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Wnt Signaling Requires Retromer-Dependent Recycling of MIG-14/Wntless in Wnt-Producing Cells

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SUMMARY

Wnt proteins are secreted signaling molecules that play a central role in development and adult tissue homeostasis. We have previously shown that Wnt signaling requires retromer function in Wnt-producing cells. The retromer is a multiprotein complex that mediates endosome-to-Golgi transport of specific sorting receptors. MIG-14/Wls is a conserved transmembrane protein that binds Wnt and is required in Wnt-producing cells for Wnt secretion. Here, we demonstrate that in the absence of retromer function, MIG-14/Wls is degraded in lysosomes and becomes limiting for Wnt signaling. We show that retromer-dependent recycling of MIG-14/Wls is part of a trafficking pathway that retrieves MIG-14/Wls from the plasma membrane. We propose that MIG-14/Wls cycles between the Golgi and the plasma membrane to mediate Wnt secretion. Regulation of this transport pathway may enable Wnt-producing cells to control the range of Wnt signaling in the tissue.

INTRODUCTION

During the development of complex multicellular organisms, uniform fields of cells are organized to form different cell types and anatomical structures. An important role in this process is played by secreted morphogens such as Wnt proteins, which provide positional information to cells in the tissue by forming a concentration gradient (Cadigan, 2002). To induce a precise and reproducible pattern, the shape and range of the Wnt gradient needs to be tightly regulated. Studies on the Wnt protein Wingless in the *Drosophila* wing imaginal disc have shown that the main kinetic parameters that control the Wingless gradient are the rate of Wingless production and the rate of Wingless diffusion and degradation (Kicheva et al., 2007). Although much attention has been focused on how Wnt spreading and degradation is regulated (Baeg et al., 2001; Lin, 2004; Piddini et al., 2005; Strigini and Cohen, 2000), the mechanism of Wnt production and secretion is still poorly understood (Coudreuse and Korswagen, 2007; Hausmann et al., 2007).

Purification and biochemical characterization of secreted Wnt has revealed that Wnt proteins are glycosylated and lipid modified (Takada et al., 2006; Willert et al., 2003). The lipid modifications are most likely attached in the endoplasmic reticulum by the membrane-bound O-acyltransferase Porcupine and are required for secretion and signaling activity (Kadowaki et al., 1996; Takada et al., 2006; Zhai et al., 2004). Several lines of evidence suggest that Wnt is not secreted via the default secretory pathway. First, the lipid modification of Wnt has been reported to function as a sorting signal that targets Wnt to specialized membrane microdomains known as lipid rafts, which may partition Wnt into a specific secretory pathway (Zhai et al., 2004). Second, Wnt localizes to multivesicular bodies and recycling endosomes, which may represent intermediate steps in a specialized secretory route (Pfeiffer et al., 2002; van den Heuvel et al., 1989). Finally, it has recently been shown that Wnt secretion depends on the Wnt binding protein Wntless (Wls; also known as Evenness interrupted [Evi] or Sprinter) (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Wls is a highly conserved multipass transmembrane protein that is specifically required in Wnt-producing cells for Wnt secretion. Based on these properties, it has been proposed that Wls functions as a chaperone or sorting receptor for Wnt (Ching and Nusse, 2006; Coudreuse and Korswagen, 2007; Hausmann et al., 2007).

In the nematode *C. elegans*, the Wnt EGL-20 is produced by a group of cells in the tail and forms a concentration gradient along the anteroposterior body axis (Coudreuse et al., 2006; Whangbo and Kenyon, 1999). Among the targets of EGL-20 are the Q neuroblasts and their descendants, which migrate in opposite directions on the left and right side of the animal (Harris et al., 1996). EGL-20 activates a canonical Wnt/β-catenin pathway in the left Q neuroblast (QL) that induces the expression of the Hox gene *mab-5* and thereby directs the migration of the QL descendants toward the posterior (Salser and Kenyon, 1992). In mutants that disrupt EGL-20 signaling, *mab-5* is not expressed and as a consequence, the QL descendants migrate in the opposite, anterior direction. We have previously shown that an intracellular protein sorting complex called the retromer complex is required for the EGL-20-dependent migration of the QL descendants (Coudreuse et al., 2006). Tissue-specific rescue and mosaic analysis demonstrated that the retromer complex is specifically required in EGL-20-producing cells (Coudreuse et al., 2006; Prasad and Clark, 2006) and knockdown studies

in *Xenopus tropicalis* showed that the function of the retromer complex in Wnt signaling is evolutionarily conserved.

The retromer consists of a core complex of Vps35, Vps26, and Vps29 that mediates cargo recognition and the sorting nexin accessory proteins SNX1 and SNX2, which are required for membrane association (Seaman, 2005). Studies in yeast and mammalian cells have shown that the retromer complex mediates the retrieval of specific sorting receptors such as the carboxypeptidase Y receptor Vps10p and the cation-independent mannose-6-phosphate (CI-MPR) receptor from late endosomes to the trans-Golgi network (Arighi et al., 2004; Haft et al., 2000; Seaman et al., 1998). In addition, the retromer complex functions in the endocytic recycling of the Fet3p-Fet1p iron receptor in yeast (Strochlic et al., 2007) and in the basal-to-apical transcytosis of the polymeric-immunoglobulin receptor-IgA complex in polarized epithelial cells (Verges et al., 2004).

The function of the retromer complex in intracellular protein trafficking suggests that it mediates a specific transport step in Wnt-producing cells that is directly or indirectly required for Wnt signaling. Here, we show that the Wnt binding protein MIG-14/Wls is a target of the retromer complex. In the absence of retromer complex function, MIG-14/Wls is degraded in lysosomes and becomes limiting for Wnt signaling. Our results indicate that the retromer-dependent sorting step is part of a transport pathway that retrieves MIG-14/Wls from the plasma membrane. Regulation of MIG-14/Wls transport and stability may enable Wnt-producing cells to control the rate of Wnt secretion and the range of Wnt signaling in the tissue.

Table 1. Rescue of the EGL-20/Wnt-Dependent Migration of the QL Descendants

	% Wild-Type QL Descendant Migration
Wild-type	100
vps-35(hu68)	0
vps-35(hu68); Phs::mig-14 - HS	0
vps-35(hu68); Phs::mig-14 + HS	27
vps-35(hu68); mig-14::gfp	100
mig-14(mu71)	0
mig-14(ga62)	0
mig-14(mu71); Pegl-20::mig-14::gfp	35
mig-14(ga62); Pegl-20::mig-14::gfp	30
mig-14(mu71); Pric-19::mig-14::gfp	0
mig-14(ga62); Pric-19::mig-14::gfp	0
dpy-23(e840)[a]	6
dpy-23(e840); Pegl-20::dpy-23	65

vps-35(hu68) mutants carrying a *Phs::mig-14* transgene were heat-shocked for 10 min at 33°C at the early L1 stage (+ HS). *vps-35(hu68)* was also combined with the *mig-14::gfp*-expressing transgene *huIs71*. Tissue-specific rescue experiments using the *egl-20* or *ric-19* promoter were as described (Coudreuse et al., 2006). The final positions of the QL descendants were scored using the *mec-7::gfp*-expressing transgenes *muIs32* or *muIs35* or by DIC microscopy. The mean of the results obtained in two to six independent experiments is shown (in each case, n > 60).

[a] Nontransgenic siblings from *dpy-23(e840); Pegl-20::dpy-23*.

RESULTS

mig-14 Is Expressed in Wnt-Producing Cells

The *C. elegans* Wls ortholog MIG-14 has an essential function in Wnt signaling, as is demonstrated by the wide range of Wnt phenotypes that can be observed in *mig-14* mutants (Eisenmann and Kim, 2000; Harris et al., 1996; Thorpe et al., 1997). Like Wls in *Drosophila* and mammalian cells, MIG-14 is specifically required in Wnt-producing cells. This has been demonstrated by mosaic analysis in the early embryo (Thorpe et al., 1997) and is supported by our observation that the EGL-20/Wnt-dependent migration of the QL descendants can be restored in *mig-14* mutants by specific expression of *mig-14* in EGL-20-producing cells (Table 1). To further investigate the function of MIG-14 in Wnt signaling, we determined the spatial and temporal expression pattern of *mig-14* by fusing the *mig-14* promoter and coding sequence to *gfp*. This fusion rescues the *mig-14* null phenotype, demonstrating that the *mig-14::gfp* transgene is expressed correctly and that the MIG-14::GFP fusion protein remains functional (data not shown). At the comma stage, during larval development, and in adult animals, *mig-14* is mainly expressed in the posterior part of the animal (see Figures S1A and S1B in the Supplemental Data available with this article online). We found that the expression of *mig-14* overlaps with the known expression patterns of *C. elegans* Wnt genes. Thus, *mig-14* is expressed in the tail hypodermis (Figure S1C), which expresses the Wnt gene *lin-44* (Herman and Horvitz, 1994); in cells in the anal region that express *egl-20/Wnt* (Whangbo and Kenyon, 1999); and in posterior body wall muscle cells that express *cwn-1/Wnt* (Gleason et al., 2006; Pan et al., 2006). In addition, *mig-14* is strongly expressed in the stomatointestinal muscle, the mesoblast cell M and its descendants, the CAN neurons, the developing vulva, the pharynx, and the pharyngeal intestinal valve (Figures S1A, S1D, and S1E). *mig-14* is also weakly expressed in a small subset of head neurons, the ventral nerve cord, and the seam cells, but is undetectable in the main body hypodermis and the intestine.

Mutation of the Retromer Complex Enhances the Wnt Phenotype of *mig-14*

We have previously shown that EGL-20/Wnt signaling requires retromer function in EGL-20-producing cells (Coudreuse et al., 2006). The shared site of action and similarity in phenotype of *mig-14* and retromer mutants suggests a functional relationship in the regulation of Wnt signaling. To investigate this possibility, we tested whether *mig-14* and retromer mutants genetically interact. The null phenotype of *mig-14* is embryonic lethal, but the hypomorphic alleles *mig-14(mu71)* and *mig-14(ga62)* are viable and show a range of postembryonic Wnt phenotypes (Eisenmann and Kim, 2000; Harris et al., 1996). We found that double mutants of *mig-14(mu71)* or *mig-14(ga62)* with *vps-35(hu68)* are mostly sterile. Double mutants with *vps-29(tm1320)*, which has a much weaker Wnt phenotype than *vps-35(hu68)* (Coudreuse et al., 2006), are however viable and produce sufficient progeny for analysis. As *mig-14(mu71)* and *mig-14(ga62)* single mutants already show a strong defect in the EGL-20/Wnt-dependent migration of the QL descendants (Table 1), we used the polarization of the PLM mechanosensory neurons, which is mediated by multiple, redundantly acting Wnts, as an assay for

Table 2. Genetic Interaction between *mig-14* and *vps-29* in PLM Polarity

	% PLM Polarity Defect
Wild-type	0
vps-29(tm120)	1 ± 2
mig-14(mu71)	0
mig-14(mu71); vps-29(tm120)	29 ± 13
mig-14(ga62)	35 ± 5
mig-14(ga62); vps-29(tm120)	67 ± 9

The polarity of the PLM neuron was scored using the *mec-7::gfp*-expressing transgene *muIs35* as described (Hilliard and Bargmann, 2006). Data are represented as mean ± SD (n > 100).

Wnt signaling (Hilliard and Bargmann, 2006; Prasad and Clark, 2006). In wild-type and most *vps-29(tm1320)* or *mig-14(mu71)* single mutants, the PLM neurons show a normal polarity (Table 2). In *mig-14(mu71); vps-29* double mutants, however, about a third of the animals show either reversal or loss of PLM polarity. We observed a similar synergistic effect on PLM polarity in *mig-14(ga62); vps-29* double mutants. Taken together, these results demonstrate that reduction of retromer function strongly enhances the Wnt phenotype of hypomorphic *mig-14* alleles. These experiments do not distinguish, however, between a function of *mig-14* and of the retromer complex in a shared or in parallel genetic pathways.

Human Wls Colocalizes with the Retromer Complex at the Golgi and Endosomes

Wls localizes to the plasma membrane in *Drosophila* wing imaginal disc cells (Bartscherer et al., 2006) and to the Golgi and endosomes in mammalian Vero cells (Banziger et al., 2006). We found that in *C. elegans*, MIG-14 localizes to the cell periphery as well as to distinct intracellular punctae (Figure 1A). To further investigate the subcellular distribution of Wls and to determine whether Wls and the retromer complex colocalize, we expressed a human Wls-GFP fusion protein in HeLa cells. Wls-GFP localizes to the cell periphery (Figure 1B), indicating that Wls is present at the plasma membrane (although localization to docked secretory vesicles cannot be excluded). Furthermore, Wls-GFP localizes to the endoplasmic reticulum (as indicated by the perinuclear staining), to the Golgi, and to endosomal structures, including EEA1-positive early endosomes (Figures 1B and 1C). In agreement with other studies, we found that the retromer complex localizes to endosomes (Figure 1C) (Arighi et al., 2004; Seaman, 2004). Importantly, staining of endogenous Vps26 in cells that express Wls-GFP showed that Wls and the retromer complex colocalize on endosomes (Figure 1C).

MIG-14/Wls Is Targeted to Lysosomes in Retromer Mutants

One of the principle functions of the retromer complex is the endosome-to-Golgi retrieval of sorting receptors such as Vps10p in yeast and the CI-MPR receptor in mammalian cells (Arighi et al., 2004; Haft et al., 2000; Seaman et al., 1998). In the absence of retromer function, Vps10p and the CI-MPR receptor are transported to lysosomes and degraded. To investigate if MIG-14 is also a target of retromer-dependent sorting, we tested whether the subcellular localization or stability of MIG-14 is affected in retromer mutants. As shown in Figure 2B, there is a striking reduction in MIG-14::GFP protein levels in *vps-35(hu68)* mutants. Western blot analysis showed that MIG-14 protein levels are also reduced in *vps-29(tm1320)* mutants (Figure 2C), but there is a marked difference in the extent of this reduction. We have previously shown that mutation of *vps-35* produces a stronger defect in EGL-20/Wnt signaling than mutation of *vps-29* (Coudreuse et al., 2006). This difference is reflected in the effect on MIG-14 protein levels, indicating that the reduction in MIG-14 correlates with the Wnt phenotype of retromer mutants. To examine if human Wls is also a target of the retromer complex, we knocked down *Vps35* and *Vps26* in human embryonic kidney (HEK293) cells and assayed the effect on endogenous Wls levels. Using a polyclonal antiserum that recognizes human Wls on western blot, we found that knockdown of both *Vps35* and *Vps26* induces a significant reduction in endogenous Wls protein levels (Figure 2D). These results demonstrate that the function of the retromer complex in MIG-14/Wls recycling is evolutionarily conserved.

To investigate why MIG-14/Wls protein levels are reduced, we tested whether MIG-14 is targeted to lysosomes in the absence of retromer function. *mig-14* is expressed in posterior body wall muscle cells, which are relatively large cells that are ideally suited for the imaging of subcellular structures. To visualize lysosomes, we used a fusion of the lysosomal protein LMP-1 with mCherry. As shown in Figure 2E, there was almost no overlap between MIG-14::GFP and LMP-1::mCherry in control animals. In *vps-35(RNAi)* animals, however, most of the MIG-14::GFP protein that remains is localized to LMP-1::mCherry-positive structures. To investigate whether human Wls is also targeted to lysosomes, we performed colocalization experiments with Wls-GFP and the lysosomal marker CD63 in HeLa cells. As shown in Figure 2F, there is a strong increase in colocalization between Wls and CD63 when *Vps35* and *Vps26* are knocked down, demonstrating that in the absence of retromer function, human Wls localizes to lysosomes as well. To test if Wls is degraded in lysosomes, we treated HeLa cells with the V-type ATPase inhibitor Bafilomycin A, which prevents the endosomal acidification that is required for the maturation of lysosomal proteases and blocks transport from late endosomes to lysosomes (van Weert et al., 1995). We found that treatment with Bafilomycin A results in a significant increase in Wls protein levels (Figure 2G). These results are consistent with the hypothesis that in the absence of retromer function, MIG-14/Wls is degraded in lysosomes.

Reduction of MIG-14 Protein Levels Limits Wnt Signaling in Retromer Mutants

The strong reduction in MIG-14 protein levels in retromer mutants suggests that MIG-14 may become limiting for Wnt signaling. To investigate this possibility, we tested whether overexpression of MIG-14 can rescue Wnt signaling in retromer mutants. To overexpress MIG-14, we generated a transgene that expresses *mig-14* under the control of an inducible heat-shock promoter (Stringham et al., 1992). *vps-35(hu68)* animals and *vps-35; hs::mig-14* animals raised at the noninducing temperature show a fully penetrant defect in the EGL-20/Wnt-dependent migration of the QL descendants (Table 1). When *mig-14* expression is induced by a brief heat pulse before EGL-20 signaling commences at the

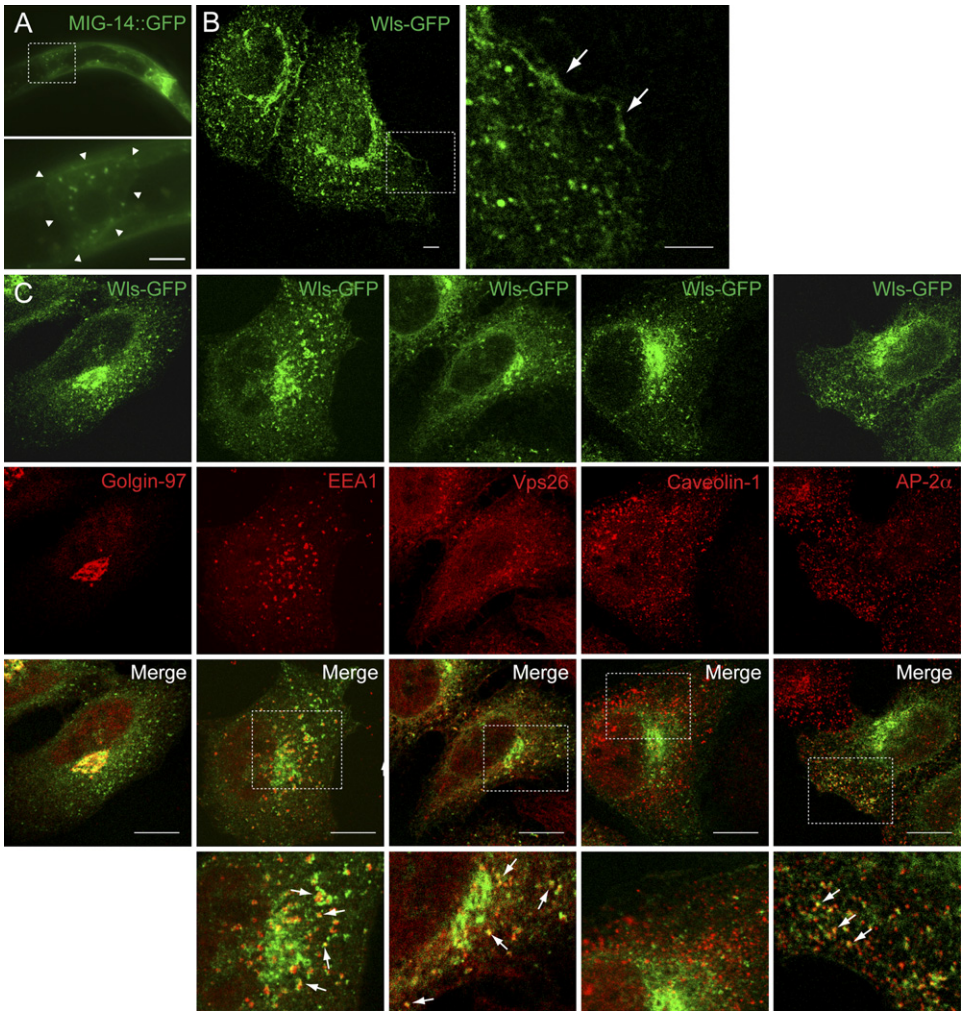

Figure 1. Subcellular Localization of MIG-14 and Wls

(A) Expression of *mig-14::gfp* (*huIs71*) in the posterior half of an early L1 larva (top panel). The boxed area shows the mesoblast cell M, which is magnified in the bottom panel. The outline of the M cell is indicated by arrowheads. Scale bar, 4 μm.

(B) HeLa cells grown on glass coverslips were transfected with Wls-GFP and fixed to determine the intracellular localization of Wls-GFP. Bars, 20 μm. Images are representative of at least three independent experiments. Dashed box is magnified and represented in the right panel. Arrows indicate plasma membrane localization of Wls.

(C) HeLa cells grown on glass coverslips were transfected with Wls-GFP, fixed, and stained for endogenous Golgin-97, EEA1, Vps26, caveolin-1, or AP-2α. Scale bars, 10 μm. Images are representative of at least three independent experiments. Dashed boxes are magnified and represented in the lower panels. White arrows indicate colocalization of Wls with EEA1, Vps26, or AP-2α.

early L1 larval stage, about a third of the animals show a wild-type pattern of QL descendant migration. In addition to heat-shock-promoter-induced overexpression, we found that overexpression of *mig-14* from its own promoter also restores EGL-20/Wnt signaling in *vps-35* mutants (Table 1). These data support the hypothesis that the Wnt phenotype of retromer mutants is a result of the reduction in MIG-14 protein levels.

Human Wls Colocalizes with the AP-2 Adaptin Complex, but Not with Caveolin

The localization of human Wls to early endosomes (Figure 1C) suggests that Wls may be internalized from the plasma membrane. To investigate this possibility, we tested whether Wls colocalizes with the AP-2 adaptin complex or caveolin. AP-2 is a heterotetrameric complex that selects specific transmembrane proteins for clathrin-mediated endocytosis (Bonifacino and Traub, 2003), whereas caveolin is part of an alternative, clathrin-independent endocytosis pathway (Razani and Lisanti, 2001). We found no overlap between endogenous caveolin-1 and Wls-GFP in HeLa cells (Figure 1C). However, staining of endogenous AP-2α showed clear colocalization between Wls and the AP-2 complex at distinct punctae (Figure 1C), indicating that Wls may be internalized through AP-2 and clathrin-mediated endocytosis.

EGL-20/Wnt Signaling Requires AP-2-Mediated Internalization of MIG-14

A function of the AP-2 complex in Wnt signaling is also suggested by the mutant phenotype of the AP-2 μ subunit gene

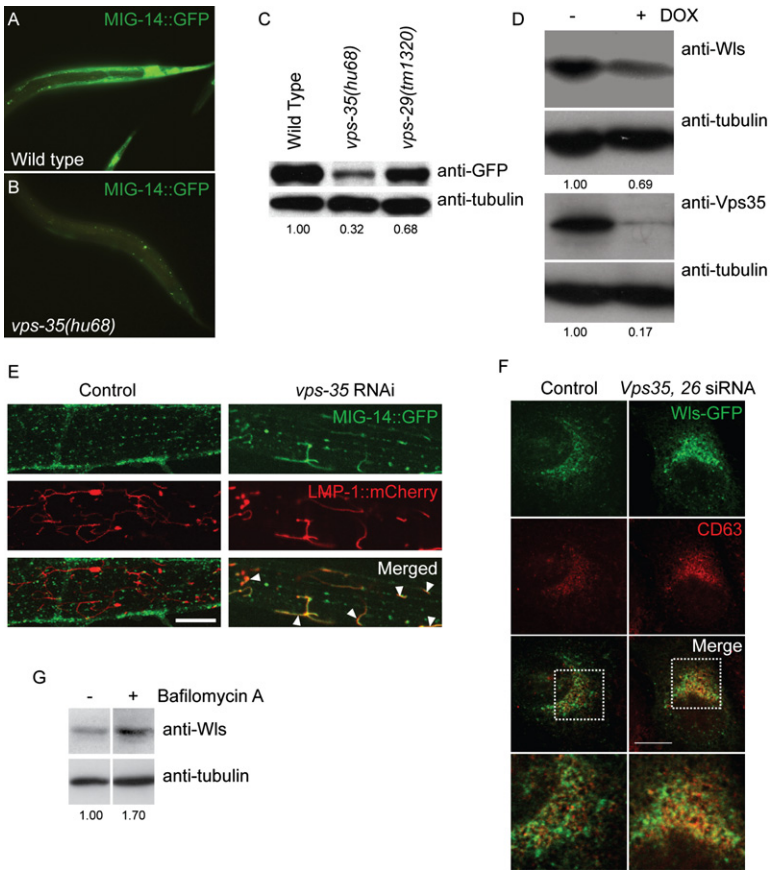

Figure 2. Reduction of MIG-14 and Wls Protein Levels and Lysosomal Targeting in the Absence of Retromer Function

(A and B) MIG-14::GFP (*huIs71*) in wild-type and *vps-35(hu68)*. Images were taken using identical camera settings.

(C) Western blot analysis of MIG-14::GFP (*huIs71*) protein levels in wild-type, *vps-35(hu68)*, and *vps-29(tm1320)* mutants. α-tubulin is used as a loading control. The ratio between MIG-14::GFP and tubulin levels is shown.

(D) Western blot detection of endogenous human Wls in HEK293 cells stably expressing a doxycycline (DOX)-inducible *Vps35* siRNA construct (Coudreuse et al., 2006). HEK293 cells were nonstimulated or stimulated with DOX and additionally transfected with a control siRNA or a combination of *Vps26* siRNA and *Vps35* siRNA for more efficient knockdown of retromer expression. α-tubulin was used as a control for equal loading. The ratio between Wls or Vps35 and tubulin is shown. Representative results of at least three independent experiments are shown.

(E) Subcellular localization of MIG-14::GFP (*huIs72*, green) and the lysosomal marker LMP-1::mCherry (red) in posterior body wall muscle cells of adult animals. Images show a single muscle cell. Scale bar, 10 μm. Arrow heads show colocalization of MIG-14::GFP and LMP-1::mCherry.

(F) HeLa cells grown on glass coverslips were transfected with Wls-GFP and control siRNA or siRNAs directed against *Vps35* and *Vps26*, fixed, and stained for endogenous CD63. Scale bar, 10 μm.

(G) HeLa cells were treated with 100 nM Bafilomycin A for 30 min before cells were lysed to detect endogenous Wls. The ratio between Wls and tubulin is shown. Representative results of two independent experiments are shown.

dpy-23 (G. Garriga, personal communication; see also Pan et al., 2008 [this issue of *Developmental Cell*]). *dpy-23(e840)* mutants are viable and show a highly penetrant defect in the EGL-20/Wnt-dependent migration of the QL descendants (Table 1). To investigate if *dpy-23* function is required in EGL-20-producing cells, we tested whether specific expression of wild-type *dpy-23* from the *egl-20* promoter can restore EGL-20 signaling in *dpy-23(e840)* mutants. As shown in Table 1, expression of *dpy-23* in EGL-20-producing cells is sufficient to rescue the migration of the QL descendants in *dpy-23(e840)* mutants. This demonstrates that AP-2 function is specifically required in EGL-20-producing cells.

To examine whether MIG-14 is a target of AP-2-mediated endocytosis, we tested the effect of RNAi-mediated knockdown of different AP-2 subunits on the subcellular localization of MIG-14::GFP. In control animals, MIG-14 was visible throughout the cell and at the plasma membrane (Figures 3A and 3B). However, in animals that were treated with RNAi against the AP-2 subunits *dpy-23*, *apa-2*, or *aps-2* or the shared AP-2 and AP-1 subunit *apb-1* (Boehm and Bonifacino, 2001), there was a striking accumulation of MIG-14 on the cell membrane, indicating that internalization of MIG-14 is impaired in the absence of a functional AP-2 complex.

Inhibition of AP-2-Mediated Endocytosis Increases MIG-14/Wls Protein Levels

We found that inhibition of AP-2 function results in an increase in MIG-14/Wls protein levels. Thus, MIG-14 protein levels were increased when AP-2 function was disrupted by *apa-2* or *apb-1* RNAi (Figure 3C). Furthermore, human Wls protein levels were increased when AP-2 function was inhibited by siRNA-mediated knockdown of *AP-2α* in HeLa cells (Figure 3D). Inhibition of AP-2 function may increase MIG-14/Wls protein levels by preventing degradation of MIG-14/Wls. Even in a wild-type background, incomplete recycling of MIG-14/Wls by the retromer complex leads to MIG-14/Wls degradation, as shown by the increase in Wls protein levels when lysosomal degradation is inhibited (Figure 2G). We propose that inhibition of AP-2 function prevents MIG-14/Wls degradation because AP-2-mediated internalization occurs before the retromer-dependent recycling of MIG-14/Wls. When internalization is blocked, the balance between de novo synthesis and degradation of MIG-14/Wls is shifted, leading to the observed increase in MIG-14/Wls protein levels. An important prediction of this model is that inhibition of MIG-14 internalization should also increase MIG-14 protein levels in a retromer mutant background. We found that this is indeed the case (Figure 3E). The increase in MIG-14 protein levels is however smaller than in wild-type animals, which is likely the result of incomplete inhibition of *apa-2* and *apb-1* function by RNAi. Taken together, these results suggest that MIG-14/Wls is first internalized through AP-2-mediated endocytosis and is then recycled by the retromer complex.

DISCUSSION

Wnt proteins are lipid-modified signaling molecules that can form long-range concentration gradients to pattern developing

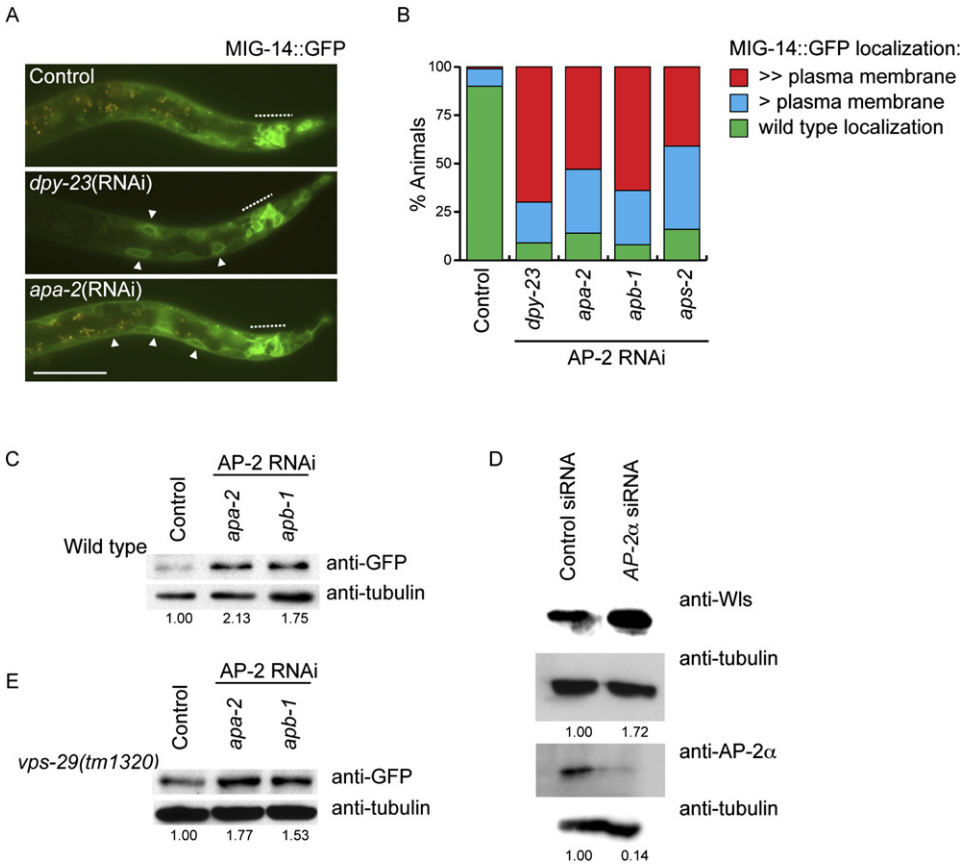

Figure 3. MIG-14 Internalization Requires AP-2-Mediated Endocytosis

(A) MIG-14::GFP (*huIs71*) expression in animals treated with control, *dpy-23*, or *apa-2* RNAi. Arrowheads indicate plasma membrane accumulation of MIG-14::GFP in body wall muscle cells. The dotted line indicates the EGL-20/Wnt-producing cells, which express a higher level of MIG-14::GFP. Scale bar, 20 μm. (B) Quantification of the effect of AP-2 RNAi on the subcellular distribution of MIG-14::GFP. In all cases n > 100, except *apa-2* (n = 58). (C and E) Western blot detection of MIG-14::GFP in wild-type or *vps-29(tm1320)* animals treated with control, *apa-2*, or *apb-1* RNAi. The ratio between MIG-14::GFP and tubulin levels is shown. (D) Western blot detection of endogenous human Wls in HeLa cells transfected with control siRNA or siRNA directed against *AP-2α*. α-tubulin was used as a control for equal loading. The ratio between Wls or AP-2α and tubulin is shown. Representative results of at least three independent experiments are shown.

tissues. A fundamental question is how the hydrophobic Wnt protein is secreted and released at the cell membrane. We have previously shown that Wnt signaling requires retromer function in Wnt-producing cells (Coudreuse et al., 2006). In this study, we show that in the absence of retromer function, the Wnt binding protein MIG-14/Wls is degraded in lysosomes and becomes limiting for Wnt signaling.

The retromer complex mediates the endosome-to-Golgi retrieval of specific cargo proteins such as Vps10p and the CI-MPR receptor (Arighi et al., 2004; Haft et al., 2000; Seaman et al., 1998). In the absence of retromer-dependent sorting, these proteins are degraded in lysosomes. Since MIG-14 is also targeted to lysosomes in retromer mutants, it is likely that the retromer complex functions in a similar endosome-to-Golgi retrieval step for MIG-14/Wls. Cargo recognition by the retromer complex is mediated by the Vps35 subunit, which directly binds to the cargo protein (Arighi et al., 2004; Nothwehr et al., 2000). Studies on the retromer-dependent transport of the CI-MPR receptor have shown that the interaction with cargo depends on a highly conserved W/F-L-M/V tripeptide motif in the target protein (Seaman, 2007). Interestingly, such a motif (F-L-M) is also present at the end of the third intracellular domain of MIG-14/Wls. Although we have not been able to demonstrate an interaction between MIG-14/Wls and the retromer in coimmunoprecipitation experiments, the presence of a conserved retromer sorting motif and the colocalization between human Wls and the retromer on endosomes suggests that MIG-14/Wls may be a direct target of the retromer complex.

Several models have been proposed for the function of MIG-14/Wls in Wnt secretion, including a role of MIG-14/Wls in Wnt folding and maturation and a function of MIG-14/Wls as a specific sorting receptor for Wnt (Ching and Nusse, 2006; Coudreuse and Korswagen, 2007; Hausmann et al., 2007). The subcellular localization of Wls at the Golgi, endosomes, and the plasma membrane and the requirement of MIG-14/Wls transport for Wnt signaling favor the latter possibility. We therefore propose a model in which MIG-14/Wls functions as a sorting receptor that transports Wnt from the Golgi to the plasma membrane for release. To maintain sufficient levels of MIG-14/Wls in the Golgi, MIG-14/Wls is recycled from the plasma membrane through

an AP-2-dependent and retromer complex-dependent pathway. In the absence of the first, AP-2-dependent step, MIG-14/Wls is trapped on the plasma membrane. When the second, retromer-dependent recycling step is disrupted, MIG-14/Wls is targeted to lysosomes and degraded. In both cases, only a limited pool of MIG-14/Wls is available in the Golgi to mediate Wnt transport and secretion. As predicted by this model, overexpression of *mig-14* rescues the Wnt phenotype of retromer mutants. Although this model explains the function of AP-2-mediated endocytosis and retromer-dependent recycling in Wnt signaling, the function of MIG-14/Wls in Wnt transport and release remains to be established. It is not known at what stage in the trafficking cycle Wnt binds to MIG-14/Wls, and it is also not clear how Wnt is released.

An important aspect of the retromer mutant phenotype is that the effect on Wnt signaling increases with distance from the source of Wnt (Coudreuse et al., 2006). We initially hypothesized that the retromer complex is required for the formation of a specific long-range acting pool of Wnt; for example, by enabling the interaction of Wnt with lipoprotein particles (Coudreuse and Korswagen, 2007; Coudreuse et al., 2006). Our results suggest that the effect on the range of Wnt signaling may also be a consequence of the decrease in MIG-14 protein levels. According to our model, the decrease in MIG-14 protein levels will lead to a reduction in the rate of Wnt secretion. Such a reduction in secretion rate will result in a shallower concentration gradient (Kicheva et al., 2007), explaining the observed defect in long-range Wnt signaling. As MIG-14 is decreased, but not eliminated, in the absence of retromer function, Wnt secretion will only be partially affected, indicating why short-range signaling by EGL-20 and other Wnt proteins is not, or only weakly, affected in retromer mutants. In conclusion, our results show that transport and recycling of MIG-14/Wls in Wnt-producing cells is essential for Wnt signaling. Future studies will examine whether regulation of this process provides Wnt-producing cells with a mechanism to control the range of Wnt signaling in the tissue.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

HeLa and HEK293 cells were maintained in RPMI 1640 medium (GIBCO) containing 10% heat-inactivated FCS (GIBCO), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. HeLa cells were transfected with control siRNA (Dharmacon) or siRNA against *AP-2α* (Motley et al., 2003) using Oligofectamine (Invitrogen). Cells were transfected three times with 24 hr intervals between each transfection. HEK293 cells stably transfected with different pools of *Vps35* siRNA constructs cloned into the pTER vector were stimulated with doxycycline to induce *Vps35* siRNA expression (Coudreuse et al., 2006). Forty-eight hours after stimulation, cells were additionally transfected with a combination of siRNA against *Vps26* (He et al., 2005) and siRNA against *Vps35* (CUGUAGGGAUGCUUUGGCU) to induce more efficient knockdown of retromer expression. The samples were analyzed by western blot 48 hr after the last transfection.

Western Blot Analysis

Cells were lysed in Laemmli sample buffer and cell lysates were separated on 10% SDS-PAGE gels, transferred onto PVDF membranes (Bio-Rad), and stained with antibodies against the indicated proteins. To quantify MIG-14::GFP protein levels, synchronized L1 larvae were lysed in 2 volumes of 10 mM Tris (pH 8), 150 mM NaCl, and 0.1% NP-40 containing protease inhibitors (Roche). For AP-2 RNAi experiments, synchronized egg populations were hatched on dsRNA expressing bacteria and grown until the L4 stage before the animals were collected for western blot analysis. Densitometric analysis was performed on scanned images using GeneTools (Syngene) analysis software.

Immunofluorescence

HeLa cells were plated on glass coverslips and transfected with plasmid containing Wls-GFP (Wls-pEGFP, 1 μg) using FuGENE Transfection Reagent (Roche). Twenty four hours after transfection, cells were fixed in 0.1 M Phosphate Buffer containing 4% paraformaldehyde for ten minutes on ice and permeabilized with 0.1% Triton X-100 for five minutes. Thereafter, cells were incubated with 0.5% BSA for 30 min followed by incubation with the indicated primary antibodies and subsequent incubation with a chicken-anti-mouse-Ig or chicken-anti-rabbit-Ig antibody labeled with Alexa 594 (Molecular Probes). Images were recorded with a Bio-Rad Radiance 2100MP confocal and multiphoton system (Zeiss/Bio-Rad).

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and one figure and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/14/1/140/DC1/.

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