Internalization is required for proper Wingless signaling in Drosophila melanogaster

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The Wnt–Wingless (Wg) pathway regulates development through precisely controlled signaling. In this study, we show that intracellular trafficking regulates Wg signaling levels. In Drosophila melanogaster cells stimulated with Wg media, dynamin or Rab5 knockdown causes reduced Super8XTOPflash activity, suggesting that internalization and endosomal transport facilitate Wg signaling. In the wing, impaired dynamin function reduces Wg transcription. However, when Wg production is unaffected, extracellular Wg levels are increased. Despite this, target gene expression is reduced, indicating that internalization is also required for efficient Wg signaling in vivo. When endosomal transport is impaired, Wg signaling is similarly reduced. Conversely, the expression of Wg targets is enhanced by increased transport to endosomes or decreased hepatocyte growth factor–regulated tyrosine kinase substrate–mediated transport from endosomes. This increased signaling correlates with greater colocalized Wg, Arrow, and Dishevelled on endosomes. As these data indicate that endosomal transport promotes Wg signaling, our findings suggest that the regulation of endocytosis is a novel mechanism through which Wg signaling levels are determined.

Introduction

During development, precisely regulated signaling pathways instruct cells to adopt particular fates. Wnt signaling mediates many developmental decisions (Wodarz and Nusse, 1998). Highlighting its importance, the misregulation of Wnt signaling causes improper fate specification, tumor formation, and early lethality (Cadigan and Nusse, 1997). Although proper Wnt signaling is essential, the mechanisms that control ligand distribution and signaling levels are not fully understood.

One process proposed to affect Wnt signaling is intracellular transport (Fig. 1 A; for review see Seto and Bellen, 2004). In endocytosis, membrane proteins are recruited to small plasma membrane invaginations. These forming endocytic vesicles are cleaved from membranes via the function of dynamin (Hinshaw, 2000), a protein encoded by shibire (shi) in Drosophila melanogaster. These vesicles then undergo Rab5-mediated fusion with the early endosome (Gorvel et al., 1991; Bucci et al., 1992). There, internalized proteins are sorted and redistributed within the cell. Proteins slated for degradation are sorted by hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs) into the inner vesicles of the multivesicular body (MVB; Lloyd et al., 2002). When MVBs fuse with lysosomes, these internalized proteins are degraded.

Work in other signaling pathways has suggested that by regulating the level and distribution of ligand, endocytosis can affect the induction of signaling (Seto et al., 2002; for review see Seto and Bellen, 2004). Indeed, studies examining the relationship between endocytosis and Wingless (Wg) signaling suggest effects on Wg levels and spread. In the wing, loss of dynamin eliminates extracellular Wg (Wg(ex)) in 50% of samples (Strigini and Cohen, 2000), suggesting that dynamin may mediate Wg secretion. However, other studies indicate that dynamin is not involved in forming secretory vesicles from the Golgi (van der Bliek et al., 1993; Altschuler et al., 1998; Kasai et al., 1999). Thus, the effect of dynamin on Wg production remains unclear.

After Wg is secreted, it must travel to reach target cells. The role of endocytosis in Wg spread is heavily debated, as Wg may spread by either diffusion or intracellular transport. Supporting extracellular spread, dynamin-mediated internalization is not required for Wg spread in the wing (Strigini and Cohen, 2000). The efficiency of diffusion has been questioned, however, because Wg interacts with proteins in the extracellular matrix (Blair, 2005). Alternatively, Wg may spread through vesicle intermediates. GFP-tagged Wg can be internalized and recycled to
the cell surface in embryonic cells (Pfeiffer et al., 2002). Visualization of membrane phospholipids also suggests that Wg may spread via vesicular structures in the wing (Greco et al., 2001). Given that evidence supporting both extracellular and intracellular transport exists, the extent to which endocytosis affects Wg spread is controversial. Thus, although previous studies suggest that endocytosis may regulate Wg levels and spread (for review see Seto and Bellen, 2004), many questions remain.

Aside from affecting ligand levels and distribution, endocytosis may also regulate signal transduction (Di Fiore and De Camilli, 2001; Miaczynska et al., 2004). Determining whether endocytosis directly affects Wg signal transduction has been complicated, however, by difficulties in distinguishing effects on protein levels from signaling levels. In shi mutant embryos, Armadillo (Arm) staining is reduced but not eliminated (Bejsovec and Wieschaus, 1995). The presence of Arm indicates that Wg signaling can occur at the cell surface; however, it is unclear whether the reduction is caused by altered Wg spread or impaired signal transduction. Although this is consistent with the facilitation of Wg signaling by dynamin, it has been suggested that signaling is negatively regulated by Rab5 (DasGupta et al., 2005), raising doubt as to the necessity of endocytosis in Wg signaling. Given these contradictory results, the effect of endocytosis on Wg signaling is unclear.

In this study, we use genetic tools to alter vesicle transport and study the effect on Wg production, transport, degradation, and signaling. In Drosophila cells treated with Wg media, the knockdown of dynamin (Shi) causes a significant reduction in luciferase ratio (\(*, P < 0.05\)). (C) Relative Super8XTOPFlash/RL ratios 8 d after transfection with Super8XTOPFlash, pCMV-RL, and dsRNA against EGFP, shi, arm, and ck1a. Wg media was added 1 d before cell lysis to induce signaling. Knockdown of dynamin (Shi) by either R51 or R53 causes a significant reduction in luciferase ratio compared with controls (\(*, P < 0.01\)). (D) Relative Super8XTOPFlash/RL ratios 4 d after transfection with Super8XTOPFlash, pCMV-RL, pMK33-Wg, and dsRNA against EGFP and Rab5. The weak knockdown of Rab5 observed at this time point was sufficient to cause a statistically significant reduction in luciferase ratio (\(*, P < 0.05\)). (E) Relative Super8XTOPFlash/RL ratios 8 d after transfection with Super8XTOPFlash, pMK33-Wg, dsRNA against EGFP and Rab5, and various RL transfection control vectors. Transfection with pCMV-RL results in the strong knockdown of Rab5 and a dramatic reduction in luciferase ratio (\(*, P < 0.01\)). Transfection with polIII-RL, however, causes an increase in luciferase ratio that is consistent with other data (\(*, P < 0.01\); DasGupta et al., 2005). Transfection with tk-RL and s-188-cc-RL both result in modest but statistically significant reductions in luciferase ratios (\(*, P < 0.05\)). Error bars represent SEM.
Results

Impaired endocytosis affects Wg signaling in cell culture

To determine endocytic effects on Wg signaling, a cell-based Wg assay was used. *Drosophila* S2R+ cells were transfected with a cytomegalovirus (CMV)-driven Renilla luciferase (RL) transfection control and Super8XTOPFlash (TOPFlash), a Wg reporter driving the expression of firefly luciferase. In response to Wg, the TOPFlash/RL ratio increases, serving as a quantitative measure of Wg signaling. Additionally, cells were transfected with double-stranded RNA (dsRNA) to determine the effect of particular genes on signaling. Knockdown of Arm, a mediator of Wg signaling, profoundly reduces TOPFlash/RL (Fig. 1, B and C; and Table S2, available at http://www.jcb.org/cgi/content/full/jcb.200510123/DC1). Conversely, knockdown of casein kinase 1α (ck1α), a negative regulator of signaling, strongly increases TOPFlash/RL (Fig. 1, B and C; and Table S2 B). Second, we transfected cells with a Wg DNA construct, as performed by DasGupta et al. (2005), in lieu of adding Wg media. We initially examined cells 4 d after transfection as performed by DasGupta et al. (2005), indicating that dynamin promotes Wg signaling.

Similarly, the effects of endosomal transport were evaluated by transfection with dsRNA against the Rab5 coding region (R51; DasGupta et al., 2005). These cells showed a 93% decrease in luciferase ratio (Fig. 1 C and Table S2 B). This is surprising because a recent study argues that R51 transfection increases Wg signaling (DasGupta et al., 2005). To understand this discrepancy, we first reduced Rab5 using a dsRNA against the highly specific 3′ untranslated region (R51′). Similar to R51, R51′-treated cells show an 82% decrease in TOPFlash/RL (Fig. 1 C and Table S2 B). Second, we transfected cells with a Wg DNA construct, as performed by DasGupta et al. (2005), in lieu of adding Wg media. We initially examined cells 4 d after transfection as performed by DasGupta et al. (2005). Although Rab5 was still present at this time point (Fig. 1 D), a 38% reduction in TOPFlash/RL was observed (Fig. 1 D and Table S2 C). At 8 d after transfection, we observe strong knockdown and an 82% reduction in luciferase ratio, which is consistent with our results (Fig. 1 E and Table S2 D). Finally, we examined TOPFlash/RL ratios upon transfection with different RL control vectors (Fig. 1 E and Table S2 D). Although cells transfected with the polIII-RL transfection control used by DasGupta et al. (2005) show a 68% increase in luciferase ratio, transfection with tk-RL and s-188-cc-RL show 34 and 25% reductions in luciferase ratio, respectively. These varied TOPFlash/RL ratios indicate that transfection control vectors can produce different RL levels that dramatically impact the quantification of Wg signaling. However, given that R51 transfections with three out of four RL vectors show reduced luciferase ratios, our data suggest that impaired Rab5-mediated endosomal fusion hinders Wg signaling.

Assessing Wg signaling activity in vivo

To determine the relevance of our cell culture data, we studied the effects of endocytosis on signaling in the wing. Wg forms a morphogen gradient in the larval wing that regulates proliferation and cell fate specification (Zecca et al., 1996; Neumann and Cohen, 1997). Wg is secreted at the dorsal–ventral (DV) boundary of the wing disc and is detected at high levels spanning approximately three cell widths (Baker, 1988; Couso et al., 1993; Williams et al., 1993). Spots of Wg are also present in the wing pouch, decreasing with distance from the DV boundary. As a morphogen, Wg can induce different target genes depending on signaling levels (Fig. 2 A). High levels of signaling induce Senseless (Sens) in cells bordering the DV boundary (Parker et al., 2002; Lin et al., 2003). Low levels of signaling are sufficient to induce Distal-less (Dll) broadly across the wing pouch (Diaz-Benjumea and Cohen, 1995; Zecca et al., 1996; Neumann and Cohen, 1997). Both Sens and Dll function in wing margin bristle development (Gorfinikel et al., 1997; Nolo et al., 2000). Formation of a normal-sized wing is also dependent on Wg signaling, as Wg mutants lack wings (Sharma and Chopra, 1976). Thus, by examining the expression of Wg targets and adult wing morphology, we can assess Wg signaling levels.

Dynamin regulates Wg protein levels

To study the effect of internalization on signaling, we expressed dominant-negative *shi* (*shi*ΔN) to impair internalization from the cell surface (Moline et al., 1999). Because it has been suggested that dynamin mediates Wg secretion (Strigini and Cohen, 2000), we used two Gal4 drivers to analyze Wg distribution and signaling. *C96-Gal4* induces expression at and near the DV boundary (Fig. 2 B; Gustafson and Boulianne, 1996), thereby permitting analysis of Wg transcription and secretion. Conversely, *C5-Gal4* (Yeh et al., 1995) induces expression throughout the wing pouch except for cells at the DV boundary (Fig. 2 C). Because this does not include Wg-expressing cells, *C5-Gal4* allows analysis of Wg spread and degradation independent of Wg production. By combining data from these drivers, we can study changes in Wg production, spread, degradation, and signaling.

When *shi*ΔN was overexpressed at the DV boundary, Wg distribution is narrow compared with controls (Fig. 2 D and E), which is indicative of altered Wg transcription or secretion. In situ show less Wg RNA (Fig. 2 I), indicating that dynamin facilitates Wg transcription likely through its regulation of Notch signaling (Diaz-Benjumea and Cohen, 1995; Rulifson and Blair, 1995; Seugnet et al., 1997). However, as shown in Fig. 2 M, *C96-Gal4/UAS-shi*ΔN discs exhibit elevated Wg(ex) levels compared with controls. Thus, our data suggest that when dynamin function is blocked, Wg transcription is reduced, but Wg is secreted and accumulates extracellularly.

To investigate the effect of dynamin on Wg spread, we expressed *shi*ΔN using *C5-Gal4*. These discs show a dramatically widened Wg distribution compared with controls (Fig. 2, F and G). Consistent with impaired internalization, this protein can be detected extracellularly (Fig. 2 O). Because Wg expression is similar to controls (Fig. 2 K), the enhanced Wg(ex) likely results from reduced Wg degradation when *shi* function is inhibited. Notably, the normal Wg expression also indicates that Wg can...
spread from the DV boundary in a dynamin-independent manner. Thus, dynamin regulates Wg levels through transcription and degradation but does not appear to be required for Wg secretion or spread.

**Wg signaling is negatively regulated by dynamin**

To determine whether dynamin affects signaling, Wg target gene expression was examined. Although both C96-Gal4 and C5-Gal4 overexpression of shiDN show enhanced levels of Wg(ex), we find that Sens expression is nearly absent (Fig. 2, P–S), indicating that dynamin is required to achieve high signaling levels. Furthermore, Dll levels in shiDN cells are decreased compared with cells outside the wing pouch that do not express shiDN (Fig. 2, T–W). Dll expression is similarly reduced in temperature-sensitive shi (shiTS1) mutant clones at the restrictive temperature (not depicted). The progressively weaker effects of dynamin on Sens and Dll are consistent with our understanding of the Wg morphogen gradient and indicate that impaired internalization reduces but does not eliminate Wg signaling. Notably, the reduced protein expression is unlikely to be the result of cell death, as little to no TUNEL-positive columnar cells are observed in the C96-Gal4/UAS-shiDN, C5-Gal4/UAS-shiDN, and shiTS1 discs studied (Fig. S1, B and C; available at http://www.jcb.org/cgi/content/full/jcb.200510123/DC1). Additionally, the differential decrease in Sens and Dll suggests that these reductions do not arise from cell death. Further supporting reduced Wg signaling, C96-Gal4/UAS-shiDN wings show a loss of margin tissue that resembles the wg mutant phenotype (Fig. 2 Y; Baker, 1988; Couso et al., 1994; Diaz-Benjumea and Cohen, 1995). C5-Gal4/UAS-shiDN adult wings are small with altered morphology (Fig. 2 AA), exhibiting bristle loss consistent with decreased Sens expression and Wg signaling (Phillips and Whittle, 1993; Couso et al., 1994). Thus, consistent with our cell culture data, these data indicate that impaired dynamin function reduces Wg signaling even when significantly more Wg(ex) is present. This effect is more obvious for Wg targets requiring high signaling levels, suggesting that Wg(ex) can induce only low signaling levels in the absence of dynamin-mediated internalization.
Endosomal trafficking promotes Wg signaling

After dynamin-mediated internalization, endocytic vesicles undergo Rab5-mediated fusion with the endosome (Gorvel et al., 1991; Bucci et al., 1992). As our cell culture data suggest that the loss of Rab5 reduces Wg signaling, we determined whether endosomal transport affects signaling in vivo by expressing dominant-negative Rab5 (Rab5DN, also called Rab5SN), a constitutively GDP-bound form that inhibits endosomal fusion (Stenmark et al., 1994; Entchev et al., 2000). In C96-Gal4/UAS-Rab5DN discs, Wg staining is more punctate but otherwise similar to controls (Fig. 3 F). Despite this, Sens expression near the DV boundary is eliminated (Fig. 3 H), indicating that high levels of signaling are blocked by impaired endosomal transport. Dll expression is also much reduced compared with levels outside of the wing pouch (Fig. 3 I). The stronger effect on Sens than Dll is similar to shiDN, further indicating that high Wg signaling levels cannot be reached when endocytosis is blocked.

Evaluating cell death, we find TUNEL-positive columnar cells upon the C96-Gal4 expression of Rab5DN (Fig. S1 D). However, this cell death likely causes only minor changes in protein expression as indicated by the large number of Wg-expressing cells present (Fig. 3 G). Additionally, C96-Gal4 overexpression of Rab5DN results in the loss of wing tissue similar to the loss of Wg (Baker, 1988; Couso et al., 1994; Diaz-Benjumea and Cohen, 1995), further suggesting that endosomal transport significantly affects Wg signaling. Similarly, we have analyzed the C5-Gal4 expression of Rab5DN. As shown in Fig. 3 K, Wg distribution is significantly expanded, which was caused, in part, by increased Wg transcription (Fig. 3 L). Despite high Wg levels, Sens expression is absent, and Dll expression is markedly reduced (Fig. 3, M and N). Again, the differential effects on Sens and Dll expression are consistent with impairment of the Wg signaling gradient. C5-Gal4 expression of Rab5DN causes almost a complete loss of wing tissue (Fig. 3 O), as documented for wg mutants (Sharma and Chopra, 1976). Notably, cooverexpression of Arm (Pai et al., 1997), a mediator of Wg signaling, partially restores bristles and wing size (unpublished data). This indicates that although some TUNEL-positive cells are observed in columnar cells expressing Rab5DN (Fig. S1 D), cell death does not account for the observed phenotypes. Together, these data suggest that early endosomal transport facilitates Wg signaling in vivo.

Although Rab5DN is constitutively inactive, wild-type Rab5 (Rab5WT) is subject to the regulation of cellular factors (Somsel Rodman and Wandinger-Ness, 2000). To examine the effect of Rab5WT, we induced clones of the Actin-Gal4 expression of Rab5WT. As shown in Fig. 4 (A–C), Rab5WT overexpression causes no change in Wg, Sens, or Dll expression. Interestingly, it has been reported that C96-Gal4 expression of Rab5WT decreases Sens levels (DasGupta et al., 2005). However, we find that the overexpression of Rab5WT by either C96-Gal4 or
C5-Gal4 causes no change in Wg, Sens, or Dll expression (Fig. 4, D–F; and not depicted). Furthermore, the adult wings are indistinguishable from controls (Fig. 4 G and not depicted). These data indicate that Rab5WT overexpression does not change Wg signaling. Given the different results obtained from Rab5DN and Rab5WT, our data also suggest that endocytic regulators can control Wg signaling by altering endosomal transport. Consistent with this, the overexpression of dominant active Rab5 (Rab5DA, also called Rab5QL) causes an enhancement in signaling that was not observed with either Rab5DN or Rab5WT. In these discs, Sens expression is detected more than two cell diameters from the DV boundary, and Dll expression is enhanced in the Gal4 expression domain (Fig. S2, D–I; available at http://www.jcb.org/cgi/content/full/jcb.200510123/DC1). Consistent with normal Wg signaling, the adult wing is indistinguishable from controls (Fig. 5).

**Traffic to the MVB reduces Wg signaling**

Upon internalization to the endosome, proteins slated for degradation are sorted into MVBs via the function of Hrs (Lloyd et al., 2002; Raiborg et al., 2002). We analyzed wings with altered Hrs function to determine whether trafficking from endosomes to MVBs affects signaling. In hrs mutant clones, Wg distribution is slightly expanded, with much of the protein localized in large puncta (Fig. 5 A; see Fig. 8 C). Wg(ex) staining fails to detect these accumulations (not depicted). Although most Wg is located intracellularly in hrs mutants, Sens is sometimes more broadly expressed within hrs mutant clones than in the internal control (Fig. 5 B). Similarly, some hrs mutant clones show enhanced Dll levels (Fig. 5 C). These changes in expression are most evident in large clones induced early in development. As Hrs is a very stable protein (Lloyd et al., 2002), small clones induced later show minor or no changes in Wg target gene expression, probably as a result of Hrs protein perdurance. Thus, although less Wg(ex) is present than in controls, the impairment of endosome to MVB transport augments Wg signaling.

Our data strongly suggest that internalization and protein localization to the early endosome play a critical role in Wg signaling. We next examined the effect of enhanced MVB transport. The overexpression of Hrs by C96-Gal4 and C5-Gal4 facilitates trafficking through MVBs as demonstrated by enlarged LAMP-positive lysosomes (not depicted). When Hrs is overexpressed at the DV boundary, Wg distribution is disrupted (Fig. 6 B). However, when Hrs is broadly expressed, Wg distribution is slightly widened (Fig. 6 C). Despite differences in Wg levels, both genotypes show a reduction in Sens and Dll (Fig. 6, D–I). Consistent with reduced signaling, C96-Gal4 UAS-hrs wings have a loss of margin tissue (Fig. 6 K), and C5-Gal4 UAS-hrs wings are reduced in size with fewer bristles near the wing margin (not depicted). Further supporting an effect on Wg signaling, the cooverexpression of Wg with Hrs largely suppresses the loss of...
margin tissue (Fig. 6 M). In canonical Wg signaling, Wg associates with Frizzled receptors and Arr coreceptors to phosphorylate the cytoplasmic protein Dsh and activate signaling (Wodarz and Nusse, 1998; Tamai et al., 2000; Wehrli et al., 2000). We find that the coexpression of Frizzled (Fig. 6 O) and myc-tagged Dsh (Fig. 6 Q) are each capable of suppressing the C96-Gal4 UAS-hrs phenotype. These data indicate that Hrs phenotypes arise specifically from changes in Wg signaling rather than other factors (Fig. S1 G). Together, these data suggest that although internalization and early endosomal transport facilitate Wg signaling, progression to the MVB negatively regulates signaling.

**Wg signaling members are localized at early endosomes**

Our data indicate that localization to early endosomes enhances Wg signaling. This is similar to receptor tyrosine kinase signaling, where the formation of endosomal signaling complexes is proposed to facilitate signaling (Lloyd et al., 2002; Miaczynska et al., 2004). To determine whether Wg signaling occurs in a similar manner, we first studied Wg localization. We find that Wg partially colocalizes with the early and late endosome marker FYVE-GFP (not depicted; Wucherpfennig et al., 2003) and the late endosomal protein Rab7-GFP (Fig. 7 A; Bucci et al., 2000). Additionally, electron microscopy was performed on wing discs expressing HRP-tagged Wg protein (Dubois et al., 2001). Based on compartment morphology, HRP activity is localized to small vesicles, endosomes, MVBs, and lysosomes (Fig. 7, B–E). Thus, Wg is internalized and trafficked through intracellular compartments.

We further examined the localization of Arr, the *Drosophila* homologue of LRP5/6, and Dsh, which are two proteins that are necessary for Wg signaling (Klingensmith et al., 1994; Theisen et al., 1994; Wehrli et al., 2000). In controls, small puncta of HA-tagged Arr (ArrHA) sometimes colocalize with...
Wg (Fig. 8 A). ArrHA and Wg also occasionally colocalize with Dsh, which is present at low levels in the cytoplasm as well as in small puncta (Fig. 8 B and not depicted). In hrs mutants, ArrHA and Wg often localize to large puncta that colocalize with the endosome/lysosome marker Benchwarmer (also called Spinster; Fig. 8, C and E; Sweeney and Davis, 2002; Dermaut et al., 2005). Intracellular ArrHA and Wg often also colocalize with accumulations of Dsh (Fig. 8, D and F; and not depicted). Thus, in hrs mutants, enhanced Wg signaling correlates with greater colocalized Wg, Arr, and Dsh on endosomes than in wild-type cells.

Discussion

Our analysis has revealed the surprising finding that intracellular transport affects the efficiency of Wg signaling. In cell culture, knockdown of dynamin, a protein essential for clathrin-mediated internalization, reduces the TOPFlash/RL ratio, which is suggestive of decreased Wg signaling. Similarly, Rab5 knockdown causes reduced TOPFlash/RL ratios under most conditions, suggesting that internalization and endosomal transport are important for Wg signaling. Interestingly, transfection with polIII-RL, a control vector used in a recent screen for modifiers of Wg signaling (DasGupta et al., 2005), produces conflicting results for Rab5 compared with other RL controls, indicating that cell culture–based Wg signaling assays are very sensitive to experimental conditions. Thus, although our cell culture results indicate an endocytic regulation of Wg signaling, in vivo validation is critically important.

In the wing, we found further evidence that Wg signaling levels are highly dependent on intracellular transport. When endocytosis is altered, ligand levels and signaling levels are uncoupled such that high Wg levels do not necessarily enhance signaling. Therefore, we have limited usage of the term morphogen gradient, which could refer to either ligand or signaling levels. We instead describe Wg distribution and signaling readouts. When internalization is inhibited in a domain that does not affect Wg production, we find high levels of Wg(ex), likely as a result of reduced degradation. However, Wg target gene expression is diminished, indicating that impaired internalization decreases Wg signaling in vivo as well as in cell culture. When early endosomal transport is impaired, Sens and Dll expression are also reduced despite abundant Wg levels. In both cases, markers of high signaling levels are especially affected, indicating that intracellular signaling is important to achieve robust Wg signaling levels. The differential decrease also argues that changes in Sens and Dll expression are not merely the result of cell death or global changes in transcription (Piddini et al., 2005). Further supporting this, we find the normal expression of other genes in the wing pouch (unpublished data). Additionally, when endosomal transport is enhanced or when transport from the endosome is impaired, Wg signaling is increased. These data suggest that protein localization to the endosome facilitates Wg signaling. Conversely, increased transport to MVBs decreases the expression of Wg readouts. This causes an adult wing phenotype that can be suppressed by Wg signaling components. Thus, we propose that in addition to low levels of cell surface signaling, intracellular Wg signaling is critical for proper signaling levels (Fig. 9).

Because endocytosis is tightly regulated, intracellular Wg signaling may allow for the rapid modulation of signaling levels. For example, endosomal transport can be regulated merely by changing the GDP/GTP state of Rab5. Our work indicates that impaired endosomal transport by GDP-bound Rab5 reduces Wg signaling, whereas enhanced endosomal fusion by GTP-bound Rab5 increases signaling. Because the GDP/GTP-binding state of Rab5 is controlled posttranslationally by GTPase-activating proteins and guanine nucleotide exchange factors, endocytic regulation likely allows more of a rapid adjustment of signaling than regulatory mechanisms requiring transcription and translation. Furthermore, because endocytic rates vary between
cell types, this regulation may allow signaling to be adjusted in particular parts of the body or cells of a tissue. Thus, regulated endocytosis allows for precise temporal and spatial control of Wg signaling.

Endocytosis is hypothesized to regulate signaling through several mechanisms. For example, lysosomal degradation of internalized active receptor tyrosine kinases serves to attenuate signaling (Lloyd et al., 2002; Seto et al., 2002). However, our data suggest that Wg signaling is enhanced by endocytosis. One theory by which intracellular transport facilitates signaling is that the internalization of ligand–receptor complexes promotes interactions with other signaling members recruited to or already present on endosomes. In MAPK signaling, ERK1 receptors form protein complexes with endosomal MP1 and p14 (Teis et al., 2002), leading to greater activation of signaling. Similarly, TGFβ signaling may be enhanced by receptor internalization to endosomes where the Smad2 anchor protein SARA is enriched (Seto et al., 2002). Although our work and that of others suggests that Wg undergoes receptor-mediated internalization in the wing (Piddini et al., 2005; Marois et al., 2006), these data alone cannot explain the enhanced Wg signaling observed. However, not only are Wg and Arr colocalized in large endosomal accumulations in hrs mutants, but they also colocalize with the cytoplasmic signaling component Dsh. The colocalization of Wg, Arr, and Dsh correlates with the increased expression of Wg readouts. These data suggest that internalization and

Figure 8. Intracellular localization of Wg signaling components. (A) Tub-Gal4/UAS-ArrHA wing discs stained for Wg (red) and the HA tag (green). Arrows point to puncta of Wg colocalization with HA-tagged Arr protein. (B) Tub-Gal4/UAS-ArrHA wing discs stained for the HA tag (red) and Dsh (green). Arrows point to colocalized HA-tagged Arr protein and Dsh. (C) hrsD28; Tub-Gal4/UAS-ArrHA wing discs stained for Wg (red), the HA tag (green), and the endosomal marker Benchwarmer (blue). Arrows point to large puncta of Wg colocalization with HA-tagged Arr protein on endosomes. (D) hrsD28; Tub-Gal4/UAS-ArrHA wing discs stained for the HA tag (red), Dsh (green), and the endosomal marker Benchwarmer (blue). Arrows point to large puncta of colocalized HA-tagged Arr protein and Dsh on endosomes. (E) Quantification of Wg and HA-tagged Arr colocalization in control and hrs mutant backgrounds. The hrsD28; Tub-Gal4/UAS-ArrHA disc (C) shows 6.7 times the amount of colocalized pixels observed in the Tub-Gal4/UAS-ArrHA control (A). (F) Quantification of HA-tagged Arr and Dsh colocalization in control and hrs mutant backgrounds. The hrsD28; Tub-Gal4/UAS-ArrHA disc (D) shows 2.8 times the number of colocalized pixels present in the Tub-Gal4/UAS-ArrHA control (B).

Figure 9. Model of intracellular Wg signaling. Based on the data obtained from altering endocytosis, Wg at the cell surface produces only low levels of Wg signaling in the wing. Wg associates with its receptors and is internalized. When endocytic vesicles fuse with the early endosome, the cytoplasmic domains of the Wg receptors Frizzled and Arr are able to associate with downstream signaling components like Dsh, thereby facilitating Wg signaling. Subsequent endosomal sorting into MVB inner vesicles sequesters the Wg–receptor complex from other signaling components, and the activation of signaling transduction is halted.
endosomal transport may promote Wg signaling by facilitating associations between the Wg–receptor complex and downstream signaling components like Dsh. Interestingly, Dsh is reportedly present on intracellular vesicles, and mutations that impair vesicular localization do disrupt canonical Wg signaling (for review see Seto and Bellen, 2004).

Axin, a protein that inhibits Wg signaling by down-regulating Arm levels (Hamada et al., 1999), has also been shown to colocalize with Dsh on intracellular vesicles (Fagotto et al., 1999). Upon Wg signaling, Axin relocates from intracellular puncta to the plasma membrane (Cliffe et al., 2003). This correlates with Arm stabilization and increased Wg signaling. Because Axin associates with Dsh and the cytoplasmic tail of Arr (for review see Seto and Bellen, 2004), we propose that internalized Wg forms an endosomal signaling complex that may relocalize Axin, thereby stabilizing Arm and facilitating signaling.

Materials and methods

Cell culture transfections

*Drosophila* S2R+ cells express all of the signaling components necessary to respond to exogenously added Wg (Tanagawa et al., 1998), making them well suited to study Wg signaling. S2R+ cells (a gift from R. Beachy, Johns Hopkins University School of Medicine, Baltimore, MD) were maintained in Schneider's Media (Invitrogen) with 10% heat-inactivated FBS. 125 μg dsRNA, and 2 and 25 ng pRL-CMV in 1.275 mL were sequentially combined with 20 μg containing or lacking Wg protein (see next section) was added 7 d after growth media. Knockdown was assessed by Western blotting at multiple time points. Strong knockdown of dynamin was observed after 8 d. To assess the effect of Rab5 on Wg signaling, Wg-conditioned media was added, and cells were lysed as described in protocol 1. To induce Wg signaling using Wg DNA rather than Wg-conditioned media, 8 d later, the cells were lysed to assess protein knockdown and to assess luciferase levels using the Dual-Luciferase Reporter Assay System (Promega). For Rab5, however, only limited knockdown was observed using this protocol even after 8 d. To test the effect of Rab5, an alternative protocol was used. (2) 2.5 μg Super8XTOPFlash or Super8XFOPFlash and 25 ng pRL-CMV (Promega) in 100 μL were sequentially combined with 3.2 μg Effectene Enhancer (QIAGEN), 10 μL Effectene (QIAGEN), and 10 μL S2R+ cells in 1.6 mL of growth media. Knockdown was assessed by Western blotting at multiple time points. Strong knockdown of dynamin was observed after 8 d. To assess the effect of shi on Wg signaling, 1 mL of media containing or lacking Wg protein (see next section) was added 7 d after transfection. 1 d later, the cells were lysed to reconfirm protein knockdown and to assess luciferase levels using the Dual-Luciferase Reporter Assay System (Promega). For Rab5, however, only limited knockdown was observed using this protocol even after 8 d. To test the effect of Rab5, an alternative protocol was used. (2) 2.5 μg Super8XTOPFlash or Super8XFOPFlash and 25 ng pRL-CMV (Promega) in 1.275 mL were sequentially combined with 20 μL Effectene Enhancer, 12.5 μL Effectene, 2.5 μL dsRNA, and 2 × 10^6 S2R+ cells in 2.5 mL of growth media. Knockdown was assessed by Western blotting at multiple time points. Strong knockdown of Rab5 was observed after 8 d. To assess the effect of Rab5 on Wg signaling, Wg-conditioned media was added, and cells were lysed as described in protocol 1. To induce Wg signaling using Wg DNA rather than Wg-conditioned media, the following protocols were used: (3) 1.25 μg Super8XTOPFlash or Super8XFOPFlash, 1.25 μg pMK33-Wg (a gift from N. Perrimon, Harvard Medical School, Boston, MA) or empty vector, and 12.5 ng pRL-CMV in 1.275 mL were sequentially combined with 20 μL Effectene Enhancer, 12.5 μL Effectene, 2.5 μL dsRNA, and 2 × 10^6 S2R+ cells in 2.5 mL of growth media. Knockdown was assessed by Western blotting and luciferase levels. To test the effect of polIII-RL (DasGupta et al., 2005) and s-188-cc-RL (Hu et al., 2003), the following protocol was used: (4) 0.625 μg Super8XTOPFlash or Super8XFOPFlash, 1.25 μg pMK33-Wg or empty vector, and 0.625 μg polIII-RL or s-188-cc-RL in 1.275 mL were sequentially combined with 20 μL Effectene Enhancer, 12.5 μL Effectene, 2.5 μL dsRNA, and 2 × 10^6 S2R+ cells in 2.5 mL of growth media. Knockdown was assessed by Western blotting and luciferase levels. To test the effect of tk-RL (Promega), the following protocol was used: (5) 1.13 μg Super8XTOPFlash or Super8XFOPFlash, 1.25 μg pMK33-Wg or empty vector, and 0.13 μg tk-RL in 1.275 mL were sequentially combined with 20 μL Effectene Enhancer, 12.5 μL Effectene, 2.5 μL dsRNA, and 2 × 10^6 S2R+ cells in 2.5 mL of growth media. By Western blotting and luciferase levels. All luciferase results are presented as the mean Super8XTOPFlash/RL or Super8XFOPFlash/RL and SEM of multiple independent trials relative to the EGFP control (Table S2). Significance was based on a two-tailed t test.

**Wg media**

To obtain media containing and lacking Wg protein, S2 Tub-Wg cells (*Drosophila Genomics Resource Center*) and S2 cells were grown in M3 Media (Sigma-Aldrich) with 1 g/L of yeast extract, 2.3 g/L lactobacitone, and 10% heat-inactivated FBS. 125 μg/ml hygromycin (Sigma-Aldrich) was added to the S2 Tub-Wg media. Cells were pelleted by centrifugation. Media was used immediately or stored at −80°C. The presence of Wg protein was confirmed by Western blotting.

**Western blot**

Cells were washed with PBS and lysed in 1× Passive Lysis Buffer (Dual-Luciferase Assay; Promega) or radioimmunoprecipitation assay lysis buffer (0.150 M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.05 M Tris, pH 8) supplemented with protease inhibitor cocktail (Complete). Proteins were quantified by Bradford assay. Blots were probed as described previously (Schulze et al., 1993) using the following antibodies: mouse antidyamin (1:2,000; BD Biosciences), mouse anti-actin (1:5,000; MP Biomedical), mouse anti-Arm (1:500; R& D Systems), mouse anti-Wg 4D4 (1:2,000; Brook and Cohen, 1996), and rabbit anti-Rab5 (1:500; Entchev et al., 2000). Secondo goat HRP-conjugated anti–mouse and anti–rabbit antibodies were used at 1:2,500 (Jackson ImmunoResearch Laboratories), and bands were visualized by Western lightning chemiluminescence plus reagent (PerkinElmer). Blots were developed in a processor (KODAK; Kodak), scanned with a scanner (ScanMaker 8700; Microtek) and the accompanying ScanWizard Pro software (Microtek), and processed for brightness using Photoshop software (Adobe).

**Drosophila strains**

Crosses were maintained at 21°C unless otherwise stated. Wing discs were equal in size to controls and morphologically normal unless otherwise stated. Other studies used the following genotypes: *w; TM3 UAS-shiDN/TM6B Tb1 C except otherwise stated. Wing discs were processed for brightness using Photoshop software (Adobe). Expression patterns of *C96-Gal4* (Gustafson and Boulianne, 1996) and *C5-Gal4* (Yeh et al., 1995) were determined by crossing to w; UAS-lacZ and staining resultant larvae for β-galactosidase activity. Patterns did not alter with the coexpression of UAS-wg/HRP/TM6 (Dubois et al., 2001). To inhibit dynamin function, the Gal4 drivers were crossed to w; TM3 UAS-shiDN/TM6B Tb1 (Moline et al., 1999). Our analysis of shi expression by C5-Gal4 was performed on discs with relatively normal morphology, as changes in gross morphology were observed in some discs. shi mutant clones were generated by crossing FRT18A shi flies to w; Ubi-GFPnls FRT18A; hsFlp males and heat shocking the progeny for 1 h at 38°C 12–26 h after egg laying. Larvae were raised at 18°C and shifted to 35°C for 7 h immediately before dissection. Female larvae were processed as in conventional antibody staining (see next section) except that dissection and fixation were performed at the restrictive temperature to maintain a blockade in endocytosis. To affect early endosomal fusion, the Gal4 drivers were crossed to UAS-Rab5*+/SMS-TM6 (Entchev et al., 2000), UAS-Rab5*/SMS-TM6 (a gift from M. Gonzalez-Gaitan, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany), and UAS-Rab5* (Entchev et al., 2000). Our analyses of Rab5* and Rab5* were performed on wing discs with relatively normal morphology, as changes in gross morphology were observed in many discs. yw UAS-Arm10*+/y+; UAS-Rab5*/+/; C5-Gal4/+; C96-Gal4/+ (Pai et al., 1997) flies were dissected from pupal cases to examine wing morphology. Wild-type Rab5 overexpression was also analyzed by crossing UAS-Rab5 to yw hsFlp; Actin-cy+<y>; Gal4-UAS-GFP/SMS-TM6 and heat shocking progeny for 1 h at 35°C 12 h after larval development. To generate hrs mitotic clones, yw hsFlp; arm-LacZ FRT40A or yw hsFlp; Ubi-GFP FRT 40A/CyO males were crossed to yw hsFlp; hrs288 FRT 40A/Gla 8c females. Progeny were heat shocked at 38°C for 1 h during early first instar development. Because maternally deposited Hrs is very stable, the phenotypes described in this study may not be evident in small clones induced late in development. The overexpression of Hrs was studied using C96-Gal4 UAS-hrs/TM6, C5-Gal4 UAS-hrs/TM6, and w; SpCy/CyO.
UAS-LampHR (a gift from H. Krämer, University of Texas Southwestern Medical Center at Dallas, Dallas, TX). Genetic interactions were examined using yw; UAS-wg (Wilder and Perrimon, 1995), UAS-zea (a gift from K. Bhat, Emory University School of Medicine, Atlanta, GA), and w; Sp/CyO; UAS-dsMyC (Penton et al., 2002). Wg signaling components were localized using the following stocks: UAS-Myc-2xFYVE-GFP/CyO (Wucherpfennig et al., 2003), UAS-Raβ7/2GFP/TM3 (Entenche et al., 2000), UAS-wg-RFP/TM6 (Dubois et al., 2001), Tub-Ga4/TM6, hts, Tub-Ga4 SM5-TM6, and UAS-ArHA/TM6 (Cull and Mann, 2003).

Immunohistochemistry and in situ hybridization

For conventional antibody staining, wandering third instar larvae were dissected in PBS, fixed in 4% formaldehyde in PBS, and incubated in primary antibody overnight. The following primary antibodies were used: mouse anti-β-tubulin (1:10, Brook and Cohen, 1996), rabbit anti-Dll (1:1,000; Cappel), guinea pig anti-Sens (1:1,000; Nolo et al., 2000), mouse anti-Dll (1:500; a gift from G. Boekhoff-Falk, University of Wisconsin, Madison, WI), rabbit anti-Dll (1:100; Panganiban et al., 1994), mouse anti-HA (1:100; Covance), guinea pig anti-Spinner/Benchwarmer (1:100; Sweeney and Davis, 2002), and rat anti-Dsh CB (1:100; Shimada et al., 2001). Samples were later incubated in fluorescent conjugated secondary antibodies (1:300; Invitrogen and Jackson Immunochemicals). Samples were mounted in Vectashield mounting medium (Vector Laboratories) and were imaged using a confocal microscope (LSM 510; Carl Zeiss Microlmaging, Inc.) and accompanying software. Additional details of image acquisition and processing are shown in Table S3 (available at http://www.jcb.org/cgi/content/full/jcb.200510123/DC1). Control and experimental samples of each figure were taken at identical confocal settings. Single confocal sections of representative samples are shown unless otherwise stated. Extra-cellular protein staining was performed as described previously (Strigini and Cohen, 2000) using tubulin as a negative control. TUNEL labeling was performed as described previously (Wang et al., 1999) except that larvae were dissected in PBS and fixed in 4% formaldehyde in PBS. The TMR red in Situ Cell Death Detection Kit (Roche) was used. Changes in the columnar cell layer were evaluated. As a positive control, y w; P{D/V} TM3 Hs-Hid Sb1 larvae underwent a 1-h heat shock at 38°C 1 d before TUNEL staining (Fig. S1 H). In situ hybridization was performed as described previously (Verstreken et al., 2002) and mounted in 50% glycerol in PBS. Images were acquired with an imaging system (Imager.Z1; Carl Zeiss Microlmaging, Inc.) and accompanying software. Additional details of image acquisition and processing are shown in Table S3 (available at http://www.jcb.org/cgi/content/full/jcb.200510123/DC1). Cell Death Detection Kit (Roche) was used. Changes in the columnar cell layer were evaluated. As a positive control, y w; P{D/V} TM3 Hs-Hid Sb1 larvae underwent a 1-h heat shock at 38°C 1 d before TUNEL staining (Fig. S1 H). In situ hybridization was performed as described previously (Verstreken et al., 2002) and mounted in 50% glycerol in PBS. Images were acquired with an imaging system (Imager.Z1; Carl Zeiss Microlmaging, Inc.) and accompanying software. Additional details of image acquisition and processing are shown in Table S3 (available at http://www.jcb.org/cgi/content/full/jcb.200510123/DC1). images were recolored using Photoshop (Adobe).

Quantification

To determine the extent of wing notching, the intact wing perimeter of each wing was measured using ImageJ software (National Institutes of Health) and the wing pouch was measured using LabelVoxel and TissueStatistics functions. The wing notching was quantified by dividing the respective total estimated wing perimeter. For each genotype, the intact wing perimeter of each wing was measured using ImageJ software (National Institutes of Health) and the wing pouch was measured using LabelVoxel and TissueStatistics functions. The wing notching was quantified by dividing the respective total estimated wing perimeter. For each genotype, images were acquired with an imaging system (Imager.Z1; Carl Zeiss Microlmaging, Inc.) and accompanying software. Additional details of image acquisition and processing are shown in Table S3 (available at http://www.jcb.org/cgi/content/full/jcb.200510123/DC1). Quantitative data from the cell culture Wg signaling assay, including increased Wg signaling, indicating that the fusion protein is functional. We thank Y. Zhou for electron microscopy; S. D’Nardo, P. Verstreken, and J. Vincent for discussions; and the Bloomington Stock Center, the University of Iowa Hybridoma Bank, P. Beachy, K. Bhat, G. Boekhoff-Falk, S. Cohen, M. Gonzalez-Gaitan, R. Nusse, N. Perrimon, and T. Uemura for reagents. H.J. Bellen is supported by the Howard Hughes Medical Institute. This work was supported by a National Institute of General Medical Sciences grant (5R01 GM068949). E. S. Seto is supported by a National Institute of Environmental Health Sciences individual National Research Service Award (5 F 32 EO11725) and is in the Baylor College of M.D./Ph.D. program.

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