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Wnt signalling requires MTM-6 and MTM-9 myotubularin lipid-phosphatase function in Wnt-producing cells

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Wnt proteins are lipid-modified glycoproteins that have important roles in development, adult tissue homeostasis and disease. Secretion of Wnt proteins from producing cells is mediated by the Wnt-binding protein MIG-14/Wls, which binds Wnt in the Golgi network and transports it to the cell surface for release. It has recently been shown that recycling of MIG-14/Wls from the plasma membrane to the *trans*-Golgi network is required for efficient Wnt secretion, but the mechanism of this retrograde transport pathway is still poorly understood. In this study, we report the identification of MTM-6 and MTM-9 as novel regulators of MIG-14/Wls trafficking in *Caenorhabditis elegans*. MTM-6 and MTM-9 are myotubularin lipid phosphatases that function as a complex to dephosphorylate phosphatidylinositol-3-phosphate, a central regulator of endosomal trafficking. We show that mutation of *mtm-6* or *mtm-9* leads to defects in several Wnt-dependent processes and demonstrate that MTM-6 is required in Wnt-producing cells as part of the MIG-14/Wls-recycling pathway. This function is evolutionarily conserved, as the MTM-6 orthologue *DMtm6* is required for Wls stability and Wg secretion in *Drosophila*. We conclude that regulation of endosomal trafficking by the MTM-6/MTM-9 myotubularin complex is required for the retromer-dependent recycling of MIG-14/Wls and Wnt secretion.

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Introduction

During the development of multicellular organisms, cells communicate with each other to coordinate complex processes, such as cell fate determination and morphogenesis. A family of secreted signalling proteins, which is used extensively in development and adult tissue homeostasis, comprises the evolutionarily conserved Wnt proteins (Clevers, 2006). Wnts are characterized by a double lipid modification with palmitic and palmitoleic acid (Willert *et al*, 2003; Takada *et al*, 2006), raising questions on how these hydrophobic proteins are secreted and released from Wnt-producing cells and how they disperse in the tissue. Although much attention has been focused on how the spreading of Wnt is mediated and regulated (for review, see Eaton, 2006; Kornberg and Guha, 2007), the mechanism of Wnt secretion is still poorly understood.

Recently, important insight into the Wnt secretion mechanism has been gained by the identification of two key players in the Wnt secretion pathway. The first is the multi-pass transmembrane protein Wntless (Wls, also known as Evenness interrupted or Sprinter; Banziger *et al*, 2006; Bartscherer *et al*, 2006; Goodman *et al*, 2006). Loss of *wls* in the *Drosophila* wing imaginal disc leads to retention of the Wnt protein Wg in producing cells and a defect in Wg target gene expression, indicating that Wnt secretion is blocked in the absence of Wls function. Wls binds Wnt in co-immunoprecipitation experiments (Banziger *et al*, 2006) and localizes to the Golgi network, endosomes and the plasma membrane (Banziger *et al*, 2006; Bartscherer *et al*, 2006; Belenkaya *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008). Furthermore, Wg accumulates in the Golgi of *wls* mutant cells (Port *et al*, 2008), indicating that Wls may function as a sorting receptor that mediates the transport of Wnt from the Golgi to the cell surface (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Pan *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008). The function of Wls is conserved across phyla, as defects in Wnt signalling are also observed in mutants of mouse Wls (Fu *et al*, 2009) and the *Caenorhabditis elegans* Wls orthologue *mig-14* (Harris *et al*, 1996; Thorpe *et al*, 1997; Banziger *et al*, 2006), which is similarly required in Wnt-producing cells (Thorpe *et al*, 1997; Yang *et al*, 2008).

The second component of the Wnt production machinery is the retromer, a multi-protein complex that mediates retrograde transport of specific cargo proteins from endosomes to the *trans*-Golgi network (Seaman, 2005). A function of the retromer in Wnt production was first noted in *C. elegans*, in which mutants of the cargo-selective subunits of the retromer (encoded by *vps-35*, *vps-26* and *vps-29*) display defects in Wnt signalling (Coudreuse *et al*, 2006; Prasad and Clark, 2006). This function is evolutionarily conserved, as loss of retromer function also disrupts Wnt signalling in *Drosophila*, *Xenopus*

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and mammalian cells (Coudreuse *et al*, 2006; Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Port *et al*, 2008; Kim *et al*, 2009). Importantly, the retromer was found to bind Wls in co-immunoprecipitation experiments, indicating that Wls is a direct cargo of retromer-dependent trafficking (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008).

Further analysis of the function of MIG-14/Wls and the retromer in Wnt-producing cells provided evidence for a model in which MIG-14/Wls cycles between the Golgi and the plasma membrane to mediate Wnt secretion (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Pan *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008). After transport to the plasma membrane, MIG-14/Wls is endocytosed and recycled back to the Golgi to take part in a new round of Wnt secretion. The endosome to Golgi recycling step is mediated by the retromer complex and mutation of the cargo-selective subunits leads to degradation of MIG-14/Wls in the lysosomal pathway. As a consequence, less MIG-14/Wls is available to mediate Wnt secretion and Wnt signalling is disrupted. The internalization of MIG-14/Wls is dependent on the AP2 endocytotic adaptor complex (Pan *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008) and when AP2- and clathrin-mediated endocytosis is blocked, MIG-14/Wls accumulates on the cell surface. Also in this case, less MIG-14/Wls is available for Wnt secretion, explaining the Wnt-signalling defect of AP2 subunit mutants (Pan *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008).

To further examine the regulation of MIG-14/Wls trafficking, we focused on the endocytotic step of the pathway. We analysed an existing panel of *C. elegans* endocytosis-defective mutants and discovered that two members of the myotubularin-related family of lipid phosphatases, MTM-6 and MTM-9, are required for efficient MIG-14/Wls recycling. We provide evidence that the role of MTM-6 in MIG-14/Wls trafficking is mediated by the sorting nexin family member SNX-3. We further show that the function of MTM-6 in MIG-14/Wls recycling is evolutionarily conserved in *Drosophila*. Our data extend the current model of regulation of Wnt secretion and provide the first functional link between the conserved MTM-6 and MTM-9 myotubularins and the Wnt signalling pathway.

Results

The myotubularin genes mtm-6 and mtm-9 are required for the EGL-20/Wnt-dependent posterior migration of the QL.d

To understand the mechanism of Wnt secretion better, we focused on the regulation of endocytosis and recycling of MIG-14/Wls. We made use of an existing collection of viable *C. elegans* mutants with known defect in endocytosis (Fares and Greenwald, 2001). These mutants are defective in the uptake of a secreted form of GFP by specialized endocytotic cells, the coelomocytes (called a Cup phenotype, for coelomocyte uptake defective). We investigated whether these mutants display a defect in Wnt signalling by assessing the position of the Q neuroblast descendants. The *C. elegans* L1 larva is born with two Q neuroblasts, on the left and right sides (QL and QR), which later generate an identical set of descendants (Q.d). The Q.d on the right side of the larva migrate in a default anterior direction, whereas the Q.d on the left side migrate to the posterior of the animal (Figure 1A). Posterior migration is regulated by the Wnt protein EGL-20 that induces the expression of the homeotic gene *mab-5* in QL and its descendants (Salser and Kenyon, 1992). In *egl-20*/Wnt, *mig-14*/Wls or *vps-35*/retromer mutants, *mab-5* fails to be expressed and as a consequence, the QL.d migrate towards the anterior (Salser and Kenyon, 1992; Harris *et al*, 1996; Coudreuse *et al*, 2006; Prasad and Clark, 2006). Among nine genes tested, we found two Cup mutants with a significant proportion of anteriorly misplaced QL.d, *mtm-6* and *mtm-9* (Figure 1B). A low but reproducible QL.d migration defect was also seen in *cup-4* mutants.

mtm-6 and *mtm-9* encode members of the myotubularin family of lipid phosphatases (Xue *et al*, 2003) and have been shown to form a heterodimer, in which MTM-6 is an active phosphatase, whereas MTM-9 lacks a critical residue in its catalytic site (Dang *et al*, 2004). We found that *mtm-6; mtm-9* double mutants do not show a significantly higher QL.d migration defect than *mtm-9* single mutant, which supports their functioning as a complex (Figure 1C). The *mtm-9 (ar479)* allele contains a premature stop (W135STOP) mutation and *mtm-6(ok330)* is a 1235-base-pair deletion spanning the central region of the protein (Supplementary Figure 1A; Dang *et al*, 2004). The lower penetrance of the *mtm-6(ok330)* QL.d migration phenotype in comparison with *mtm-9(ar479)* might be caused by residual function of the truncated protein. We therefore tested two other *mtm-6* alleles: *ar513*, a stop mutation causing a C-terminal truncation that removes the FYVE domain, and *ar515*, a missense mutation in the catalytic site (Fares and Greenwald, 2001; Dang *et al*, 2004). Both alleles show a reproducible QL.d migration phenotype; however, in neither of them the penetrance reaches the levels observed with *mtm-9* (15 and 25%, respectively, compared with 62% in *mtm-9* mutants; $n > 100$). An alternative explanation may therefore be that *mtm-6* acts partially redundantly with other myotubularin-related genes.

There are three other myotubularin genes encoded in the *C. elegans* genome, *mtm-1*, *mtm-3* and *mtm-5* (Xue *et al*, 2003). To test whether the incomplete penetrance of the QL.d migration defect in *mtm-6* mutants is due to functional redundancy among the family members, we scored QL.d migration in *mtm-1* and *mtm-5* mutants and in animals in which *mtm-1*, *mtm-3* and *mtm-5* were knocked down by RNAi (Figure 1C). None of them displayed a defect in QL.d migration. However, in *mtm-6; mtm-5* double mutants the QL.d migration defect was similar to that observed in *mtm-6; mtm-9* double mutants. A small enhancement was also observed with *mtm-5* and *mtm-3* RNAi in an *mtm-6* mutant background (although only the enhancement by *mtm-3* RNAi was statistically significant). A more pronounced enhancement was observed with *mtm-1* RNAi in the *mtm-6* background but not in *mtm-1(op309); mtm-6* double mutants. This may be due to the hypomorphic nature of the *op309* allele (M Hengartner, personal communication). Taken together, our data indicate that the myotubularins *mtm-6* and *mtm-9* are required for proper migration of the QL descendants. Other myotubularins may have a minor role in QL.d migration by acting partially redundantly with *mtm-6* and *mtm-9*. In most of the following study, we focused on *mtm-6* as the enzymatically active part of the complex.

Next, we investigated whether other *cup* mutants act in parallel with *mtm-6* in regulating QL.d migration. We therefore constructed double mutants between *mtm-6* and other *cup* mutants and assessed the penetrance of the QL.d migration phenotype. Out of the four mutants tested, only *cup-4*

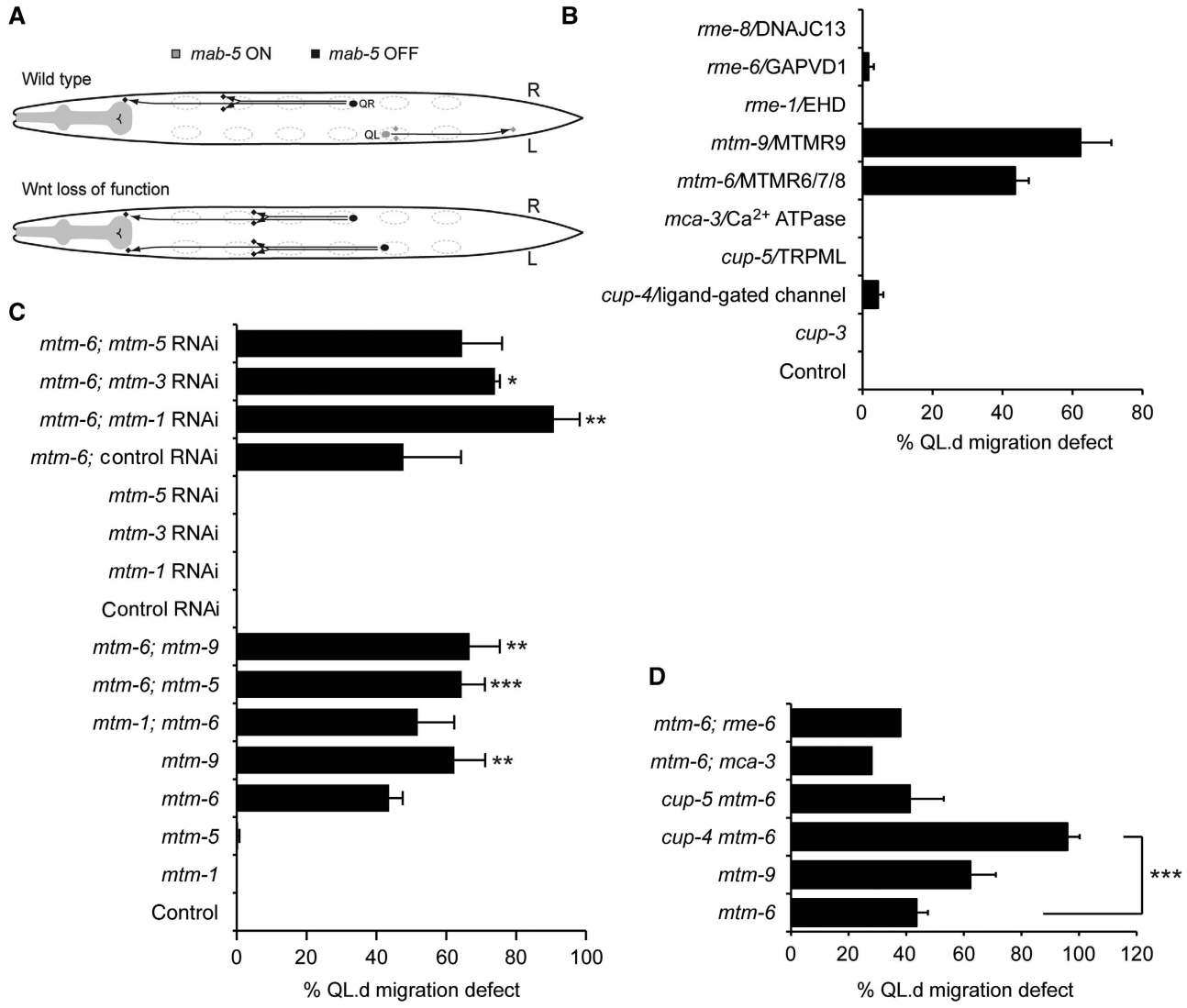

Figure 1 The myotubularin-related genes *mtm-6* and *mtm-9* are required for correct QL descendant migration. (**A**) Schematic representation of the migration of Q neuroblast descendants in wild-type L1 larvae and in animals with impaired EGL-20/Wnt signalling. In wild-type animals, the QR.d migrate to the anterior, whereas the QL.d activate the Wnt target gene *mab-5* and migrate to the posterior. Loss of Wnt signalling abrogates *mab-5* expression in the QL.d and results in their anterior migration. Note that the Q.d migrate less far into the anterior in mutants that are defective in EGL-20/Wnt secretion. Dorsal view: dashed circles represent the positions of the epidermal seam cells (**B**) QL.d migration defects in Cup mutant strains. (**C**) QL.d migration defects in single and double myotubularin mutants, wild-type animals treated with RNAi against different myotubularins and *mtm-6(ok330)* animals treated with RNAi against different myotubularins. (**D**) QL.d migration defect in double mutants between *mtm-6* and other Cup mutants. All strains in (**B**–**D**) also contain the *muIs32* transgene expressing *mec-7*::*gfp* (Ch'ng *et al*, 2003). All error bars represent s.d. values of at least three independent assays. Full genotypes and total numbers of animals scored are shown in Supplementary Table 1. $*P<0.02$, $**P<0.005$, $***P<0.001$, unpaired *t*-test (compared to *mtm-6* or *mtm-6*; control RNAi).

enhanced the phenotype of *mtm-6* (Figure 1D). This suggests that *mtm-6* and *cup-4* function synergistically in the regulation of Q cell migration.

mtm-6 mutants display defects in several Wnt-dependent processes

EGL-20/Wnt controls the posterior migration of the QL.d through induction of the homeotic gene *mab-5* (Salser and Kenyon, 1992; Harris *et al*, 1996). To investigate whether the defect in QL.d migration is caused by a loss of *mab-5* expression in the QL lineage, we analysed the expression of a *mab-5*::*lacZ* reporter that has been shown to closely mimic the expression of the endogenous *mab-5* gene (Cowing and Kenyon, 1992). In wild-type animals, *mab-5* was strongly expressed in QL and its descendants, but in a significant fraction of *mtm-6(ok330)* mutants, no *mab-5* expression was observed in the QL lineage (Figure 2A). Importantly, the EGL-20-independent expression of *mab-5* in other cells, such as the posterior ventral nerve cord neurons (Salser and Kenyon, 1992), was not affected. These results indicate that *mtm-6* is required for the EGL-20/Wnt-dependent induction of *mab-5* expression. This conclusion is further supported by the observation that the QL.d migration defect of *mtm-6* is rescued by EGL-20/Wnt-independent activation of *mab-5*. Thus, the QL.d localized at their normal posterior position in double mutants between *mtm-6* and the *mab-5* gain-of-function mutation *e1751* (Figure 2B) (Salser *et al*, 1993). Furthermore, posterior localization of the QL.d was partially restored in double mutants between *mtm-6* and *pry-1*/Axin (Figure 2B), a negative regulator of the canonical

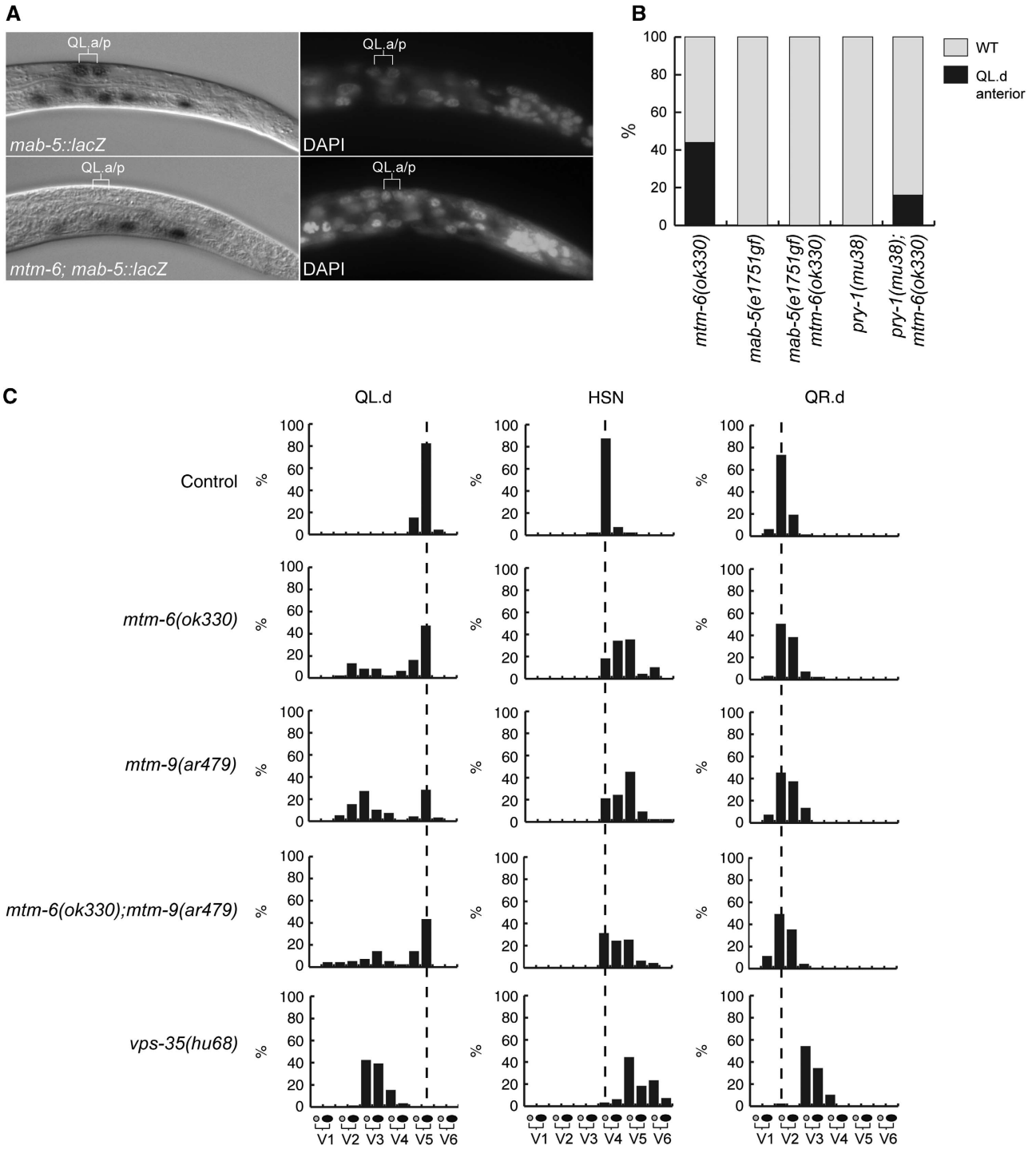

Figure 2 Characterization of the Wnt-related phenotypes of *mtm-6* mutants. (**A**) Activation of a *mab-5*::*lacZ* transcriptional reporter in the QL.a/p daughter cells. *mab-5* expression is not activated in *mtm-6* mutants, even though QL.a/p are present. (**B**) Suppression of the QL.d migration phenotype of *mtm-6* mutant by the *mab-5(e1751)* gain-of-function allele and by a mutation in the negative Wnt pathway regulator *pry-1*/Axin. In each case, $n > 150$. (**C**) Quantification of the Wnt-related positioning defects of the QL and QR descendants and the HSN neurons in *mtm-6*, *mtm-9* and *mtm-6; mtm-9* mutants. Phenotypes of the retromer mutant *vps-35* are shown for comparison. Positions are displayed relative to the positions of the V1–V6 epidermal seam cells (black circles) and their anterior hyp7 daughters (small grey circles). All strains except for *vps-35* also contain the *muIs32* transgene expressing *mec-7*::*gfp* (Ch'ng *et al*, 2003). Strains carrying the *mtm-9* mutation also contain the *arIs37* transgene expressing *Pmyo-3*::*ssgfp* for easier tracking of the mutation. In each case, $n > 80$.

Wnt/β-catenin pathway that is triggered by EGL-20/Wnt in the QL lineage (Maloof *et al*, 1999; Korswagen *et al*, 2002).

We next examined whether loss of *mtm-6* and *mtm-9* also affects other Wnt-dependent processes during *C. elegans* development, such as the anterior migration of the HSN neurons and the QR descendants and the polarization of the ALM and PLM mechanosensory neurons, which depends on the simultaneous action of several Wnt proteins (Pan *et al*,

Table I ALM and PLM polarity defects in myotubularin and *mig-14*/Wls mutants

	% ALM	% PLM
Wild type	0	0
mtm-6(ok330)	2	0
mtm-9(ar479)	3	0
mig-14(mu71)	27	0
mig-14(ga62)	73	34
mig-14(mu71); mtm-6(ok330)	61	43
mig-14(ga62); mtm-6(ok330)	62	73

The polarity of the ALM and PLM neurons was assayed using the *mec-7*::*gfp* expressing transgenes *muIs32* or *muIs35* (Ch'ng *et al*, 2003). Both reversal and loss of polarity were scored as defective polarization. In each case, $n>150$.

2006; Prasad and Clark, 2006). We found that the HSN neurons and the QR.d were significantly under-migrated in the *mtm-6* and *mtm-9* single and double mutants (Figure 2C). Furthermore, there was a weak but reproducible defect in the polarity of the ALM neurons (Table I), but no defect was observed in the Wnt-dependent polarization of the seam cell V5 or in the fate specification of the P11/12 ventral epithelial cells. Taken together, these results demonstrate that *mtm-6* and *mtm-9* are required for several Wnt-dependent processes.

MTM-6 is required in EGL-20/Wnt-producing cells

It has previously been reported that *mtm-6* is predominantly expressed in the intestine (Xue *et al*, 2003), which is in disagreement with the function of *mtm-6* in EGL-20/Wnt signalling. *mtm-6* is part of a large operon (Supplementary Figure 1B), which complicates the determination of its expression pattern. The promoter element used by Xue *et al* (2003) encompasses only part of the operon sequence and may therefore lack essential regulatory regions. We were also unable to generate a reporter construct that includes the complete *mtm-6* operon. We therefore determined the expression pattern of *mtm-9*, as MTM-6 and MTM-9 act as a complex and are thus likely to be co-expressed (Dang *et al*, 2004). We found that a transcriptional *mtm-9*::*gfp* reporter was expressed in a broad range of tissues, including the muscle, intestine, hypodermis and neurons (Supplementary Figure 2; data not shown). *mtm-9* was also expressed in the rectal epithelial cells that are the major source of EGL-20/Wnt (Whangbo and Kenyon, 1999; Figure 3A). The widespread expression of *mtm-9* is consistent with the function of the MTM-6–MTM-9 complex in several Wnt-dependent processes.

To investigate whether MTM-6 is required in Wnt-responding or -producing cells, we expressed MTM-6 tagged with GFP under the control of different tissue-specific promoters in a *mtm-6(ok330)* mutant background and assayed the EGL-20/Wnt-dependent migration of the QL.d. We found that expression of MTM-6 under the control of the *egl-20* promoter (Coudreuse *et al*, 2006) was sufficient for a complete rescue of the QL.d migration defect (Figure 3B). Expression of MTM-6 from the *cdh-3* promoter (Pettitt *et al*, 1996), which overlaps in expression with the *egl-20* promoter in only one cell, resulted in a partial rescue. Importantly, when we made a phosphatase-inactive version of MTM-6 by mutating a conserved cysteine in the catalytic domain (C335S) (Dang *et al*, 2004) and expressed it from the *egl-20* promoter, we did not observe any rescue. These results demonstrate that MTM-6 is required in Wnt-producing cells

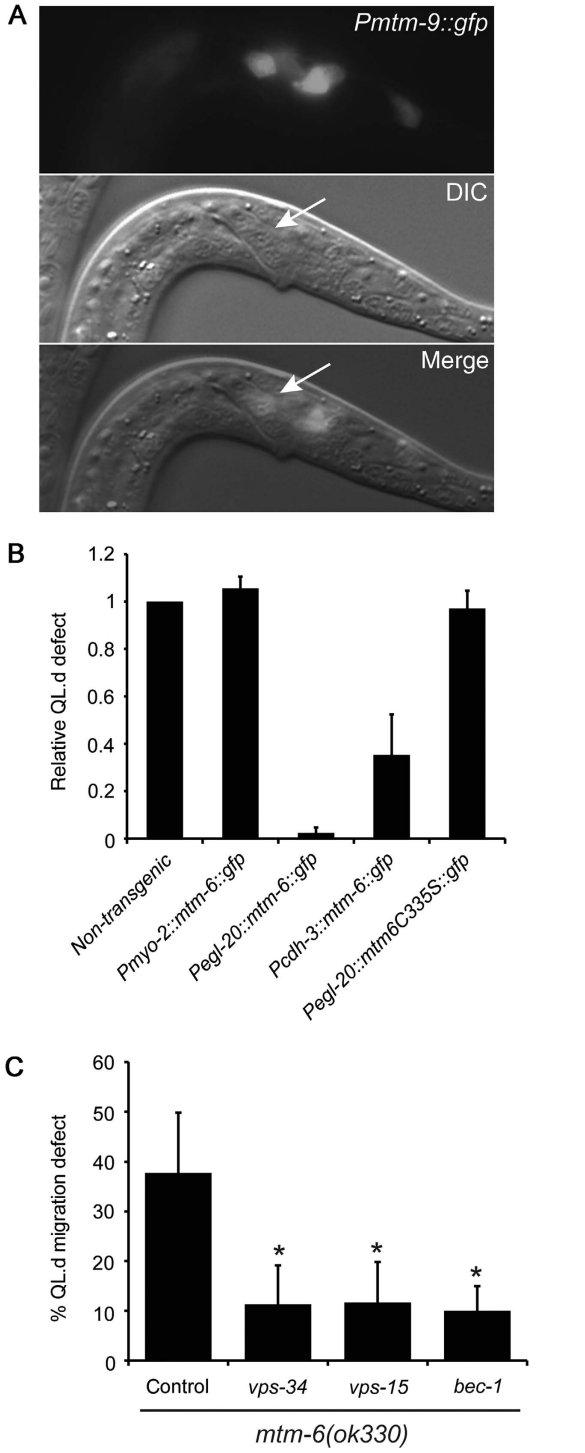

Figure 3 Tissue specificity of *mtm-6* and its interaction with the PI3 kinase complex. (**A**) Expression of GFP under the control of *mtm-9* promoter in the posterior of an early L2 larva. Arrow points to the EGL-20-expressing F cell. (**B**) Transgenic rescue of the *mtm-6* QL.d migration defect. Defects in each strains were normalized to control siblings that did not carry the transgene. Error bars represent the s.d. values of three independent experiments, $n>100$. (**C**) Rescue of the QL.d migration phenotype of *mtm-6* mutants by RNAi against components of the VPS-34–PI3 kinase complex. Error bars represent s.d. values (three independent experiments, $n>120$), $^*P<0.02$ (unpaired *t*-test, compared with control RNAi). All strains in (**B**) and (**C**) also contain the *muIs32* transgene expressing *mec-7*::*gfp* (Ch'ng *et al*, 2003).

and that the phosphatase activity is indispensable for its function.

Knockdown of the Vps34 PI3 kinase complex restores EGL-20/Wnt signalling in mtm-6 mutants

Phosphatidylinositol-3-phosphate (PI3P), the substrate of MTM-6, is generated by the phosphatidylinositol-3-kinase VPS-34 (Schu *et al*, 1993). To further investigate the importance of phosphoinositide turnover in EGL-20/Wnt signalling, we examined the function of *vps-34* in QL.d migration. As *vps-34* mutants are embryonic lethal (Xue *et al*, 2003), we used RNAi to partially knock down *vps-34* expression. Knockdown of *vps-34* produced no defect in the localization of the QL.d in wild-type animals. However, *vps-34* RNAi partially rescued the QL.d migration defect of *mtm-6* mutants (Figure 3C). A similar effect was achieved with RNAi against *ZK930.1* and *bec-1*, genes encoding two subunits of the Vps34 kinase complex, Vps15 and Vps30/Beclin1, respectively (Figure 3C; Stack *et al*, 1993; Kihara *et al*, 2001). These results support the idea that the primary cause of the Wnt-signalling defect in *mtm-6* mutants is an excess of PI3P and that a proper balance between PI3P synthesis and degradation is required for efficient Wnt signalling.

MIG-14/Wls protein levels are reduced in mtm-6 mutants

As *mtm-6* is required in Wnt-producing cells, we examined whether *mtm-6* genetically interacts with *mig-14*/Wls, the putative sorting receptor for Wnt that is an essential component of the Wnt secretion pathway (Banziger *et al*, 2006; Bartscherer *et al*, 2006; Goodman *et al*, 2006). Animals carrying the *mig-14* null allele *or78* die during early embryogenesis (Thorpe *et al*, 1997), so to test for genetic interactions, we made use of two viable reduction-of-function alleles, *mu71* and *ga62* (Yang *et al*, 2008). As these mutants show a fully penetrant defect in QL.d migration, we focused on the Wnt-dependent polarization of the ALM and PLM neurons. Whereas *mtm-6(ok330)* animals displayed only a weak defect in ALM polarity and no defect in PLM polarity (see above), the *mtm-6* mutation strongly enhanced both the ALM and PLM polarization defect of *mig-14(mu71)* and the PLM polarization defect of *mig-14(ga62)* (Table I). Although these results do not distinguish between a function of *mtm-6* and *mig-14* in shared or parallel genetic pathways, they are consistent with a functional interaction between MTM-6 and MIG-14 in Wnt signalling.

MIG-14/Wls cycles between the Golgi complex, the plasma membrane and endosomes to mediate Wnt secretion (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Pan *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008). To investigate whether *mtm-6* affects the endocytosis or trafficking of MIG-14/Wls, we examined the level and subcellular localization of a functional MIG-14::GFP fusion protein in *mtm-6(ok330)* mutants. As shown in Figure 4A and B, there was a significant reduction in MIG-14::GFP protein levels, with most of the remaining MIG-14::GFP protein localizing to punctate structures, which most likely represent localization of MIG-14::GFP to the endolysosomal system. A similar effect on MIG-14::GFP level and subcellular localization is observed in mutants of the cargo-selective retromer sub-complex (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Pan *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008). Comparison between MIG-14::GFP in *mtm-6* and *vps-26(tm1523)* mutants showed, however, that MIG-14 protein levels were about three-fold lower in *vps-26* than in *mtm-6* mutants. The stronger reduction in MIG-14 protein levels in *vps-26* mutants is in agreement with the observation that *vps-26* mutants (Coudreuse *et al*, 2006) have a more penetrant defect in EGL-20/Wnt signalling than *mtm-6* mutants.

To investigate whether MIG-14/Wls becomes limiting for Wnt signalling in *mtm-6* mutants, we tested whether *mig-14* overexpression can rescue EGL-20/Wnt signalling. To overexpress *mig-14*, an extrachromosomal transgene was used that expresses *mig-14* under the control of the heat-shock promoter (Yang *et al*, 2008). A short heat shock during the early L1 stage, which is before the EGL-20/Wnt-dependent activation of *mab-5* expression, fully rescued QL.d migration in animals carrying the transgene, but not in their non-transgenic siblings (Figure 4C). The rescue of Wnt signalling by *mig-14* overexpression is consistent with a function of *mtm-6* in MIG-14/Wls trafficking and suggests that the defect in Wnt signalling is caused by a reduction in the pool of MIG-14/Wls that is available to mediate Wnt secretion.

Dmtm6 is required for Wg secretion and Wls stability in Drosophila

To investigate whether the function of *mtm-6* in MIG-14/Wls trafficking is evolutionarily conserved, we turned to another well-studied model of Wnt signalling, which is the *Drosophila* wing imaginal disc. In the wing disc, a stripe of cells at the dorsoventral boundary of the disc expresses the Wnt protein Wingless (Wg; Zecca *et al*, 1996). When we knocked down the *mtm-6* orthologue *Dmtm6* in the posterior compartment of the disc by transgene-mediated RNAi (Supplementary Figure 3), we observed the accumulation of Wg protein in the Wg-expressing cells in the posterior compartment, but not in the control anterior compartment, suggesting that Wg is not efficiently secreted upon *Dmtm6* knock-down (Figure 4D, Supplementary Figure 4). Importantly, *Dmtm6* knockdown did not affect secretion of Hedgehog, another lipid-modified morphogen (Figure 4E, Supplementary Figure 4) showing that loss of *Dmtm6* does not lead to a general defect in protein secretion. This conclusion is further supported by the *mtm-6* mutant phenotype in *C. elegans*: apart from a defect in Wnt signalling, *mtm-6* mutants have no other obvious developmental defects (data not shown).

To address whether *Dmtm6* is also required for the control of Wls levels, we stained endogenous Wls protein in discs in which *Dmtm-6* was knocked down. As shown in Figure 4D and Supplementary Figure 4, there was a significant reduction of Wls staining in the Wg-producing cells. Interestingly, Wls levels were also strongly reduced outside of the Wg-producing cells. This phenotype is different from that of *Dvps35*, where a reduction in Wls levels is only observed in the *wg*-expressing cells (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Port *et al*, 2008).

MTM-6 regulates binding of SNX-3 to MIG-14/Wls-containing endosomes

The substrate of the enzymatic activity of MTM-6, PI3P, is a phosphoinositide species that is highly enriched on endosomal membranes and has an important role in many aspects of endosomal biology. One of its functions is to serve as a docking mark for proteins that regulate endosomal trafficking

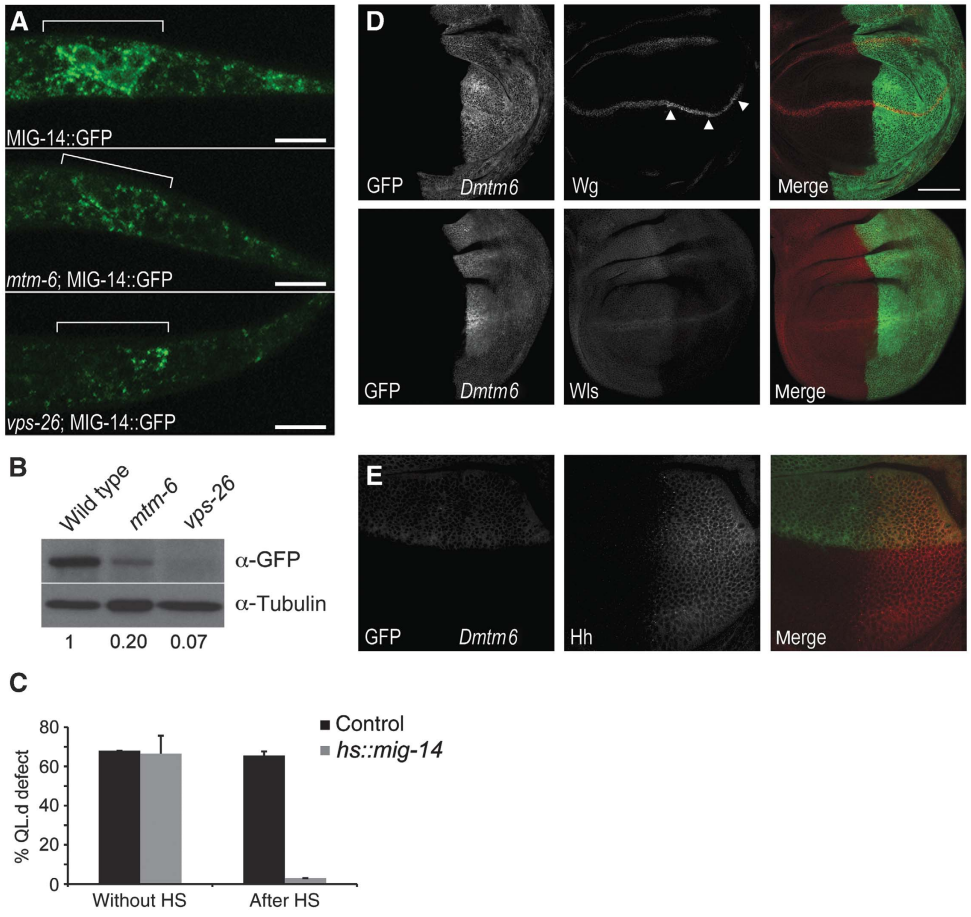

Figure 4 *mtm-6* regulates MIG-14/Wls protein levels in *C. elegans* and *Drosophila*. (**A**) Levels of MIG-14::GFP expressed from its own promoter from the single copy transgene *huSi2* are reduced in *mtm-6* and *vps-26* backgrounds compared with wild type. Images are maximum projections of 10 confocal sections taken at 0.2-μm *z*-steps and depict the posterior body region of L1 larvae. White brackets mark the position of the EGL-20-expressing cells. Anterior to the left, dorsal up. Scale bar is 10 μm. (**B**) Western blot analysis of MIG-14::GFP (*huSi2*) levels in L1 larvae of wild type, *mtm-6(ok330)* and *vps-26(tm1523)* animals. α-tubulin is used as a loading control. (**C**) Rescue of the *mtm-6* QL.d migration defect by expression of *mig-14* from a heat-shock promoter. Error bars represent s.d. values (three independent experiments, $n>70$). (**D**) *Dmtm6* influences Wg secretion by regulating Wls levels in the *Drosophila* wing disc. *Dmtm6* RNAi was expressed in the posterior compartment (marked by *UAS-CD8GFP*) using *hh-Gal4*. In the upper panel, an anti-Wg staining is shown. Arrowheads indicate accumulation of Wg in producing cells. Lower panel shows an anti-Wls staining. Wls levels are reduced in the *Dmtm6* expressing posterior compartment. Scale bar is 50 μm. (**E**) *Dmtm6* does not interfere with the secretion of Hedgehog in the *Drosophila* wing disc. *Dmtm6* RNAi was expressed in the dorsal compartment using *ap-Gal4* (marked by *UAS-CD8GFP*). Quantifications are shown in Supplementary Figure 4.

(for review, see Di Paolo and De Camilli, 2006). Among these are the sorting nexins (SNX), a family of proteins containing a PI3P-binding Phox-homology (PX) domain (Cullen, 2008), which are required for the recruitment of the cargo-selective retromer sub-complex to cargo-containing endosomes (Griffin *et al*, 2005; Rojas *et al*, 2007; Wassmer *et al*, 2007). We hypothesized that by regulating PI3P levels, MTM-6 may affect membrane association of the retromer and consequently MIG-14/Wls recycling. We have found that the sorting nexin SNX-3 functions together with the retromer complex in MIG-14/Wls recycling and Wnt secretion (M Harterink *et al*, in preparation) and therefore examined the sub-cellular localization of SNX-3 tagged with GFP in the *egl-20*-expressing cells of wild-type and *mtm-6* mutant animals. Localization of SNX-3 in wild-type animals was mostly cytoplasmic with only a few punctate structures, whereas in *mtm-6* mutants the punctate localization became much more prominent (Figure 5A and B). A similar phenotype was observed in the *Drosophila* wing imaginal disc, in which DSnx3 localized much more frequently to punctate structures in posterior cells expressing a D*mtm6* RNAi transgene compared with cells in the anterior where the RNAi transgene was not expressed (Figure 5C). This is consistent with the hypothesis that a defect in *mtm-6* leads to increased PI3P levels and therefore to increased recruitment of PI3P-binding proteins to the membrane. To confirm that the observed SNX-3 accumulation is due to binding to endosomal PI3P, we mutated a single amino acid in the SNX-3 PX domain that abrogates PI3P binding (Pons *et al*, 2008). We again expressed this SNX-3(R70A)::GFP mutant protein from the *egl-20* promoter and compared its localization in wild-type and *mtm-6* mutant animals. In this case, we did not observe any punctate localization in either wild-type or *mtm-6* mutants (Figure 5A), confirming that the SNX-3 accumulation in *mtm-6* mutants is indeed dependent on PI3P binding.

To investigate whether the SNX-3-positive vesicles contain MIG-14, we co-expressed MIG-14::GFP and SNX-3::mCherry in EGL-20/Wnt-producing cells. In wild-type animals, MIG-14 showed a more widespread sub-cellular distribution than SNX-3, but there was clear co-localization between the two

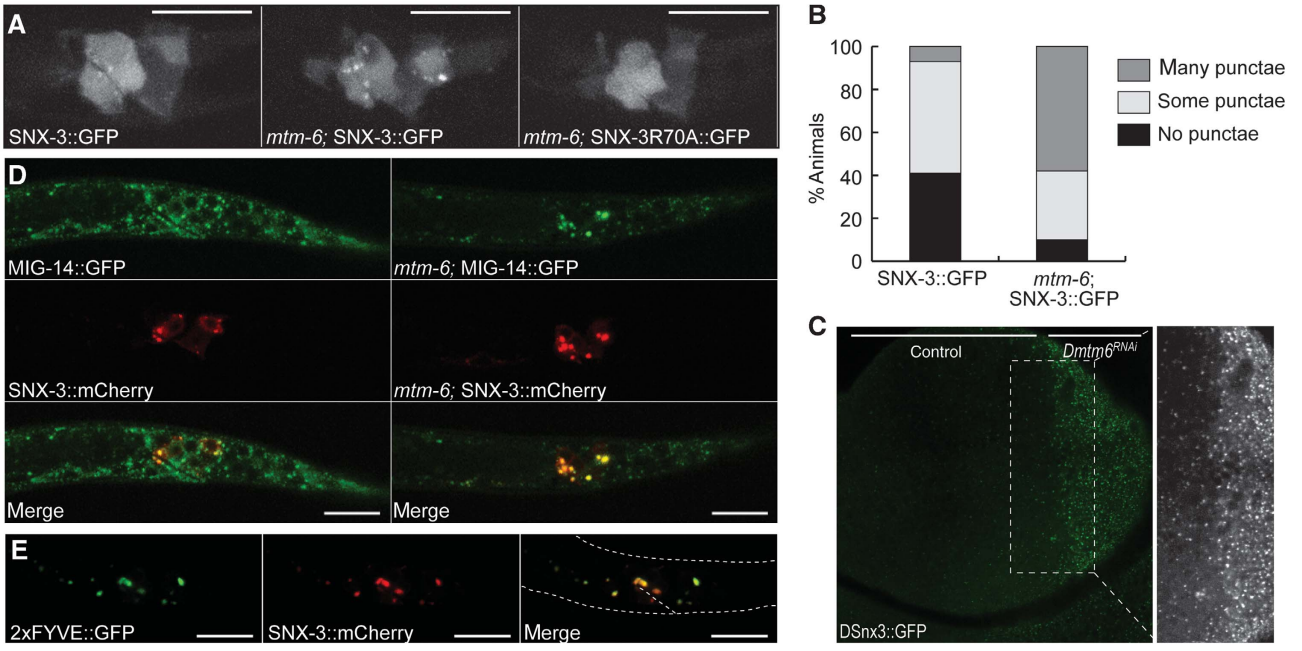

Figure 5 MTM-6 controls the endosomal localization of SNX-3. (**A**) Localization of SNX-3::GFP in the EGL-20-expressing cells of a wild-type and *mtm-6* L1 larva. *mtm-6* mutants display more punctuate localization of SNX-3 that is lost upon introduction of the R70A mutation in the PI3P-binding domain of SNX-3. Images are maximum projections of ten confocal sections taken at 0.2-μm *z*-steps. Scale bar is 10 μm. (**B**) Quantification of the differences in SNX-3 localization between wild-type and *mtm-6* mutants ($n = 50$). Legend: no punctae (only diffuse cytoplasmic localization), some punctae (one to five distinct punctae) many punctae (more than five distinct punctae) (**C**) Localization of DSnx3::GFP in *Drosophila* wing imaginal discs expressing a *UAS-Dmtm6* RNAi transgene in the posterior compartment under the control of *hh-Gal4*. DSnx3::GFP is expressed under the control of the β-tubulin promotor. DSnx3 localizes more frequently to punctate structures in cells with reduced *Dmtm6* expression. A confocal section of the apical region of the wing pouch is shown and a magnification is provided to the right. (**D**) Co-localization between MIG-14 and SNX-3 in wild-type animals and in *mtm-6* mutants. MIG-14::GFP was expressed from its own promoter using the single copy transgene *huSi2*, SNX-3::mCherry was expressed under the control of the *egl-20* promoter from an extrachromosomal array. Co-localization is observed in wild-type animals, but is much more prominent in the *mtm-6* mutant background. The remaining GFP signal in the *mtm-6* mutants is more concentrated in the presence of SNX-3::mCherry (compare with Figure 4A). Scale bar is 10 μm and images are maximum projections of four confocal sections taken at 0.2-μm *z*-steps (**E**) Co-localization between SNX-3 and the endosomal marker 2 × FYVE::GFP in *mtm-6* mutants. Both proteins were expressed under the control of the *egl-20* promoter from an extrachromosomal array. Co-localization is observed on almost all vesicles. Outline of the animal and position of the rectum are indicated with the dashed line. Images are single confocal sections, scale bar is 10 μm. Images in (**D**) and (**E**) depict the posterior body region of L1 larvae. Anterior to the left, dorsal up.

on intracellular punctae, with most of the SNX-3-positive vesicles also containing MIG-14::GFP (Figure 5D). In *mtm-6* mutants, the remaining MIG-14::GFP co-localized with SNX-3. Interestingly, the MIG-14-positive vesicles in *mtm-6* mutants appeared larger and stained more intensely for MIG-14::GFP in the SNX-3::mCherry-expressing transgenic animals than in animals not carrying the *snx-3* transgene (Figures 4A and 5D), suggesting that overexpression of SNX-3 in a *mtm-6* mutant background may partially block MIG-14 trafficking. Consistently, we found that the *mtm-6*-induced defect in the EGL-20/Wnt-dependent migration of the QL.d was strongly enhanced by SNX-3 overexpression (18% in *mtm-6(ok330)* versus 92% in *mtm-6(ok330); Pegl-20::snx-3::mcherry*, whereas overexpression of SNX-3::mCherry had no effect in wild-type animals, $n > 200$). To investigate whether the enlarged SNX-3-decorated vesicles in *mtm-6* mutants are endosomes, we co-expressed SNX-3::mCherry with the endosomal marker 2 × FYVE::GFP (Roggo *et al*, 2002). As shown in Figure 5E, SNX-3 and 2 × FYVE::GFP localized to the same vesicles, confirming that these structures are endosomes. Taken together, these results demonstrate that *mtm-6* regulates the PI3P-dependent association of SNX-3 to MIG-14-containing endosomes (Supplementary Figure 5). Disruption of this regulation induces a defect in MIG-14/Wls trafficking, which interferes with its recycling and ultimately leads to its degradation. We propose that a proper balance of SNX-3 recruitment and release at the endosomal membrane is critical for the efficient retromer-dependent recycling of MIG-14/Wls and the secretion of Wnt.

Discussion

The discovery of the putative Wnt-sorting receptor, MIG-14/Wls, has shown that secretion of Wnt proteins requires a dedicated pathway that provides a previously unanticipated level of regulation to the Wnt signalling machinery (Banziger *et al*, 2006; Bartscherer *et al*, 2006; Goodman *et al*, 2006). An important aspect of this secretion pathway is that MIG-14/Wls cycles between the Golgi and the plasma membrane to mediate Wnt secretion. In the absence of plasma membrane-to-Golgi transport, Wnt secretion is diminished, as is the case in mutants of the retromer complex and mutants of the AP2 endocytic adaptor complex (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Pan *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008). Proteins directing Wls recycling are therefore important regulators of Wnt secretion.

In this study, we show that the myotubularin lipid phosphatase family members, MTM-6 and MTM-9, regulate

MIG-14/Wls recycling in *C. elegans* and *Drosophila*. Myotubularin phosphatases have been shown to specifically dephosphorylate PI3P or PI(3,5)P2 (Taylor *et al*, 2000; Walker *et al*, 2001; Schaletzky *et al*, 2003), two important phosphoinositide species involved in regulation of membrane trafficking (for review, see Vicinanza *et al*, 2008). As several myotubularins act on the same substrate, they may either act redundantly or may dephosphorylate specific sub-pools of PI3P. We show that of the five *C. elegans* myotubularins genes, only *mtm-6* and *mtm-9* significantly affect Wnt signalling, suggesting that individual myotubularins have distinct roles. Indeed, minor functions of the other myotubularins in Wnt signalling are only observed in the absence of *mtm-6* and *mtm-9*, indicating that they can only partially substitute for MTM-6/MTM-9 function. The specificity of myotubularins can be achieved either by tissue-specific expression, differential localization within the cell or by specific protein–protein interactions. Although initial studies hinted at the first possibility, showing non-overlapping patterns of *mtm-1*, *mtm-3* and *mtm-6* expression (Xue *et al*, 2003), later reports demonstrated broader and overlapping expression domains for *mtm-1* and *mtm-3* (Ma *et al*, 2008; Zou *et al*, 2009). Our own data also show a broad expression pattern of *mtm-9*, suggesting that like in mammals (Laporte *et al*, 2002), *C. elegans* myotubularins are expressed ubiquitously and their specificity is achieved differently. Localization of myotubularins to distinct sub-cellular compartments has been demonstrated by some in mammalian cells (Lorenzo *et al*, 2006; Cao *et al*, 2007), whereas others have reported diffuse cytoplasmic localization (Blondeau *et al*, 2000; Taylor *et al*, 2000; Laporte *et al*, 2002). An intriguing example of differential localization has been described for human MTM1 and MTMR2, in which MTM1 localizes to Rab5-positive early endosomes and only partially to Rab7-positive late endosomes (Cao *et al*, 2007), whereas MTMR2 localizes predominantly to Rab7-positive endosomes (Cao *et al*, 2008). Furthermore, corresponding changes in early or late endosomal PI3P pools have been achieved by knockdown of MTM1 or MTMR2, respectively (Cao *et al*, 2008). *C. elegans* MTM-6 and MTM-1 have been shown to localize to the plasma membrane in various tissues (Xue *et al*, 2003; Zou *et al*, 2009). In our hands, GFP-tagged MTM-6 showed a predominantly diffuse cytoplasmic localization, arguing against localization-driven specificity, although we cannot rule out the possibility that localization to specific sub-cellular compartments is masked by protein overexpression.

Apart from the finding that MTM-6 and MTM-9 (MTMR6 and MTMR9 in mammals) form a heterodimer (Mochizuki and Majerus, 2003; Dang *et al*, 2004), little is known about their protein interaction partners. MTMR6 can interact with and modulate the activity of the calcium-activated potassium channel $K_{Ca}3.1$ (Srivastava *et al*, 2005). This interaction is important for the regulation of Ca^{2+} signalling during T cell activation (Srivastava *et al*, 2006). Although *C. elegans* MTM-6 interacts with the $K_{Ca}3.1$ orthologue KCNL-2 in a yeast two-hybrid assay (Srivastava *et al*, 2005), the physiological relevance of this interaction in *C. elegans* is not clear. Knockdown of *kcnl-2* did not produce any defect in QL.d migration in wild-type animals, nor did it enhance the penetrance of the phenotype in *mtm-6* mutants (data not shown), suggesting that this interaction is unlikely to be relevant for the role of MTM-6 in Wnt secretion.

The major defect we observe in *mtm-6* mutants is a reduction in MIG-14/Wls protein levels, a phenotype reminiscent of the effect of loss of retromer and thus the absence of MIG-14/Wls recycling. How could an excess of PI3P lead to a decrease in levels of MIG-14? We show that SNX-3, a member of the sorting nexin family of PI3P-binding proteins, accumulates on vesicles in *mtm-6* mutants. SNX-3 interacts with the retromer complex to mediate endosome-to-Golgi transport of MIG-14/Wls (M Harterink *et al*, in preparation). Although the exact mechanism remains to be determined, we speculate that a tight balance of SNX-3 recruitment and release is necessary for the efficient retromer-dependent recycling of MIG-14/Wls (Supplementary Figure 5). In the absence of MTM-6, SNX-3 accumulates on endosomes and the efficiency of the retromer-dependent retrieval of MIG-14/Wls is reduced, which ultimately leads to its degradation. This model is consistent with our observation that Wnt signalling in *mtm-6* mutants can be restored by MIG-14/Wls overexpression.

Although mutation of *mig-14*/Wls affects all Wnt-dependent processes in *C. elegans* (Thorpe *et al*, 1997; Pan *et al*, 2008; Yang *et al*, 2008), loss of *mtm-6* results in a more restricted spectrum of Wnt phenotypes. This is similar to the phenotype observed in mutants of the cargo-selective retromer subunits, which affect some, but not all Wnt-dependent processes (Coudreuse *et al*, 2006; Prasad and Clark, 2006). We have previously shown that blocking MIG-14/Wls recycling results in a reduction, but not a full block of Wnt secretion (Yang *et al*, 2008). Interfering with MIG-14/Wls recycling will therefore mainly affect Wnt-dependent processes that depend on a high level of Wnt secretion. Consistently, there is a large degree of overlap in the phenotype of *mtm-6* and mutants of the cargo-selective retromer subunits (Coudreuse *et al*, 2006; Prasad and Clark, 2006). The Wnt-signalling phenotype of *mtm-6* mutants is, however, weaker than observed in retromer mutants, which is consistent with the less severe reduction in steady state MIG-14/Wls protein levels. A likely explanation of this difference is that loss of *mtm-6* only partially interferes with the retromer-dependent recycling of MIG-14/Wls; a conclusion that is supported by our observation that the Wnt-signalling defect of *mtm-6* can be strongly enhanced by partial knockdown of the cargo-selective retromer subunits (data not shown).

We provide evidence that the role of *mtm-6* in MIG-14/Wls recycling is evolutionarily conserved. Experiments in *Drosophila* confirm that *Dmtm6* is required to maintain Wls levels in the wing disc. Interestingly, unlike in *Drosophila* retromer mutants, knockdown of *Dmtm6* leads to a drop in Wls levels in the whole disc, not only in the stripe of Wnt-producing cells (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Port *et al*, 2008). So far we do not have an explanation for this observation. A recent study has found that in the *Drosophila* neuromuscular junction, Wls has an unanticipated role in Wnt-receiving cells (Korkut *et al*, 2009). A possibility is therefore that the trafficking and function of Wls in the Wnt-receiving cells is different from that in Wnt-producing cells, and whereas retromer is required only in the producing cells, *Dmtm6* might be required in both.

Our results suggest that the MTM-6/9 myotubularin complex is particularly important for MIG-14/Wls recycling and Wnt secretion. Apart from the coelomocyte uptake defect, *mtm-6* mutants do not show obvious defects in

other signalling pathways. Furthermore, we found that the secretion of Hh is not affected by *Dmtm6* knockdown in *Drosophila*. As loss of retromer function also mainly affects Wnt signalling (Port *et al*, 2008; Yang *et al*, 2008), these results further support our hypothesis that MTM-6 and the retromer complex function together in the MIG-14/Wls recycling pathway. The specificity of the *mtm-6* and retromer mutant phenotype for Wnt signalling suggests that other developmentally important signalling pathways are less dependent on retromer-dependent endosome-to-Golgi transport than the Wnt secretion pathway.

In humans, members of the myotubularin family have been implicated in several disorders, such as myotubular myopathy (Laporte *et al*, 1996), peripheral neuropathies (Bolino *et al*, 2000; Azzedine *et al*, 2003; Senderek *et al*, 2003) and azoospermia (Firestein *et al*, 2002). A common mechanism underlying these pathologies is defective membrane transport and homeostasis (for review, see Nicot and Laporte, 2008). However, the exact mechanism through which these myotubularin family members cause the different pathologies is not understood. We provide a new link between myotubularin phosphatases and the Wnt-signalling pathway, raising the interesting possibility that some of the pathologies associated with myotubularin mutations may be caused by defects in this essential signalling pathway.

Materials and methods

C. elegans strains and culture

General methods for culture, manipulation and genetics of *C. elegans* were performed as described previously (Lewis and Fleming, 1995). Strains, mutations and integrated arrays used in this study were Bristol N2 and LGI: *arIs37[Pmyo-3::ssgfp; dpy-20(+)]* (Fares and Greenwald, 2001), *mtm-1(op309)*, *pry-1(mu38)* (Maloof *et al*, 1999), *rme-8(b1023)* (Fares and Greenwald, 2001); LGII: *cup-3(ar498)* (Fares and Greenwald, 2001), *mig-14(mu71)* (Harris *et al*, 1996), *mig-14(ga62)* (Eisenmann and Kim, 2000), *muIs32[Pmec-7::gfp; lin-15(+)]* (Ch'ng *et al*, 2003), *vps-35(hu68)* (Coudreuse *et al*, 2006), *huSi2[Pmig-14::mig-14::gfp]*; LGIII: *cup-4(ok837)* (Fares and Greenwald, 2001), *cup-5(ar465)* (Fares and Greenwald, 2001), *mtm-6(ar513)* (Fares and Greenwald, 2001), *mtm-6(ar515)* (Dang *et al*, 2004), *mtm-6(ok330)*; LGIV: *mab-5(e1751)* (Salser and Kenyon, 1992), *mca-3(ok2048)*, *muIs2[Pmab-5::lacZ; unc-31(+)]* (Salser and Kenyon, 1992), *vps-26(tm1523)* (Coudreuse *et al*, 2006); LGV: *mtm-9(ar479)* (Dang *et al*, 2004), *muIs35[Pmec-7::gfp; lin-15(+)]* (Ch'ng *et al*, 2003), *rme-1(b1045)* (Grant and Hirsh, 1999); LGX: *mtm-5(ok469)*, *rme-6(b1014)* (Grant and Hirsh, 1999).

Molecular biology, germline transformation and RNA interference

C. elegans cell-specific expression constructs and transcriptional reporters were generated using standard molecular biology techniques and vectors provided by Dr A Fire. cDNA of the *mtm-6* A isoform was amplified by PCR from Bristol N2 mixed stage cDNA. To construct *Pegl-20::mtm-6::gfp*, 4.4 kb of *egl-20* promoter and the *mtm-6* cDNA were cloned in frame with GFP in the pPD95.81 vector. To construct *Pcdh-3::mtm-6::gfp*, the *mtm-6* cDNA was inserted behind a 6-kb fragment of the *cdh-3* promoter in pJP38 (Pettitt *et al*, 1996). To construct *Pmyo-2::mtm-6::gfp*, the *myo-2* promoter was cut from pPD118.33 and was inserted together with the *mtm-6* cDNA into pPD95.81. To construct *Pmtm-9::gfp*, 4-kb of *mtm-9* promoter sequence was PCR-amplified and inserted into pPD95.81. To construct *Pegl-20::snx-3::gfp*, 4.4 kb of *egl-20* promoter sequence was inserted together with the *snx-3*-coding sequence into pPD95.75. *Pegl-20::snx-3::mcherry* was derived from *Pegl-20::snx-3::gfp* by replacing the *gfp* sequence with the *mcherry* sequence. To construct *Pegl-20::gfp::2xFYVE*, the GFP::2 × FYVE fragment was released from the 2 × T10G3.5(FYVE) construct described previously (Roggo *et al*, 2002; a kind gift of Dr Fritz Müller, University of Fribourg, Switzerland) and cloned behind 4.4 kb of *egl-20* promoter sequence in vector pPD49.26. To generate *Pegl-20::mtm-6(C335S)::gfp* and *Pegl-20::snx-3(R70A)::gfp*, point mutations were introduced into the corresponding constructs using the QuikChange Site-Directed Mutagenesis kit (Stratagene). To generate stable extrachromosomal arrays, constructs were injected into the germline of young adults at a concentration of 1–10 ng/μl (Mello and Fire, 1995). *Pmyo-2::tdTomato* was used at a concentration of 10–15 ng/μl as a co-injection marker. RNAi was performed by feeding using clones from the Ahringer or Vidal libraries (Kamath *et al*, 2003; Rual *et al*, 2004). L4 larvae were placed on RNAi plates and phenotypes were scored in their progeny after 6–10 days incubation at 15 or 20°C.

Heat-shock experiments

Experiments with *Phsp16-2::mig-14* were performed on synchronized L1 larvae as described (Yang *et al*, 2008). In short, L1 larvae were collected that had hatched within a 1-h interval. Each batch was divided into two groups, one was exposed to a 10-min heat shock at 33°C, the other was left as a control. Animals were left to develop at 20°C and the position of the QL.d was scored at the L4 or early adult stage.

β-galactosidase detection

Detection of *mab-5::lacZ* expression was performed essentially as described previously (Fire *et al*, 1990). Briefly, synchronized L1 larvae were collected 4–5 h after hatching in M9 buffer, washed with distiled water and dried. Larvae were fixed with cold acetone and incubated in β-galactosidase staining solution (Fire *et al*, 1990) at room temperature. Larvae were counterstained with DAPI for better cell recognition.

Western blotting

To obtain synchronized populations of L1 larvae, populations of gravid hermaphrodites were subjected to hypochlorite treatment, embryos were allowed to hatch overnight in M9 buffer and the synchronized larvae were allowed to feed for 5–6 h. Larvae were then collected in TX-114 buffer (25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5 mM $CaCl_2$, 1% Triton X-114), frozen in liquid nitrogen and subsequently ground in liquid nitrogen to break open the cuticle. Lysates were centrifuged at 20 800 *g* at 4°C for 30 min, mixed with Laemmli sample buffer, separated on 8% SDS–PAGE and transferred onto PVDF membrane (Bio-Rad). GFP was detected with monoclonal anti-GFP antibody (clone JL-8, Clontech) and equal loading was assessed by staining with monoclonal anti-α-tubulin antibody (clone DM1A, Sigma). Densitometric analysis was performed on scanned images using the Gels plug-in of ImageJ (NIH).

C. elegans phenotypes and microscopy

QL.d migration and polarity of the ALM and PLM neurons were assessed in L4 larvae or young adults using the *mec-7::gfp* expressing transgenes *muIs32* or *muIs35* (Ch'ng *et al*, 2003). All strains were grown at 20°C, except for *rme-8(b1023)*, which was grown at 15°C. For *rme-8(b1023)*, newly hatched L1 larvae were shifted to the restrictive temperature of 25°C and the final positions of the QL.d was determined in L3 larvae. QL.d migration was scored as defective when the PVM daughter of QL was positioned at or anterior to the posterior edge of the vulva. The precise positions of the HSN and Q descendants were scored by DIC microscopy in synchronized L1 larvae as described (Coudreuse *et al*, 2006). For epi-fluorescence and DIC imaging, animals were mounted on 2% agarose pads containing 10 mM sodium azide. Images were obtained with a Zeiss Axioscop microscope equipped with a Zeiss Axiocam digital camera. For confocal microscopy, animals were immobilized by 10 mM sodium azide or 0.1% tricaine/0.01% tetramisole in M9 and observed with a Leica TCS SPE confocal microscope. For quantification of the SNX-3 vesicular localization, samples were scored blindly under the microscope and animals were assigned into one of three categories: having only cytoplasmic signal with no vesicular structures, having one to five distinguishable vesicles or having more than five clearly visible vesicles.

Drosophila stocks and immunostaining

Flies carrying an inducible *Dmtm-6* RNAi transgene were obtained from the Vienna *Drosophila* RNAi Center (Stock 26216). The hhGal4 and apGal4 driver lines were obtained from the Bloomington

Drosophila Stock Center and combined with a *UAS-CD8GFP* transgene to mark Gal4-expressing cells. DSnx3::GFP-expressing flies were generated by inserting a *ptub-snx3-GFP* plasmid into an attB landing site at cytological position 51D using the ϕ31 integrase system. Immunostaining was performed using standard protocols. Briefly, third instar larva were dissected in ice-cold Ringers solution. Discs were fixed and permeabilized in PBS containing 4% paraformaldehyde and 0.05% Triton X-100 for 25 min at room temperature (RT). Discs were washed in PBS containing 0.05% Triton X-100 (PBT) for 1 h at RT and then incubated in primary antibody solution at 4°C overnight. Afterwards, discs were washed in PBT containing 1% goat serum for 1 h at RT and subsequently incubated in secondary antibody solution for 2 h at RT. After a final wash for 1 h in PBT, discs were mounted on cover slips using double-sided tape as a spacer to avoid compression of the discs. Antibodies were diluted in PBT. Snx3::GFP was visualized in living discs immediately after dissection. Images were collected on a Zeiss LSM710 or Leica SP5 confocal microscope using the sequential scanning mode. Images were analysed using ImageJ (NIH).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author's contributions: MS, MH and HCK designed and carried out the *C. elegans* experiments, FP and KB designed and carried out the *Drosophila* experiments, MS and HCK wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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