp protein is undetectable and extracellular Wg fails to spread in dorsal cells. (G,H) Apical extracellular Wg staining of discs in which Rab5SN has been expressed for 5 hours in the dorsal compartment (indicated by brackets), either in the presence (G) or absence (H) of Dlp protein. (H') shows depletion of Dlp by RNAi in the dorsal compartment of a disc also expressing Rab5SN. (I-L) Disc ubiquitously expressing YFP-Rab7, overexpressing Dlp in the dorsal compartment (indicated by bracket) and stained for Wg. A projection of three sections 3  $\mu$ m below the apical membrane is shown. (I) Wg imaged with high gain, emphasizing plasma membrane recruitment. (J) Wg imaged with low gain, emphasizing punctate endosomal staining. (K) YFP-Rab7-labeled endosomes. (L) Overlay of Wg and YFP-Rab7.



than wild type in cells near the source ( $1.3 \pm 0.3$ -fold within the first four cell rows,  $n=3$ ). This suggests that much of the laterally accumulating Wg on Dlp-overexpressing cells is inaccessible to internalization.

## DISCUSSION

The mechanisms that promote and inhibit the spread of morphogens play important roles in pattern formation and have been the subject of intense study. Although planar transcytosis (González-Gaitán et al., 1994) has been proposed to explain Wg spread (Bejsovec and Wieschaus, 1995), other data suggest that, at least in imaginal discs, Wg travels extracellularly. Loss of Dynamin activity in cell clones within receiving tissue causes extracellular Wg accumulation (Strigini and Cohen, 2000), suggesting that its range of movement is restricted by a Dynamin-dependent mechanism. Other studies have suggested roles for both degradation and recycling in controlling the spread of Wg (Dubois et al., 2001; Pfeiffer et al., 2002). In this study, we have used dominant negative Rab GTPases to perturb specific branches of the endocytic pathway and address these questions.

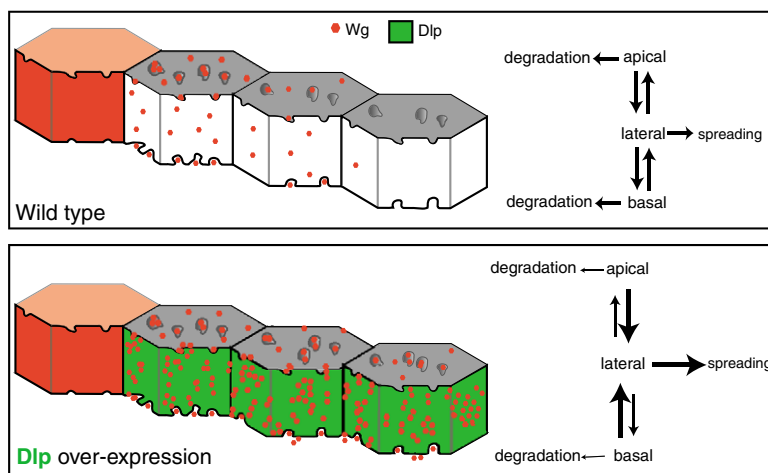
Our data suggest that the spread of Wg is controlled, like that of zebrafish FGF-8, by restrictive clearance (Scholpp and Brand, 2004). Preventing Rab5-dependent internalization increases the spread of Wg through disc tissue. While this may also elevate extracellular Wg levels by indirect mechanisms (for example by modulating extracellular Wg proteolysis or release from disc tissue), we favor the possibility that the gradient is shaped by internalization of Wg itself, for several reasons. First, Wg is actually found in Rab5- and Rab7-positive endosomes. Second, treating living discs with protease inhibitors does not cause Wg to accumulate (E.M and S.E, unpublished). Finally, differential centrifugation experiments suggest that only about 6% of Wg is not membrane-associated (Panáková et al., 2005).

Some ligands signal from endocytic compartments after internalization (Bivona and Philips, 2003; González-Gaitán, 2003; Miaczynska et al., 2004). Therefore, we initially wondered whether the high levels of Wg accumulating after Rab5SN expression could increase signal transduction. While Rab5SN-expressing cells both accumulate Armadillo and reduce Senseless expression (not shown), we did not further investigate whether internalization of Wg is required for signaling because of the striking and unexpected transcriptional changes caused by blocking Rab5 activity. For example, transcription of both *Fz2* and

*Dlp* increases and that of *Arrow* plummets within a few hours of initiating Rab5SN expression – any of these changes by themselves could alter Wg signaling. Although we do not yet understand this phenomenon, one might imagine that coupling transcriptional regulation of endocytic receptors to their actual endocytosis and/or degradation would be an effective homeostatic mechanism.

Studies in tissue culture cells have shown that inhibiting Rab7-dependent lysosomal degradation can increase recycling of some proteins to the cell surface (Edinger et al., 2003). In imaginal discs, we find that Rab7TN expression increases the abundance of Rab11 recycling endosomes, and that inhibiting recycling via Rab11SN enlarges the Rab7-positive degradative compartment. Thus, the recycling and degradative pathways may compete for some cargo in discs as they do in cultured cells. This raises the possibility that changing the balance of degradation and recycling might affect the pool of extracellular Wingless available for spreading. Indeed, Wg appears to be recycled in embryos (Pfeiffer et al., 2002), and inhibiting lysosomal degradation in the embryonic ectoderm extends its range (Dubois et al., 2001). However, our data suggest that the increase in the range of Wg caused by inhibiting degradation (at least in imaginal discs) is not the result of increased recycling and extracellular spread. Although Wg protein is detected in endosomes over a broader range in imaginal disc tissue expressing Rab7TN, there is no increase in the range of extracellular Wg. Furthermore, neither extracellular nor intracellular Wg distribution is affected by inhibiting the Rab4- or Rab11-dependent recycling pathways. Inhibiting degradation probably extends the apparent range of Wg by stabilizing internalized protein, raising its levels above the threshold of detectability in more distant cells.

The idea that apical-basal polarity of epithelial cells might play a role in regulating morphogen trafficking has been suggested by the observation that *wg* mRNA is enriched apically in the embryonic ectoderm. Changing mRNA localization alters the distribution of Wg protein in receiving tissue (Simmonds et al., 2001), raising the intriguing possibility that Wg might be trafficked differently depending on whether it is secreted apically or basal-laterally. In support of this idea, our data show that Wingless is internalized specifically from the apical and basal (but not lateral) surfaces of the disc epithelium. Indeed, the distribution of Rab5- and Rab7-positive endosomes in general suggests that the apical and basal surfaces are more endocytically active than other regions. The apical and basal internalization mechanisms may be distinct; the known receptors for



**Fig. 8. A model for Wingless gradient formation.**

Cartoon of Wg trafficking through wild-type tissue (upper panel) and Dlp overexpressing tissue (lower panel). Wg is released from producing cells (red) and moves into receiving tissue, where it is endocytosed from apical and basal surfaces. Dlp overexpression (green in lower panel) biases Wg localization to the lateral surface, where it can diffuse without being endocytosed. This causes both elevation of Wg protein levels and extension of its spreading range (illustrated in flow charts). This model presupposes that Wg moves freely between apical, lateral and basal surfaces. If apical junctions establish a fence preventing diffusion of membrane proteins between apical and basal-lateral domains, Wg movement would have to occur via apical/basal-lateral transcytosis.

Wingless, Fz2 and Arrow are internalized mainly from the apical surface (despite their steady-state basal-lateral localization), suggesting that basal Wingless endocytosis must be independent of these proteins. One possibility is that membrane association of Wg via Palmitate is sufficient to allow its endocytosis – perhaps by mechanisms similar to those used by GPI-linked proteins (Sabharanjak et al., 2002). Alternatively, Wg bound to Lipoprotein particles (Panáková et al., 2005) might be internalized via Lipoprotein receptors.

We were surprised to observe that the Wg accumulating on the basal side of disc epithelial cells after Rab5SN expression did not spread onto the lateral membrane, as no barrier to diffusion has been identified between these domains. Three possible explanations occur to us. (1) There may indeed be a ‘fence’ separating the lateral and basal sides of disc epithelial cells. Neurons have a fence that prevents diffusion of lipid and lipid-linked proteins between the axon and the cell body, although it does not resemble a classical intercellular junction (Kobayashi et al., 1992; Nakada et al., 2003). (2) It may be that the receptor(s) that normally internalize Wg basally are linked to cytoskeletal components, or interact with extracellular matrix (ECM), and are not free to diffuse. If these Wg receptors were of sufficiently high affinity, they might trap Wg before it could move laterally. (3) Perhaps Wg itself interacts efficiently with basal ECM components.

While it seems that endocytosis restricts the spread of Wg on the apical and basal surfaces, it is not yet clear which receptors might be responsible. A simple model would predict that removing such a receptor should produce phenotypes similar to Rab5SN expression, i.e. increased and more extensive extracellular Wg, and less Wg in endosomes. Conversely, overexpression might be expected to compress the range of Wg distribution and decrease extracellular Wg. None of the known receptors behaves in this way. Previous studies showed that at least a fraction of Wg is still internalized in the absence of both Fz1 and Fz2 (Baeg et al., 2004). Furthermore, overexpression of Fz2 causes extracellular Wg accumulation over longer distances (Cadigan et al., 1998; Baeg et al., 2004). We have shown here that loss of Arrow actually increases the amount of Wg present in Rab5- and Rab7-positive endosomes – more consistent with a role in Wg degradation after endocytosis. The complexity of these observations may reflect different mechanisms of Wg endocytosis on the apical and basal sides of the cell – understanding both these pathways and their interplay will be necessary to understanding how the Wg gradient forms.

While internalization limits the range of Wg accumulation, the glypican Dlp extends it. It has been proposed that Dlp allows Wg to interact with disc cells, increasing local Wg concentration and restricting its diffusion to the epithelium (Han et al., 2005). Our data, however, show that disc cells can accumulate high levels of Wg on their surface in the absence of Dlp as long as Rab5-dependent internalization is blocked. This observation is not consistent with a model in which Dlp traps Wg on the cell surface or helps it transfer from cell to cell. Instead, it suggests that Dlp normally stabilizes Wg at the cell surface by antagonizing the effects of Rab5-dependent internalization (see model in Fig. 8). While Wg is normally internalized from the apical- and basal-most surfaces of disc cells, Dlp overexpression recruits Wg to the lateral cell surface. This raises the possibility that Dlp stabilizes Wg and increases its range by changing its subcellular localization to protect it from endocytosis. Polarizing the distribution of morphogens within an epithelium may have a key regulatory role in the trafficking events leading to gradient formation.

### Note added in proof

A recent paper (Piddini et al., 2005) from the Vincent Laboratory has demonstrated that Arrow promotes Wingless degradation after internalization by Frizzled 2.

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### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/2/307/DC1>

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