UNRAVELING THE WNT SECRETION PATHWAY

Martin Harterink

The work described in this thesis was performed at the Hubrecht Institute for Developmental Biology and Stem Cell research within the framework of the Graduate School of Cancer Genomics and Developmental Biology and financially supported by the Boehringer Ingelheim Fonds and the Dutch Cancer Society.

Printing of this thesis was financially supported by the Dutch Cancer Society and the J.E. Jurriaanse Stichting.

ISBN: 978-94-90371-88-3

Cover: Stacked image of a DiO filled bag of worms

Layout and printing: Off Page, www.offpage.nl

UNRAVELING THE WNT SECRETION PATHWAY

Ontrafeling van de Wnt secretie route
(Met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op woensdag 29 juni 2011 des middags te 2.30 uur

door

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geboren op 26 juli 1981
te Utrecht

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CONTENTS

PREFACE

INTRODUCTION TO WNT SIGNALING

The Wnt pathway, like several other signaling systems, is a major molecular mechanism that controls animal development. Moreover, deregulation of Wnt signaling is tightly linked to human disease, such as multiple forms of cancer and bone malformation (Clevers, 2006; Klaus and Birchmeier, 2008). In sporadic colorectal cancer, the most common form of intestinal cancer, mutations in multiple Wnt signaling components have been found, ectopically activating the Wnt pathway. This is best illustrated by the gene adenomatous polyposis coli (APC) which is mutated in more than two-thirds of the cases (Segditsas and Tomlinson, 2006). More recently, Wnt signaling received additional attention for its important role in specification and maintenance of stem cells in various tissues and organs (Wend et al., 2010). Therefore, it is of critical importance to have a good understanding of this pathway. Over the last few decades much work has focused on the different signal transduction mechanisms initiated by binding of the Wnt ligand to receptors of Wnt responsive cells. More recently, also the Wnt posttranslational modifications and the secretion mechanism in the Wnt producing cells have received much attention. This will a major focus of this thesis and is further introduced in Chapter 1.

Wnt proteins were originally identified in Drosophila (Nusslein-Volhard and Wieschaus, 1980) and mice (Nusse and Varmus, 1982), which were called Wingless (Wg) and Int1, hence the name Wnt. These proteins are characterized by a high number of conserved cysteine residues and are glycosylated and lipid modified at two conserved residues, which makes Wnt proteins highly hydrophobic. Surprisingly, however, Wnt proteins have been shown to diffuse over several cell diameters in the extracellular space. Several mechanisms have been proposed to mediate this spreading to form a morphogenetic gradient, which will be further discussed in the next chapter. Additional factors in the extracellular space are able to regulate the activity of the Wnt morphogenetic gradient. Proteins of the secreted frizzled related protein (SFRP) family and the Wnt interacting factor 1 (Wif1) can bind Wnts and are therefore seen as Wnt inhibitors. Other factors act at the level of the Wnt receptors such as the Wnt signaling inhibitor Dickkopf (Dkk) and Wnt activator R-spondin.

Wnt receiving cells can induce several signaling cascades in response to the morphogenetic Wnt gradient. The best studied signal transduction mechanism is the Wnt/β-catenin pathway, which is also known as the canonical Wnt pathway (Fig. 1)(Clevers, 2006). Here, binding of Wnt to its receptors induces the stabilization of β-catenin, which is otherwise targeted for proteasomal degradation. Stabilized β-catenin translocates into the nucleus and activates TCF-LEF depended transcription of Wnt target genes. Other forms of Wnt signaling are collectively known as non-canonical Wnt signaling and include the Wnt-PCP (planar cell polarity) and the Wnt-calcium pathways. Although incompletely understood, it is likely that the combination of specific Wnt receptors in combination with the Wnt itself determines which signaling pathway is activated (Grumolato et al., 2010).

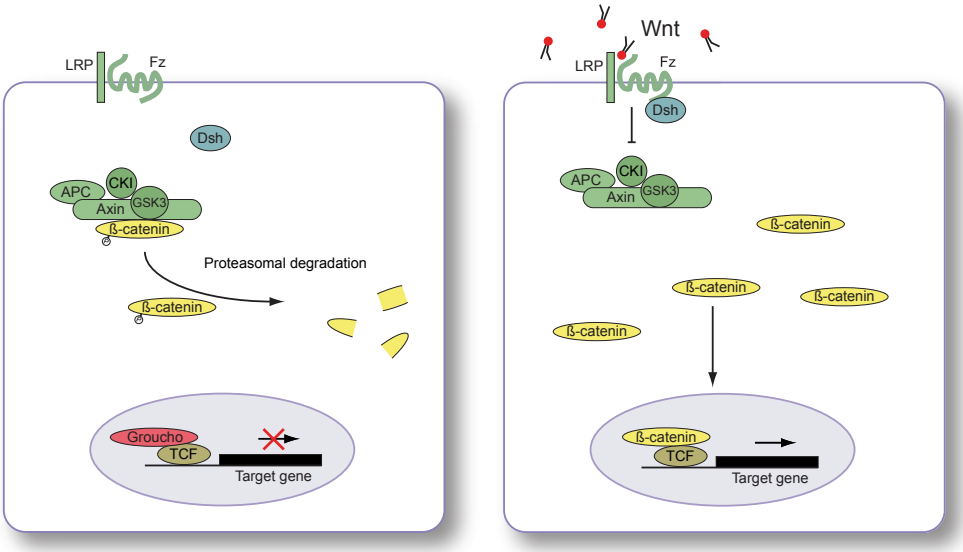

Figure 1. The canonical Wnt signaling pathway. In the absence of the Wnt ligand (left), β-catenin is phosphorylated by a destruction complex composed of Axin, APC and the kinases GSK-3β and CKIα, which will target it for proteasomal degradation. Furthermore, Wnt target genes are repressed by binding of the co-repressor Groucho to TCF (T-cell factor)-LEF (lymphoid enhancer factor) transcription factors. Upon binding of Wnt to the Frizzled receptor and the Lrp5/6 co-receptor (right), Disheveled (Dsh) is recruited to the plasma membrane and activated, which inhibits the destruction complex. This leads to increased cytoplasmic levels of β-catenin and translocation of β-catenin into the nucleus, where it displaces Groucho and forms a transcriptionally active complex with TCF-LEF.

WNT SIGNALING IN *C. ELEGANS*

In this thesis we have used the nematode *C. elegans* to study Wnt signaling. Its invariant cell lineage, ease of culturing and effective gene inactivation by RNA interference (RNAi) or mutagenesis are a few of the characteristics which make it a popular model organism to study biology.

The *C. elegans* genome contains five Wnt genes, which are important for anteroposterior (AP) pattering of the animal (Silhankova and Korswagen, 2007). One of these, EGL-20, is expressed in the tail region of the animal and forms a posterior to anterior concentration gradient (Coudreuse et al., 2006). This signaling gradient is essential for the characteristic migration of the Q neuroblasts and their descendents. EGL-20/Wnt induces a canonical Wnt signaling pathway in the left Q cell, which leads to posterior migration (Fig 2)(Whangbo and Kenyon, 1999). Note that the canonical Wnt signaling pathway is highly conserved in *C. elegans* (Fig 2B)(Korswagen, 2002). The migration of the right Q neuroblast descendants is dependent on non-canonical Wnt signaling by multiple partially redundant

acting Wnts. We have extensively used the migration of these neuronal cells as a readout for Wnt signaling in the work described in this thesis.

In addition to cell migration along the AP axis, *C. elegans* Wnt signaling was found to be important for multiple other processes. Non-canonical Wnt signaling is, for example, important for the polarity of the ALM and PLM neurons, which is often reversed or symmetric in Wnt mutants (Prasad and Clark, 2006). Also several asymmetric cell divisions were found to be regulated by Wnt signaling (Sawa, 2010). Although Wnt signaling is important for AP patterning of *C. elegans*, only the MOM-2/Wnt is essential for viability, since it regulates the asymmetric division of the EMS cell at the four cell stage (Rocheleau et al., 1997; Thorpe et al., 1997).

On the whole, Wnt signaling acts similarly in *C. elegans* as compared to other organisms. There are, however, some important differences. For example, none of the *C. elegans* LRP family member has yet been implicated in Wnt signaling. Furthermore, the *C. elegans* genome does not contain any Dkk or Wif1 orthologs. Despite these discrepancies, *C. elegans* has proven to be a valuable tool to study Wnt signaling, since multiple new Wnt components have first been identified in this model system.

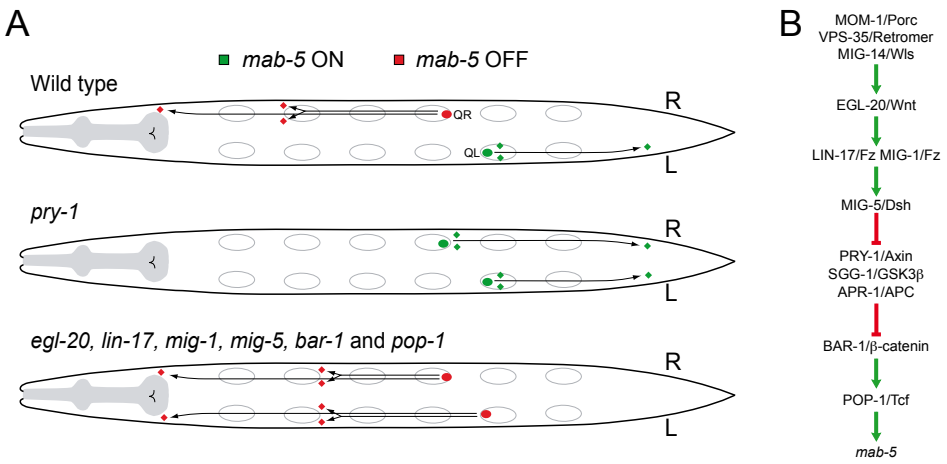

Figure 2 Q neuroblast migration (A) Two Q neuroblasts are born on the left (QL) and right (QR) side of the animal and give rise to the same set of daughter cells (QL.d and QR.d). However, due to a difference in sensitivity to the posteriorly expressed EGL-20/Wnt, only QL will induce canonical Wnt signaling. This results in the activation of the Hox gene *mab-5*, which induces posterior migration (Salser and Kenyon, 1992). The QR.d do not express *mab-5* and migrate towards the anterior, which is mediated by the partially redundantly acting Wnts, CWN-1, CWN-2 and EGL-20 (Zinovyeva et al., 2008). Upon ectopic activation of canonical Wnt signaling, such as in *pry-1*/Axin mutants, *mab-5* is expressed in the QR.d and therefore these cells also migrate in posterior direction. Conversely, upon loss of Wnt signaling, the QL.d loose *mab-5* expression and migrate in the anterior direction. Anterior is left and posterior is right; grey oval represent the hypodermal seam cells V1 to V6 along the A-P axis.

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SCOPE OF THE THESIS

With the recent identification of several important components required in Wnt producing cells for Wnt secretion, it has become apparent that Wnt secretion is tightly controlled. In Chapter 1 we will further introduce the current ideas about how Wnt proteins are produced and secreted. Additionally we will point out essential remaining question in this field, such as the function of lipid modification of Wnt as well as the mechanism of Wnt release into the extracellular environment.

In order to gain more insight into Wnt signaling, we have used a genome wide RNAi library to search for genes involved in *C. elegans* Q neuroblast migration. In Chapter 2 we report the identification of a number of interesting genes, and describe their initial analyses.

In Chapter 3 we describe the analyses of one of the genes identified in the screen, the sorting nexin *snx-3*, which is required for Wnt signaling. We found that SNX3 associates with the retromer for efficient Golgi retrieval of the Wnt sorting receptor MIG-14/Wls, whereas the classical retromer sorting nexins are dispensable for Wnt signaling. This suggests that multiple endosome to Golgi retrieval routes are taken by the retromer, requiring specific sorting nexin adaptors for membrane tethering.

Sorting nexins can bind the endosomally enriched lipid, phosphatidylinositol 3-monophosphate (PI3P), which has important roles in endosomal function. In Chapter 4 we describe the analyses of the myotubularin PI3P phosphatase family in Wnt signaling. We have found that *mtm-6* and *mtm-9*, which form a complex, are required for efficient Wnt secretion. In the Wnt producing cells MTM-6 and MTM-9 are required for the Golgi retrieval of the Wnt sorting receptor MIG-14/Wls, possibly by regulating the membrane attachment of the retromer via SNX-3.

Finally, in Chapter 5, we describe the expression patterns of the *C. elegans* Wnt genes and the single SFRP family member, *sfrp-1*, using single mRNA molecule fluorescent in situ hybridization (smFISH). We found that most Wnt genes are posteriorly expressed in a partially overlapping pattern whereas *sfrp-1* is expressed in the anterior. Furthermore, the analyses of a *sfrp-1* mutant shows that it is required to inhibit multiple Wnts for the correct anteroposterior migration of several neuronal cells. Our results suggest that the Wnts and SFRP-1 form opposing gradients to mediate anteroposterior patterning. Our results support the notion that posterior Wnt signaling and anterior Wnt inhibition is an ancient and highly conserved mechanism of primary body axis patterning.

1 DISSECTING THE WNT SECRETION PATHWAY: KEY QUESTIONS ON THE MODIFICATION AND INTRACELLULAR TRAFFICKING OF WNT PROTEINS

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Acta Physiologica, in press

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ABSTRACT

The Wnt family of signaling proteins has essential functions in development and adult tissue homeostasis throughout the animal kingdom. Although signaling cascades triggered by Wnt proteins have been extensively studied, much remains to be learned about how Wnts are produced and secreted. Over the past few years it has become clear that the secretion of Wnt proteins requires a specialized trafficking pathway. As this pathway has been discussed in two recent reviews (Lorenowicz and Korswagen, 2009; Port and Basler, 2010), we will focus our discussion on the key questions that need to be addressed to gain a more complete understanding of the mechanism and regulation of this essential secretion pathway.

The Wnt family of secreted signaling proteins is involved in virtually all aspects of development, with essential functions in the regulation of cell proliferation and differentiation, cell polarity and migration and nervous system development. Tight control of Wnt signaling is crucial for the correct orchestration of development, but is also essential during adult life, as illustrated by the fact that Wnt signaling is deregulated in a wide range of human diseases, most notably in cancer (Clevers, 2006; Logan and Nusse, 2004).

Wnt proteins are characterized by a high number of conserved cysteine residues (Miller, 2002) and are post-translationally glycosylated and lipid modified. Secreted Wnt proteins form concentration gradients in the extracellular space, to which cells expressing the appropriate receptors respond in a concentration dependent manner. This raises several interesting question: how is such a hydrophobic lipid modified protein secreted and released from Wnt producing cells and how can the Wnt protein form a concentration gradient that extends over several cell diameters? Although much research has focused on the signaling cascades that are triggered by Wnt proteins, the importance of Wnt producing cells in the Wnt signaling pathway is only recently becoming apparent. With the identification of several components required in the Wnt producing cells for Wnt signaling, the idea evolved that the production and secretion of Wnt molecules requires a specialized secretory machinery that offers an additional layer of control to the Wnt signaling pathway. Here, we will discuss the function of the post-translational modifications of Wnt and the dedicated Wnt secretory pathway in controlling the activity and range of Wnt signaling.

What is the function of the lipid modification of Wnt proteins?

When Wnt proteins were first isolated, a surprising finding was that they are highly hydrophobic (Bradley and Brown, 1990). This was explained by the identification of two lipid modifications (Fig. 1); a saturated acyl chain, palmitate, attached to a conserved cysteine residue (C77 in mouse Wnt3a) (Willert et al., 2003) and a mono-unsaturated acyl chain, palmitoleate, on a conserved serine residue (S209 in mouse Wnt3a) (Takada et al., 2006). For simplicity, we will refer to these positions as C77 and S209, although the exact positions may differ in other Wnt proteins. Mutating the C77 position in Wnt5a also led to a loss of hydrophobicity, suggesting that the lipid modification on this residue is conserved (Kurayoshi et al., 2007), although it is not known whether the same type of acyl chain is used.

Mutational analysis has shown that lipid modification is required for the secretion as well as the signaling activity of Wnt proteins. In mammalian Wnt proteins, mutating C77 to alanine strongly interferes with signaling activity, while secretion is largely unaffected (Galli et al., 2007; Komekado et al., 2007; Kurayoshi et al., 2007; Willert et al., 2003). Mutating the S209 residue, on the other hand, has a strong effect on secretion, resulting in accumulation of the

mutant Wnt protein in the endoplasmic reticulum (ER) (Takada et al., 2006). The effect of mutating the lipid modified residues is however less clear in *Drosophila*. In S2 tissue culture cells, both C77 and S209 mutants of the Wnt protein Wg are secreted, while in transgenic animals, there is accumulation of the C77A mutant Wg protein in the ER (Franch-Marro et al., 2008a). Despite these discrepancies, the current consensus is that lipidation is important for the exit of Wnt from the ER. The observed accumulation of Wnt proteins in the ER might be the result of defects in the folding of the mutated protein. Alternatively, membrane tethering might be required for the interaction of Wnt with the ER exit machinery. It cannot be excluded, however, that the ER accumulation is caused by overexpression of the mutated protein. Therefore, to better understand these results, it will be important to analyze these mutant Wnt proteins under physiological conditions. In *C. elegans* as well as in *Drosophila*, alleles of Wnt were isolated in which the C77 residue is mutated. This leads to a strong loss of Wnt signaling, although not as severe as observed in Wnt null mutants (Coudreuse et al., 2006; Couso and Martinez Arias, 1994; Willert et al., 2003), suggesting that C77 mutated Wnts still have residual signaling activity. The subcellular distribution of the mutated Wnt proteins has however not been studied. Interestingly, genetic screens have not recovered mutants with mutations in the S209 residue. Is this just coincidence or would this mutation lead to less penetrant phenotypes that were missed in genetic screens? The only functional data to compare the two lipid modification mutants is the above mentioned S2 cell secretion assay (Franch-Marro et al., 2008a). Here it was found that, in agreement with the *C. elegans* and *Drosophila* mutants, the C77 mutant has only residual activity, while the S209 mutant has retained more activity. It would therefore be very interesting to generate an endogenously expressed S209 Wnt mutant to address its in vivo importance, whether or not it is still C77 lipid modified and secreted and whether it retains signaling activity. This is also interesting because in *Drosophila* WntD, the only Wnt that is known to lack any lipid modification, C77 is conserved but S209 is not (Ching et al., 2008). Is the S209 residue critical for further lipidation or is the structure of WntD too different to be recognized by the lipidation machinery, as it does not have four conserved cysteine residues that are characteristic of all other Wnt proteins (Miller, 2002)?

Another interesting question is the biological significance of the S209 acylation with a mono-unsaturated fatty acid. The resulting double bond will induce a kink in the acyl chain, which may negatively influence the interaction with lipid ordered membrane domains (Moffett et al., 2000). It was shown for proteins such as Fyn, Annexin II and Gai that acylation with unsaturated fatty acids results in displacement of proteins from membrane domains with ordered lipid structure (Liang et al., 2001; Moffett et al., 2000; Zhao and Hardy, 2004). Therefore, this unsaturated lipid modification could play an important role in targeting of Wnt to specific membrane domains during secretion. Furthermore, modification with

an unsaturated acyl chain will slightly decrease membrane affinity compared to a saturated acyl chain, which may be important for the extracellular spreading of the secreted Wnt protein

An intriguing observation was made by Komekado and colleagues (Komekado et al., 2007). They used a Triton X-114 phase separation assay to test the hydrophobicity of Wnt3a, and similar to previous reports (Willert et al., 2003), found that while secreted Wnt partitions to the detergent phase, Wnt(C77A) partitions to the aqueous phase because of a loss of hydrophobicity (Komekado et al., 2007). Surprisingly, they found that in lysates of Wnt producing cells, a fraction of the C77A mutated Wnt is found in the Triton fraction, exactly like wild type Wnt3a, an observation that was also made by Galli and colleagues (Galli et al., 2007). This hydrophobic fraction was lost upon addition of the general O-acyltransferase inhibitor 2-bromopalmitate, suggesting that there is a pool of intracellular Wnt that is differently lipid modified as extracellular Wnt. Are these additional lipid modifications that are lost during Wnt maturation and secretion? Or could it be due to the recently identified protein Oto, which was shown to enhance Wnt hydrophobicity and ER retention (Wada et al., 2008; Zoltewicz et al., 2009)? Oto is a deacetylase that acts on the GPI membrane anchor precursor. Since Oto overexpression modifies Wnt in such a way that it cannot be further processed and is retained in the ER, the authors suggest that Wnts are GPI anchored for ER retention (Zoltewicz et al., 2009). It will be interesting to test whether 2-bromopalmitate also inhibits the Oto mediated GPI modification of Wnt.

A good candidate for mediating the lipid modification of Wnt is Porcupine (Porc), which was originally identified as a segment polarity gene in *Drosophila* (van den Heuvel et al., 1993) and encodes a member of the membrane-bound O-acyltransferase (MBOAT) family. Porc localizes to the ER, where Wnts are thought to be lipid modified (Zhai et al., 2004), and interacts with Wnts in a region that includes the C77 residue (Tanaka et al., 2002; Tanaka et al., 2000). Depletion of Porc leads to a complete block in Wnt secretion and accumulation of Wnt in the ER (van den Heuvel et al., 1993), similar as observed with the murine Wnt3a(S209) mutant. Furthermore, Porc depletion strongly reduces the hydrophobicity of Wnts (Zhai et al., 2004), whereas Porc overexpression increases hydrophobicity (Galli et al., 2007). Given the similarity in phenotype, it is likely that Porc is responsible for the S209 O-esterification of Wnt with palmitoleic acid. Whether Porc is also responsible for the C77 lipid modification is less clear, since this might be masked by the S209 modification and subsequent ER retention. Furthermore, one might question whether a single enzyme can specifically catalyze both oxyester (S209) as well as thioester (C77) formation using two different substrates (palmitoyl and palmitoyleoyl CoA) (Jing and Trowbridge, 1987; Rose et al., 1984). A possible explanation could be that Porc is responsible for the S209 acylation of Wnt and that subsequent C77 acylation is dependent on a different enzyme. But then why does Porc bind to the C77 region? Another

explanation could be that both C77 as well as S209 get palmitoylated by Porc and that the S209 palmitate gets de-saturated to palmitoleate by another enzyme. Experimental evidence for this possibility is however lacking.

In addition to a role in Wnt maturation and secretion, lipidation is also important for the signaling activity of Wnt proteins, as illustrated by the S2 reporter assays and genetic mutants discussed above and by the observation that enzymatic de-lipidation of purified secreted Wnt strongly inhibits signaling activity. Lipid modification is thought to restrict the spreading of Wnt in the extracellular environment and to concentrates it at the membrane for signaling. In addition, the lipid modifications are also required for the interaction with its receptors, as de-lipidation or mutation of C77 also results in a strong reduction in the affinity of Wnt for the Wnt binding domains of its receptors LRP and Frizzled (Cong et al., 2004; Franch-Marro et al., 2008a; Komekado et al., 2007; Kurayoshi et al., 2007).

What is the function of Wnt glycosylation?

Wnts are modified by multiple N-linked glycosylations, which, like the lipid modifications, are not well understood and are likely involved in regulating both secretion and signaling. For example, upon enzymatic de-glycosylation of secreted Wnt, signaling activity is strongly reduced, even though it still interacts with the Frizzled receptor (Komekado et al., 2007). Moreover, mutating the presumed modified residues or inhibiting the modification by a glycosylation inhibitor strongly affects Wnt secretion (although this may not be the case for all Wnts) (Komekado et al., 2007; Kurayoshi et al., 2007). These effects on secretion could be caused by folding defects, as one of the functions of glycosylation is to facilitate the folding of proteins in the ER (Caramelo and Parodi, 2007). Surprisingly, overexpression of Porc stimulates both lipidation and glycosylation and Porc depletion was reported to decrease glycosylation (Galli et al., 2007; Tanaka et al., 2002). It seems unlikely that Porc is directly responsible for the glycosylation of Wnt. It may, however, be involved in the recruitment of the oligosaccharide transferase (OST) complex. Alternatively, Porc overexpression may facilitate glycosylation by promoting membrane tethering of Wnt, making it better accessible to the OST complex.

Mouse Wnt3 is glycosylated on two asparagines (Fig. 1) (Komekado et al., 2007). Even though the corresponding residues in mouse Wnt5a and *Drosophila* Wg are also glycosylated, the first asparagine residue in mouse Wnt1 is not (Mason et al., 1992). This suggests that glycosylation is variable between the different Wnts. This conclusion is further supported by the fact that the second asparagine residue is not conserved in all human and *Drosophila* Wnt proteins. Differences in glycosylation could explain some of the discrepancies found in the functions of glycosylation in different Wnts.

The Wnt sorting receptor Wls

Three independent groups identified the multi-pass transmembrane protein Wls (also known as Evenness interrupted/Evi or Sprinter) as a critical component of the Wnt secretion machinery (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Wls is specific for Wnt secretion, since its depletion does not affect other secreted proteins. It is a highly conserved multi-pass transmembrane protein that binds Wnt in co-immunoprecipitation experiments (Banziger et al., 2006; Coombs et al., 2010; Fu et al., 2009). Wls localizes most prominently to the Golgi, the plasma membrane and endosomes (Banziger et al., 2006; Bartscherer et al., 2006; Belenkaya et al., 2008; Franch-Marro et al., 2008b; Port et al., 2008; Yang et al., 2008), indicating that it functions downstream of Porc in the secretory pathway. Analysis of Wls mutants in *Drosophila* showed that in the absence of Wls, Wnt accumulates in the Golgi, suggesting that Wls functions as a sorting receptor that transports Wnt from the Golgi to the cell surface for release. It was found that upon inhibition of clathrin mediated endocytosis (by depletion of the AP2μ subunit DPY-23 in *C. elegans* or dynamin in *Drosophila*) Wls accumulates at the plasma membrane (Belenkaya et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008). As this leads to a strong defect in Wnt signaling, it was proposed that Wls is endocytosed and is recycled to take part in multiple rounds of Wnt secretion (through a pathway that will be discussed below) (Fig. 2a). In this model, Wls plays a central role in the Wnt secretion pathway. Regulation of the trafficking of Wls therefore represents a mechanism to closely control Wnt secretion.

A key question that remains to be addressed is how Wnt is released from Wls. It has recently been shown that endosomal acidification is essential for the dissociation of Wnt form Wls, as inhibition of acidification by treatment with bafilomycin, a V-ATPase inhibitor, interferes with Wnt secretion (Coombs et al., 2010). Intracellular and plasma membrane levels of both Wnt and Wls were increased and importantly, Wnt and Wls remained in complex together. This suggests that vesicular acidification is somehow involved in the release of Wnt from Wls. However, a decrease in pH was not enough to dissociate a purified Wls-Wnt complex *in vitro*.

The Wnt binding domain of Wls was mapped to the first intraluminal loop and modeling of the structure of this domain revealed that it may fold into a lipocalin-like structure (Coombs et al., 2010). Lipocalins are a family of secreted proteins that can bind a range of hydrophobic molecules, including palmitate (Flower, 1996), indicating that the lipocalin-like domain of Wls might bind to the Wnt lipid modifications. In support of this possibility is the recent observation from the Nusse laboratory that soluble Wnt isolated from tissue culture medium is in complex with the lipocalin family member Swim (Nusse et al., 2008). It was shown that the S209 lipid modification is essential for the binding of Wnt to Wls, whereas the palmitoylated C77 residue is not (Coombs et al., 2010), indicating

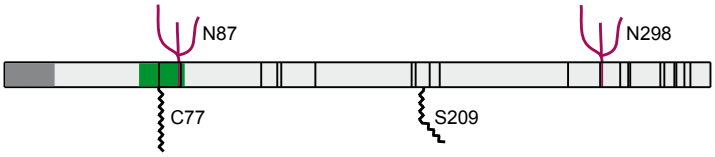

Figure 1. Schematic representation of mouse Wnt3a, showing the lipid modifications on C77 and S209, and the glycosylation modifications on N87 and N298. The conserved cysteines (black vertical bars), the Porc binding domain (green) and the signal sequence (dark grey) are indicated as well.

that in the case of Wls the lipocalin domain may bind the mono-unsaturated palmitoleic acid residue instead of the palmitate residue. Interestingly, it has been shown that a plant lipocalin family member dimerizes upon vesicular acidification (Arnoux et al., 2009). This suggests the intriguing possibility that vesicular acidification en route to the plasma membrane induces the release of Wnt from Wls by triggering dimerization of the Wls Wnt binding domains.

Endosomal sorting of Wls

In parallel to the discovery of Wls, it was found that an intracellular trafficking complex called the retromer is specifically required in the Wnt producing cells for Wnt signaling (Coudreuse et al., 2006; Prasad and Clark, 2006). The retromer was identified in yeast and was shown to mediate endosome to Golgi trafficking of membrane proteins, such as the vacuolar protein sorting receptor Vps10p (Seaman et al., 1998). Subsequently, it was shown that the retromer also transports Wls from endosomes to the Golgi as part of the recycling pathway that ensures that Wls can take part in multiple rounds of Wnt secretion (Belenkaya et al., 2008; Franch-Marro et al., 2008b; Pan et al., 2008; Port et al., 2008; Yang et al., 2008). In retromer mutants, Wls fails to be transported to the Golgi and is instead degraded in the lysosomal system, explaining the strong Wnt signaling defect in *C. elegans* and *Drosophila* retromer mutants. The retromer may therefore be at a key control point in the Wnt secretion pathway, deciding whether Wls is recycled or degraded and thereby setting the level of Wls available for Wnt secretion. This raises several important questions, such as how the retromer recognizes Wls and sorts it away from the degradative pathway and how the function of the retromer is regulated.

The retromer directly binds to the cytoplasmic tails of cargo-proteins through a cargo-selective subcomplex that consists of the subunits VPS26, VPS29 and VPS35 (Seaman et al., 1998). The retromer also binds Wls in co-immunoprecipitation experiments, but it is not known whether this is a direct interaction. Since no obvious retromer binding sequence can be identified in Wls (Seaman, 2007) and since it is a multi-pass transmembrane protein, it remains to be established which cytoplasmic domains are responsible for retromer dependent Golgi retrieval.

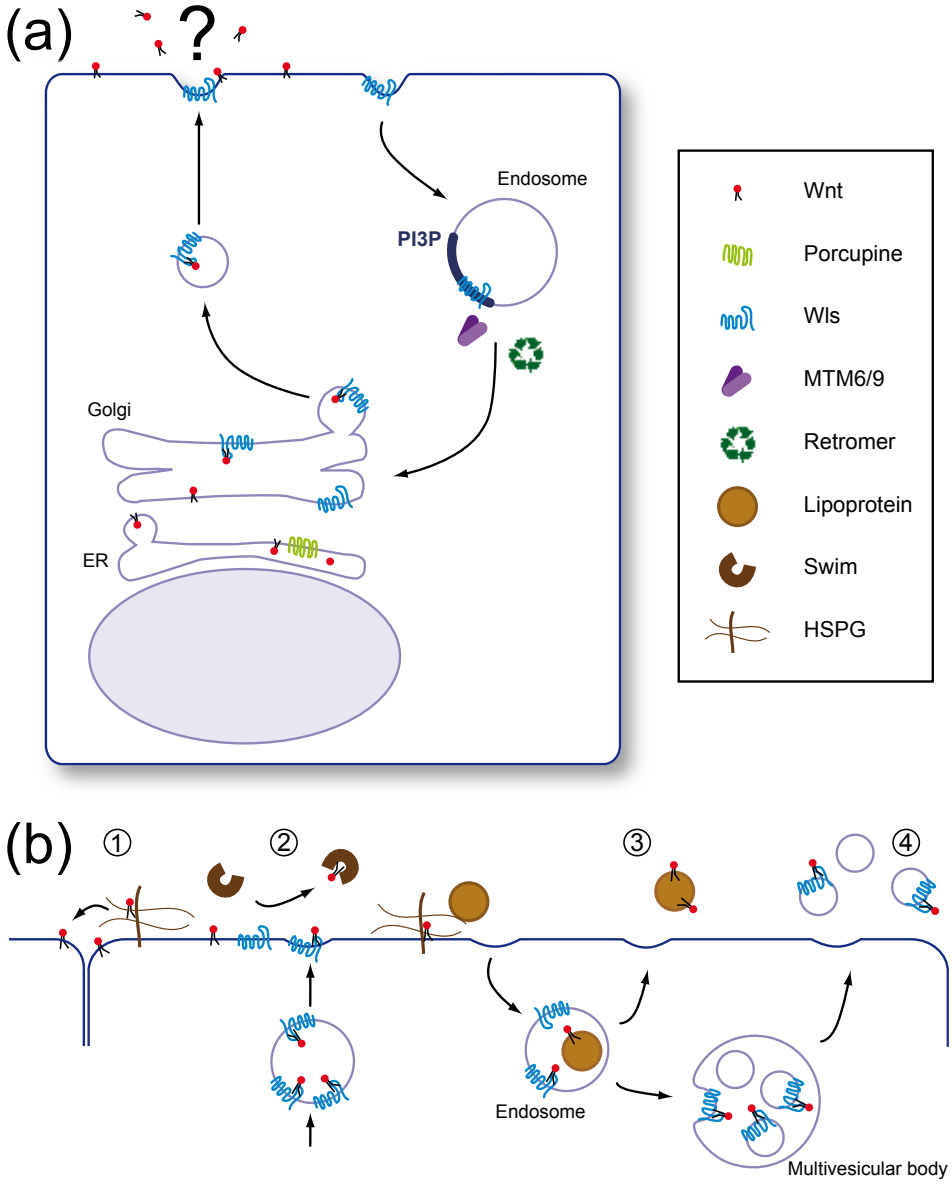

Figure 2. Model of the Wnt secretion pathway. (**A**) Wnt proteins are lipid modified in the ER, which requires the activity of Porcupine. Upon translocation to the Golgi, the sorting receptor Wls binds to Wnt and escorts it towards the plasma membrane. In order to perform multiple rounds of Wnt secretion, Wls is trafficked back to the Golgi by endocytosis followed by retromer dependent endosome to Golgi trafficking. Wls is depicted as an 8-pass transmembrane protein, since the signal sequence may not be processed and cleaved off (Jin et al., 2010) (**B**) Schematic representation of several possible mechanisms for Wnt release at the plasma membrane: by spreading (1), by association with the lipocalin Swim (2), lipoproteins (3) or exocytic vesicles (4).

In addition to the cargo-selective subunits, the retromer also includes an accessory complex that consists of sorting nexin family members, which are though to aid membrane tethering of the complex by binding to the endosomally enriched lipid phosphatidylinositol 3-phosphate (PI3P) (Seaman, 2005). Interestingly, we recently reported that mutants of specific myotubularin PI3P phosphatases have a strong defect in Wnt signaling and Wnt secretion in *C. elegans* and *Drosophila* (Silhankova et al., 2010). Since these myotubularins are required in Wnt producing cells and since in myotubularin mutants Wls levels are strongly reduced, it was proposed that myotubularins function with the retromer in Wls retrieval. Surprisingly, we found that the canonical retromer sorting nexin *snx-1* is not required for Wnt signaling (Coudreuse et al., 2006). However, another sorting nexin, SNX-3, which functions together with the retromer in retrieving Wls (M. Harterink, in preparation), accumulates on endosomes in the myotubularin mutants. Although the exact mechanism needs to be determined, these results suggest that a tight balance of sorting nexin recruitment and release is essential for correct Golgi retrieval of Wls. This is further supported by the fact that the myotubularin mutant phenotype could be suppressed by depleting components of the PI3P kinase complex. Importantly, although there are multiple myotubularins that are broadly expressed in *C. elegans*, only MTM-6 and MTM-9, which form a complex *in vivo*, are required for Wnt signaling (Silhankova et al., 2010). This suggests that the MTM-6/MTM-9 complex targets a specific pool of PI3P that is essential for the correct retromer dependent recycling of Wls. There is however an interesting difference between the effect the retromer and the myotubularin mutant have on Wls, which is not understood. Thus, knock down of retromer subunits in the *Drosophila* wing imaginal disc leads to a loss of Wls in Wnt producing cells but not in cells that do not express Wnt (Port et al., 2008), suggesting that Wls is differently trafficked in non-Wnt producing cells. Upon myotubularin depletion, however, Wls is lost in both the Wnt producing and the non-Wnt producing cells (Silhankova et al., 2010).

How is Wnt released from producing cells?

Since Wnts are highly hydrophobic and therefore tightly associated with membranes, the question arises how Wnt proteins are released from producing cells to spread in the tissue. Several mechanisms have been proposed that may act in parallel to mediate the solubilization of Wnt (Fig. 2b). One possibility is that Wnt simply spreads by diffusion, transferring laterally from cell to cell. Interactions with cell surface proteins such as heparin sulphate proteoglycans (HSPGs) or Wnt receptors modulates this diffusion and shapes the concentration gradient that Wnt forms in the tissue (Yan and Lin, 2009). A second possibility is that Wnt proteins are solubilized by shielding their hydrophobic lipid tails by binding to extracellular carriers. In *Drosophila*, Wnts have been proposed to insert into lipoproteins (Panakova et al., 2005). Furthermore, Wnt3a was found on

lipoprotein particles in the medium of mammalian Wnt producing tissue culture cells (Neumann et al., 2009). Lipoproteins have been shown to bind to HSPGs (Eugster et al., 2007), which might facilitate transfer of Wnt to and from cells. Finally, Wnts bind to the lipocalin family member Swim, which may also facilitate the spreading of Wnt in the tissue (Nusse et al., 2008).

Even though it is generally thought that the transfer of Wnts to these extracellular carriers occurs at the plasma membrane, it is interesting to consider that traffic through an endosomal compartment may be required for this transfer. The endocytic uptake of Wnts and extracellular carriers might bring them in close proximity for transfer, which would be followed by secretion of the carrier-Wnt complex. It is difficult, however, to address this *in vivo*, since endocytosis has several other functions in Wnt signaling. A recent study provides an example where Wnt may be required to traffic through the endocytic pathway for release. Korkut and colleagues describe trans-synaptic Wnt signaling at the *Drosophila* neuromuscular junction where, surprisingly, Wls is secreted along with Wg from the presynaptic terminal (Korkut et al., 2009). Since Wls is a multi-pass transmembrane protein, it may be secreted on exocytic vesicles. Such vesicles can be formed by fusion of multi-vesicular bodies with the plasma membrane, resulting in the release of the internal vesicles (Simons and Raposo, 2009). The presence of the Wnt-Wls complex on such intraluminal vesicles (which has also been observed by electron microscopy) (Korkut et al., 2009), can be explained by endocytosis of the complex at the cell surface, after which the complex is internalized from the endosomal limiting membrane in multi-vesicular bodies. However, whether this is indeed the case still remains to be established. In the *Drosophila* wing, Wnts have also been proposed to be secreted on exocytic vesicles, called argosomes (Greco et al., 2001). Although the presence of Wls on these argosomes was not addressed, it might require the same mechanism as described for signaling in the neuromuscular junction.

The fact that most of these Wnt release mechanisms have been identified in the *Drosophila* wing imaginal disc indicates that multiple mechanisms function in parallel to facilitate and control the release and spreading of Wnt in the tissue. Even though technically difficult, it will be interesting to determine to what degree endocytosis is required for these mechanisms and how the requirement of vesicular acidification for the dissociation of Wnt from Wls is linked to the subsequent release of Wnt from producing cells.

Finally, the idea that the Wnt producing cell itself might promote Wnt spreading by regulating its own plasma membrane composition is appealing. Expression of Wnt3a was shown to downregulate the expression of an enzyme involved in the biosynthesis of chondroitin sulfate proteoglycans (CSPGs) (Nadanaka et al., 2010). CSPG, like HSPG, binds Wnts and is thought to restrict Wnt diffusion for efficient signaling (Nadanaka et al., 2008). Therefore, autocrine Wnt signaling may lower the membrane affinity for Wnt by altering the CSPG

coat and thereby promoting Wnt release, but this has not been tested *in vivo* yet. Another mechanism that may assist the release and spreading of Wnt depends on Reggie-1/flotillin, which is a major component of membrane microdomains (Katanaev et al., 2008). Overexpression of Reggie-1 strongly expands the signaling range of Wnt by facilitating the capacity of Wnt to diffuse in the tissue. The mechanism by which Reggie-1 achieves this increase in Wnt spreading is however still unknown.

Regulation of Wnt secretion

Since Wnt signaling is such a key mechanism of development and adult tissue homeostasis, close regulation of Wnt signaling is essential. It is clear that there are several layers of regulation of the Wnt secretion process. First, there is transcriptional control of Wnt, but also of Wnt secretion factors. This is illustrated by Wls in the mouse, which is a target of the canonical Wnt/β-catenin pathway. This suggests a positive feedback mechanism in which Wnt stimulates Wnt secretion through an autocrine or paracrine mechanism (Fu et al., 2009). The induction of Wls expression by canonical Wnt signaling is however not evolutionarily conserved, as constitutive activation of canonical Wnt/β-catenin signaling in the Drosophila wing disc did not alter Wls levels (Port et al., 2008). Second, Wls translation in Drosophila and mouse cells is regulated by the miRNA miR-8 (Kennell et al., 2008).

Next to regulating the expression and translation of Porc and Wls, multiple other scenarios can be envisioned for the control of Wnt secretion. Although no regulators of Porc activity have so far been reported, is seems likely that its enzymatic activity is directly or indirectly regulated. Another important level of control is the Wls trafficking pathway, which offers several layers of regulation for Wnt trafficking and release. It enables the delivery of Wnt to specific regions of the plasma membrane, as illustrated by the proposed polarized secretion of Wg in the *Drosophila* wing imaginal disc (Gallet et al., 2008; Strigini and Cohen, 2000). In addition, regulation of Wls stability will have a large impact on the rate of Wnt secretion. Such regulation can be at the level of the choice between retromer dependent recycling or lysosomal degradation, for example by controlling the recruitment of retromer to Wls containing endosomes.

Further perspectives

In this review we have tried to give an overview of how Wnts are produced and secreted, focusing on several key aspects that need to be addressed to gain further insight into Wnt secretion and release. The Wnt secretion mechanism is surprisingly specific and is highly conserved throughout evolution, as illustrated by Wls, which is present in organisms ranging from simple cnidarians to mammals (Banziger et al., 2006; Bartscherer et al., 2006; Guder et al., 2006). Although there is now a widely accepted model of the Wnt secretion pathway,

it is clear that important variations on this pathway exist. For example, Korkut and colleagues have convincingly shown that Wls is secreted along with Wnt in trans-synaptic signaling at the neuromuscular junction (Korkut et al., 2009). Perhaps even more surprisingly, they show by cell specific Wls depletion that Wls also plays a role in the Wnt receiving cells. This is the first and only example of Wls functioning in Wnt responding cells. In the *Drosophila* wing imaginal disc Wls is also observed in the Wnt receiving cells, but no defect in Wnt signaling is observed upon Wls depletion in these cells (Port et al., 2008). Further research will be needed to better understand this novel function of Wls.

Wnt signaling is essential for human development and its deregulation is closely linked to disease. Even though most disease causing mutations in the Wnt signaling pathway are found downstream of Wnt secretion, also Porc and Wls are associated with disease. Mutations or deletions in Porc are associated with several developmental disorders, such as focal dermal hypoplasia and osteopathia striata (Behninger and Rott, 2000; Grzeschik et al., 2007; Wang et al., 2007) and the Wls locus was found associated with bone-mineral-density defects (Rivadeneira et al., 2009). Furthermore, Porc is overexpressed in several cancer cell lines and in lung cancer and importantly, downregulation of Porc in these cells leads to a loss of Wnt signaling and apoptosis, demonstrating the importance of Porc for tumor cell growth and survival (Chen et al., 2008). Recently, potent inhibitors of Porc were identified which potentially could be used to treat such cases (Chen et al., 2009). Interestingly these inhibitors were identified in a screen searching for Wnt/ß-catenin signaling inhibitors in cells harboring mutations in APC. This suggests that such drugs might be effective even when downstream components are mutated. Interfering with Wnt secretion therefore offers an important possibility for therapeutic intervention in Wnt dependent tumorigenesis.

ACKNOWLEDGEMENTS

We thank Dr. M. Lorenowicz for critically reading the manuscript. This work was funded by a grant from the Dutch Cancer Society (HUBR 2008-4114) and a NWO VIDI fellowship (016.076.317) to H.C.K. and a Boehringer Ingelheim Foundation fellowship to M.H.

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2

IDENTIFICATION OF NOVEL WNT PATHWAY COMPONENTS THROUGH GENOME-WIDE RNAI SCREENS IN *C. ELEGANS*

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Manuscript in preparation

ABSTRACT

Wnt proteins are signaling molecules that play a central role in development and adult tissue homeostasis. Wnt proteins trigger different intracellular signaling pathways that control target gene transcription or directly interface with regulators of the cytoskeleton to modulate cell polarity and cell migration. To gain further insight into these signaling mechanisms, we performed genome-wide RNAi screens to identify genes that are required for the Wnt dependent migration of the Q neuroblasts in *C. elegans*. Using screens in both wild type and sensitized genetic backgrounds, we found a total of 56 genes that significantly affect Q neuroblast migration. These include known Wnt pathway components, but also genes with functions in intracellular trafficking, protein degradation, signal transduction and gene expression. An initial analysis indicates that some of these genes may encode novel regulators of Wnt signaling and cell migration.

INTRODUCTION

Wnt proteins are members of a highly conserved family of signaling proteins with essential functions in development and adult tissue homeostasis (Clevers, 2006). Wnt signaling has been extensively studied, but important questions remain about the signaling mechanism and the regulation of the signaling pathway *in vivo*. Here, we took a genetic approach in the nematode *C. elegans* to identify novel components and regulators of the Wnt signaling pathway.

The Q neuroblast lineage of the nematode *C. elegans* offers a system in which Wnt signaling can be studied at single cell resolution. At the end of embryogenesis, two Q neuroblasts are born at equivalent positions on the left (QL) and right (QR) lateral side of the animal (Fig. 1) (Chalfie and Sulston, 1981; Sulston and Horvitz, 1977). During the first stage of larval development, the two Q neuroblasts generate an identical set of descendants that migrate in opposite directions between the two sides: on the left side, the QL descendants (QL.d) migrate towards the posterior, whereas on the right side, the QR.d migrate towards the anterior. This difference in migration direction is specified during an initial polarization phase in which the two Q neuroblasts are instructed to respond differently to the Wnt protein EGL-20 (Honigberg and Kenyon, 2000; Whangbo and Kenyon, 1999). The QL neuroblast becomes highly sensitive to EGL-20 and activates a canonical Wnt/β-catenin pathway to induce the expression of the target gene *mab-5*, which in turn directs the migration of the QL.d towards the posterior (Harris et al., 1996; Maloof et al., 1999; Salser and Kenyon, 1992). The QR neuroblast, on the other hand, becomes much less sensitive to canonical Wnt/β-catenin pathway activation by EGL-20 and fails to induce *mab-5*, resulting in migration of the QR.d in the default anterior direction. The final position of the Q cell descendants therefore provides a sensitive measure of canonical Wnt/β-catenin pathway activity. In mutants that disrupt Wnt/β-catenin signaling, QL fails to induce *mab-5* expression and the QL.d migrate in the default anterior direction (Harris et al., 1996; Maloof et al., 1999; Salser and Kenyon, 1992). Mutants that constitutively activate the pathway, such as mutants of the negative regulator *pry-1*/Axin, induce EGL-20 independent expression of *mab-5* in QR and therefore posterior migration of the QR.d. Finally, in mutants that disrupt the specification of the left right asymmetry in Wnt signaling response, the Q descendants migrate randomly between the anterior and posterior (Honigberg and Kenyon, 2000). In addition to Wnt signaling components, also mutation of genes that function in the guidance and migration of the Q descendants will affect the final positioning of the cells.

Here, we have used genome-wide RNAi screens to identify genes that are required for the highly stereotypic migration of the Q cell descendants. In addition to known Wnt pathway components we found genes involved in a variety of cellular pathways. An initial analysis suggests that this set includes novel regulators of Wnt signaling.

RESULTS

2 A genome-wide RNAi screen for genes that affect the stereotypic migration of the Q cell descendants

To identify novel genes that are required for Q cell migration, we performed a genome-wide RNAi screen using a library which covers about 85% of the predicted genes of *C. elegans* (Fraser et al., 2000; Kamath et al., 2003). To rapidly score the final positions of the Q descendants, we used an integrated *mec-7::gfp* reporter transgene (Ch'ng et al., 2003), which is expressed in the six touch receptor neurons, including the Q descendants AVM (QR.paa) and PVM

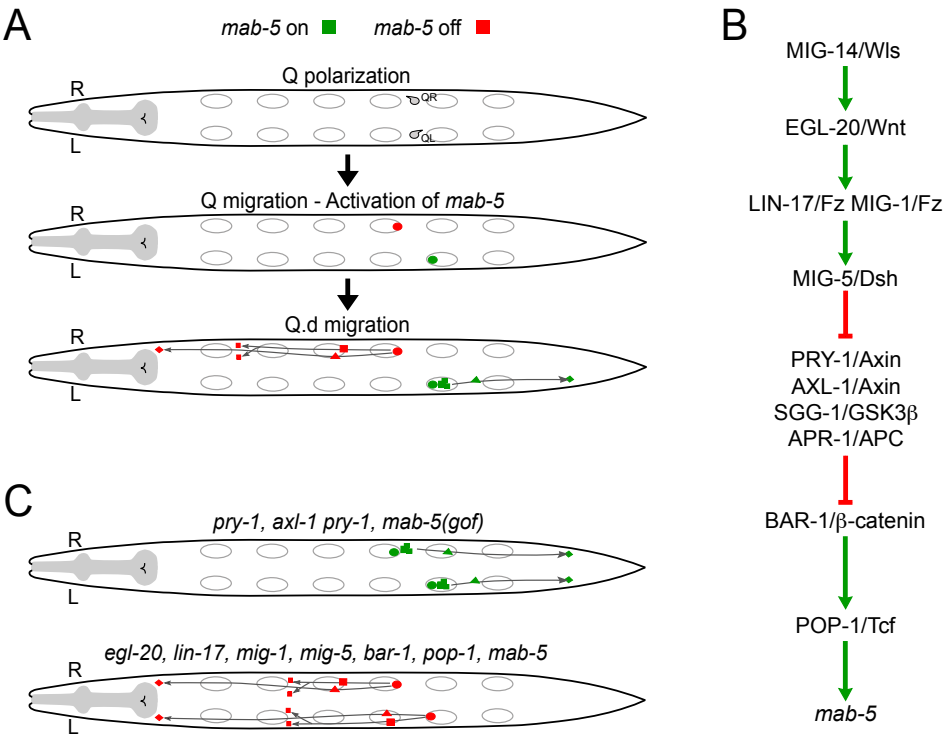

Figure 1 Q cell migration and EGL-20/Wnt signaling. (A) Three principal steps determine the migration of the Q daughter cells. On the left side, QL polarizes and shortly migrates posteriorly, whereas on the right side, QR polarizes and migrates anteriorly. This results in activation of the homeobox gene *mab-5* only in QL and its descendant. In turn, *mab-5* activation drives posterior migration of the QL daughter cells (QL.d). (B) The C. elegans EGL-20/Wnt triggers a canonical Wnt pathway that controls *mab-5* expression. (C) Ectopic activation of EGL-20 signaling in the QR daughter cells results in *mab-5* expression and posterior migration, whereas inhibition of the pathway in the QL.d leads to anterior migration. All the mutants are loss- or reduction-of-function, except when specified. gof: gain-of-function.

(QL.paa). Three categories of phenotypes were expected in the screen (Fig. 1). First, silencing of positive regulators of *mab-5*, such as components of the EGL-20/Wnt pathway, will result in anterior migration of the QL.d (QL.d phenotype). Second, silencing of negative regulators of the EGL-20/Wnt pathway will result in ectopic expression of *mab-5* in QR and posterior migration of the QR.d (QR.d phenotype). In addition, such a phenotype could result from disruption of non-canonical Wnt signaling in QR (see Chapter 5) or from disruption of cell migration itself. Finally, the simultaneous observation of both phenotypes suggests a role for the encoded protein in the initial specification of the left right asymmetry in Wnt signaling response (random phenotype). We identified genes in all three categories (first column Table 1). As expected, we found multiple components of the Wnt/β-catenin signaling pathway: the Frizzled *mig-1*, the Dishevelled *mig-5* and the β-catenin *bar-1*. In addition, we found 10 genes that have not previously been implicated to regulate Q cell migration. Silencing of four of these genes resulted in anterior migration of the QL.d; three showed posterior localization of the QR.d and four displayed both phenotypes. We have previously reported the identification of one of these genes, *vps-35* (Coudreuse et al., 2006), which encodes a subunits of the retromer, a protein complex which traffics cargo from endosomes to the *trans*-Golgi network (TGN) (Seaman, 2005). We and others have shown that the retromer is required in Wnt producing cells, where it mediates trafficking of the Wnt sorting receptor MIG-14/Wls, a process that is essential for the efficient secretion of Wnt (Coudreuse et al., 2006; Prasad and Clark, 2006).

Genome-wide RNAi screen for QL.d migration defects in sensitized genetic backgrounds

The fact that we did not find all known Wnt pathway components in our screen illustrates the high false negative rate in genome-wide RNAi screens in *C. elegans*. Based on comparisons between phenotypes of genetic mutants and phenotypes induced by RNAi, it has been estimated that only 45% of genes affecting post-embryonic development and function induce a phenotype by the feeding RNAi approach used in genome-wide RNAi screens (Fraser et al., 2000). This indicates that genes affecting Q cell migration have potentially been missed in our RNAi screen. To enhance the likelihood of finding these genes, we repeated the screen in sensitized genetic backgrounds. To sensitize for defects in the EGL-20/Wnt dependent posterior migration of the QL.d, we used a hypomorphic allele of the TCF transcription factor *pop-1* and a null allele of *vps-29*, a retromer subunit that is only partially required for endosome to TGN recycling of MIG-14/Wls and thus EGL-20/Wnt secretion (Yang et al., 2008). In both backgrounds, EGL-20 signaling is reduced, resulting in a partially penetrant loss of *mab-5* expression and anterior migration of the QL.d (20-40% of the animals show anterior localization of the QL.d). To verify the efficiency of the two sensitized genetic backgrounds, we compared the effect of *vps-35* RNAi in wild type, *pop-1(hu9)* and

Locus	gene		Screen		Description
		wildtype	*vps-29*	*pop-1*	
Wnt signaling					
mig-1	Y34D9B.1	QL.d	+	+	Frizzled
mig-5	T05C12.6	QL.d	n.d.	(+)	Dishevelled
bar-1	C54D1.6	QL.d	n.d.	(-)	beta-cathenin
Intracellular Traffic					
vps-35	F59G1.3	QL.d	+	+	VPS35
dab-1	M110.5	QL.d	+	-	Disabled2
apb-3	R11A5.1	QL.d	n.d.	(-)	adaptor protein complex 3, subunit Beta1/2
vps-26	T20D3.7		+	+	retromer subunit
snx-3	W06D4.5		+	+	SNX3/SNX12
vps-34	B0025.1		+		VPS34 PI3-kinase
dhc-1	T21E12.4		+		Dynein heavy chain
wdfy-2	D2013.2		+		WD40 and FYVE-domain containing protein
mtm-3	T24A11.1		+		Myotubularin PI3P phosphatase
	ZK930.1		+		VPS15
bec-1	T19E7.3		+		Beclin1
sec-10	C33H5.9		+		Exocyst subunit
vha-2	R10E11.2		+		Vacuolar H+-ATPase V0 domain subunit
vha-3	Y38F2AL.4		+		Vacuolar H+-ATPase V0 domain subunit
obr-4	C32F10.1		+		Oxysterol-binding protein
clh-5	C07H4.2		+		Chloride channel
	C42C1.4		+		VPS8, CORVET tethering complex subunit
cup-4	C02C2.3		+		Ion channel
	F46F11.9		+		TRAPP complex subunit Trs85
Signaling					
	F01F1.13	random	+	+	Semaphorin-5A precursor
ceh-20	F31E3.1	QR.d	(+)	(mix)	hox co-factor
let-502	C10H11.9		+	+	Rho-binding Ser/Thr kinase
kin-10	T01G9.6		+	+	Casein kinase II, beta subunit
rack-1	K04D7.1		+	+	Receptor of Activated C Kinase
ruvb-1	C27H6.2		+		RuvB-like
max-2	Y38F1A.10		+		p21-activated serine/threonine protein kinase
Transcription/mRNA maturation/protein synthesis					
nhr-25	F11C1.6	QR.d	n.d.	(mix)	Nuclear hormone receptor betaFTZ-F1
pab-1	Y106G6H.2		+	+	PolyA binding protein
let-607	F57B10.1		+	+	CREB/ATF family transcription factor
hrp-1	F42A6.7		+	+	Ribonucleoprotein
	C08H9.2		+	+	vigilin
cpf-1	F28C6.3		+		Cleavage stimulation factor subunit 1
rpb-11	W01G7.3		+		RNA polymerase, subunit L
rps-27	F56E10.4		+		40s ribosomal subunit S27
	Y65B4BR.5		+		Nascent polypeptide-associated complex subunit
emb-4	Y80D3A.2		+		Intron-binding protein aquarius
rsp-1	W02B12.3			+	Protein splicing factor
ntl-2	B0286.4			+	CCR4-NOT transcription complex subunit 2
Protein degradation					
csn-2	B0025.2		+	+	COP9 signalosome subunit
dcn-1	H38K22.2		+		DCN1-like protein
apc-2	K06H7.6			+	Anaphase-promoting complex subunit 2
Extracellular matrix					
	W03F8.10	QR.d	n.d.	(mix)	Laminin subunit beta
sqt-3	F23H12.4	random	(-)	(+)	Collagen
lam-2	C54D1.5	QR.d	n.d.	(mix)	laminin subunit gamma
Others/unknown					
	F53F4.11	QL.d	n.d.	(+)	Ribosomal L1 domain-containing protein
imp-2	T05E11.5	random	+	+	IntraMembrane Protease (IMPAS) family
	Y17G7B.18		+		7SK snRNA methylphosphate capping enzyme
	R148.2		+		Hepatitis B virus X-interacting protein
vit-3/4/5	F59D8.1/2;C04F6.1		+		vitellogenin
din-1	F07A11.6		+		Msx2-interacting protein
	D2013.3		+		Novel protein
npl-4.1/2	F59E12.5		+		Nuclear protein localization protein 4
ogt-1	K04G7.3			+	O-linked N-acetylglucosamine transferase

◀ **Table 1** Genes identified in the genome-wide RNAi screens. The screen in the wildtype background resulted in three types of phenotypes: anterior migration of the QL daughter cells (QL.d phenotype), posterior migration of the QR daughter cells (QR.d phenotype) and a combination of both (random phenotype). The genes identified in the sensitized genetic backgrounds *vps-29* and *pop-1* resulted in at least a twofold enhancement of the QL.d phenotype and are marked by + (also see Table S1). Phenotypes between brackets are genes which were identified in the wildtype screen but not in the sensitized background screen and were later tested. The sequences of the genes *vit-3*, *vit-4* and *vit-5* are very similar; therefore the RNAi is predicted to deplete all three genes. mix = both QL.d as well as QR.d migration defect is observed, n.d. = not determined.

vps-29(tm1320). Whereas *vps-35* RNAi induced a 31% penetrant QL.d migration phenotype in wild type animals, QL.d migration was almost fully defective in the sensitized genetic backgrounds. These results clearly demonstrate that the *pop-1* and *vps-29* backgrounds strongly sensitize for defects in EGL-20/Wnt signaling.

Rescreening of the genome-wide RNAi library for enhancers of the sensitized mutant backgrounds resulted in 46 genes which reproducibly enhance the phenotype of either of these backgrounds at least twofold (Table 1 and Table S1). 42 of these genes were not previously identified, either because they were missed in the first screen or because they only produce a QL.d migration phenotype in the sensitized genetic backgrounds. This latter possibility is illustrated by the retromer subunit *vps-26* and the sorting nexin *snx-3* that were identified in the second screen. Neither of these genes produced a significant QL.d migration phenotype in a wild type background (Table S1), explaining why they were not picked up in the first screen. Null mutants of *vps-26* and *snx-3* are however fully defective in QL.d migration (Table 2), indicating that these genes are inefficiently targeted by RNAi. These results validate our approach of using sensitized genetic backgrounds to reduce the false negative rate in genome-wide RNAi screens.

Similar to *snx-3* and *vps-26*, most of the other genes identified in the sensitized RNAi screen did not result in substantial defects in a wild type genetic background (Table S1). To test whether this is because of poor targeting by RNAi or because of a synthetic interaction with *pop-1* or *vps-29*, we tested available genetic mutants of these genes. Null mutations of 9 genes were homozygous viable and 4 of these produced a significant QL.d migration phenotype (Table 2). These included the retromer subunit *vps-26* and the sorting nexin *snx-3* discussed above, as well as the Disabled2 ortholog *dab-1* and the uncharacterized ion channel gene *cup-4*. The five other genes did not induce a defect in QL.d migration. As knock down of these genes enhanced the *vps-29* but not *pop-1* QL.d migration phenotype, we speculated that the enhancement is the result of a synthetic genetic interaction with *vps-29*. To test this, we constructed double mutants of null alleles of these genes with *vps-29*. We found that *obr-4* and *wdfy-2* induced a clear enhancement of the *vps-29* induced QL.d migration phenotype, indicating that these genes are only required for QL.d migration when the retromer complex is partially defective.

Table 2 Analyses of the QL.d migration defect of several genetic mutants for genes identified in the screen

genotype	QL.d (%)
wildtype	0
apb-3(ok429)	8
C08H9.2(ok1071)	0
cup-4(ok837)	4
dab-1(gk291)	12
din-1(dh149)	0
obr-4(hu189)	0
ogt-1(ok430)	0
snx-3(tm1595)	99
vps-26(tm1523)	100
vps-35(hu68)	100
wdfy-2(tm3806)	0
bec-1(ok691) (F1)	0
hrp-1(ok963) (F1)	2
ntl-2(ok974) (F1)	0
rack-1(tm2262) (F1)	0
sec-10(tm3437) (F1)	0
vps-29(tm1320)	17
C08H9.2(ok1071);vps-29(tm1320)	6
obr-4(hu189);vps-29(tm1320)	76
wdfy-2(tm3806);vps-29(tm13420)	74
bec-1(ok691);vps-29(tm1320) (F1)	38

The QL.d migration defect (in %) was scored using the *mec-7::GFP* transgene or by Nomarski optics (n>100, except for *vps-26* (n=58), *din-1* (n=26), *hrp-1* (n=42), *sec-10* (n=51), *ntl-2* (n=30) and *bec-1*;*vps-29* (n=55)).

Several of the genes that were identified in the sensitized RNAi screen are essential genes, with null alleles showing sterility or embryonic or larval lethality. Mutants of these genes were grown as heterozygous animals and scored in the first homozygous generation (F1). We could confirm the synthetic interaction of *bec-1* with *vps-29*, but none of the five genes tested produced a significant QL.d migration defect as F1 homozygous mutant. It should be noted, however, that maternally contributed mRNA or protein may be sufficient for normal QL.d migration in the F1 generation. This is illustrated by *vps-35* and *snx-3*, which are maternally rescued in the first generation, but show a fully penetrant QL.d

migration phenotype in the second generation (results not shown). As we could not assay effects of essential genes in second generation homozygotes, it cannot be ruled out that some of these genes may have a function in Q cell migration that is independent of the sensitized genetic background.

Genes required for the posterior migration of the QL.d

In our first genome-wide RNAi screen we identified four genes which displayed a QL.d migration defect. In the sensitized screen, which focused solely on QL.d migration, we identified 43 more genes which reproducibly enhanced the QL.d migration defects of the *vps-29* or *pop-1* mutant backgrounds (Table 1). Interestingly, a considerable proportion of these genes has known functions in intracellular trafficking: 3 of the genes identified in the initial screen and approximately a third of the genes identified in the sensitized screen. The genes *vps-35*, *vps-26* and the sorting nexin *snx-3*, are components of an alternative retromer pathway that traffics MIG-14/Wls from endosomes to the Golgi (Chapter 3). Trafficking of MIG-14/Wls between the Golgi and the plasma membrane is probably the most important function for trafficking in Wnt signaling. It is therefore tempting to speculate that at least some of the other trafficking genes identified in our screens are involved in MIG-14/Wls transport. However, a role in the Wnt responsive cells cannot be excluded, as endosome acidification and ESCRT mediated sequestering of GSK3 are essential for Wnt signaling as well (Cruciat et al., 2010; Taelman et al., 2010).

In addition to the retromer, we also identified the Disabled2 ortholog *dab-1* and the adaptor protein complex 3 (AP3) beta1/2 subunit, *apb-3* in our RNAi screen. The AP3 complex is believed to play a role in the transport of proteins to lysosomes. *apb-3* RNAi resulted in a mild but reproducible anterior migration of the QL.d, which to a large extent was linked to a small and dumpy phenotype. Analysis of an *apb-3* deletion mutant confirmed the results obtained by RNAi. Thus, *apb-3* mutants showed 8% anterior migration of the QL.d (n=400) and up to 27% when only small and dumpy animals were scored (n=200) (Table 3 and results not shown). Also *dab-1* is required for posterior localization of the QL.d (Table 3). In *dab-1(hu186)* and *dab-1(gk291)* mutants, the QL.d are anteriorly located in up to 80% of the animals (Table 3). Disabled2 is a widely expressed adaptor protein involved in signaling and cellular trafficking. *C. elegans dab-1* was shown to be required for cell-type specific endocytosis (Holmes et al., 2007) and to bind the LDL receptor-related proteins LRP-1 and LRP-2 to mediate secretion of the FGF protein EGL-17. As the migration of the QL.d is independent of EGL-17, our results indicate that *dab-1* has an additional function in EGL-20/Wnt signaling. We found that the *dab-1* induced QL.d migration phenotype is fully suppressed by a gain of function mutation of *mab-5* (Table 3), indicating that *dab-1* functions upstream of *mab-5* in the QL.d migration processes. Interestingly, human Disabled2 has also been linked to Wnt signaling, but in

Table 3 *dab-1* is required for *mab-5* dependent QL.d migration

genotype	QL.d (%)
dab-1 RNAi	5
rrf-3(pk1426); dab-1 RNAi	54
dab-1(hu186)	12
*dab-1(hu186)**	83
*dab-1(gk291)**	78
*dab-1(hu186);dab-1::gfp**	6
*dab-1(hu186);mab-5(e1751)gf**	0

The QL.d migration defect (in %) was scored using the *mec-7::GFP* transgene (n>100). Worms were grown and analyzed at 20 °C except those marked by an asterisk, for which embryos were collected by bleaching and grown at 15 °C.

contrast to *C. elegans dab-1*, human Dab2 was shown to function as a negative regulator of Wnt signaling (Hocevar et al., 2003). It is not clear why human Dab2 and *C. elegans* DAB-1 have such different functions in Wnt signaling. In contrast to human Dab2, DAB-1 does not bind to Axin (data not shown), indicating that DAB-1 has a different function in *C. elegans* Wnt signaling. One possibility is that Disabled functions both in Wnt sending and responding cells, with a more dominant function of human Dab2 in Wnt signaling and *C. elegans* DAB-1 in Wnt secretion. Cell type specific rescue experiments are needed to distinguish between these two scenarios.

We also identified several genes which encode proteins that are involved in phosphatidylinositol 3-monophosphate (PI3P) formation or turnover. We found the PI 3-kinase gene *vps-34* and its regulatory subunits *vps-15* and *bec-1*, as well as a member of the PI3P phosphatase family, the myotubularin *mtm-3*. In addition, we found proteins with a PI3P binding domain, such as the PX domain containing sorting nexin SNX-3 and the FYVE domain containing protein WDFY-2. PI3P is enriched on endosomes and is a key regulator of endosomal trafficking (Lindmo and Stenmark, 2006). PI3P also plays an important role in retromer function. Members of the sorting nexin family (SNX-3 in the case of MIG-14/Wls recycling, see Chapter 3 and 4) are recruited to cargo containing endosomal membranes by binding to PI3P. Tight regulation of PI3P formation and degradation is therefore essential for the recruitment and dissociation of the retromer complex. Indeed, we found that the myotubularins MTM-6 and MTM-9, which de-phosphorylate PI3P, are required for maintaining a proper balance of SNX-3 recruitment and dissociation (Chapter 4). Although we found *mtm-3* in our screen, this myotubularin only plays a minor role in MIG-14/Wls recycling and Wnt signaling.

Other genes implicated in intracellular trafficking are the vacuolar proton pump (V-ATPase) components, *vha-2* and *vha-3*. The V-ATPase plays an important role

in endosomal acidification and has been proposed to function in Wnt signaling, both in Wnt producing cells for Wnt secretion (Coombs et al., 2010) and the Wnt receiving cells for signaling (Cruciat et al., 2010). We also found *sec-10*, a component of the exocyst complex, which mediates polarized secretion in organisms ranging from yeast to mammals (Hsu et al., 2004). Depletion of other exocyst components also enhanced the *vps-29* induced QL.d migration defect (Fig. 2A), providing further support for a function of this complex in Wnt signaling. No defect was observed in the *sec-10* mutant in the F1 generation, however this might be masked by maternal contribution, since sec-10 like the other exocyst components are essential genes. Finally, we found two genes with functions in vesicle tethering: C42C1.4, which encodes the *C. elegans* ortholog of the VPS8 subunit of the CORVET complex (Markgraf et al., 2009) and F46F11.9, a subunit of the TRAPP complex (Sacher et al., 2008). We found that knock down of the other TRAPP subunit genes also enhanced the sensitized genetic backgrounds (Fig. 2B), consistent with a function of this complex in QL.d migration.

In addition to trafficking genes, we also found genes with functions ranging from cellular signaling to gene expression and protein synthesis. One example is the casein kinase II (CK2) ortholog *kin-10*. CK2 has been shown to phosphorylate TCF transcription factors as well as ß-catenin and to function as a positive regulator of Wnt signaling (Dominguez et al., 2004; Song et al., 2003; Wang and Jones, 2006). Whether KIN-10 also acts at the level of POP-1/TCF or ß-catenin remains to be established. Another example is *ntl-2*, which encodes a subunit of the CCR4/NOT complex. Knock down of the other NOT subunits also induced a QL.d migration defect in the sensitized genetic background (Fig. 2C), but this was not the case for the CCR4 subunits of the complex. It has recently been

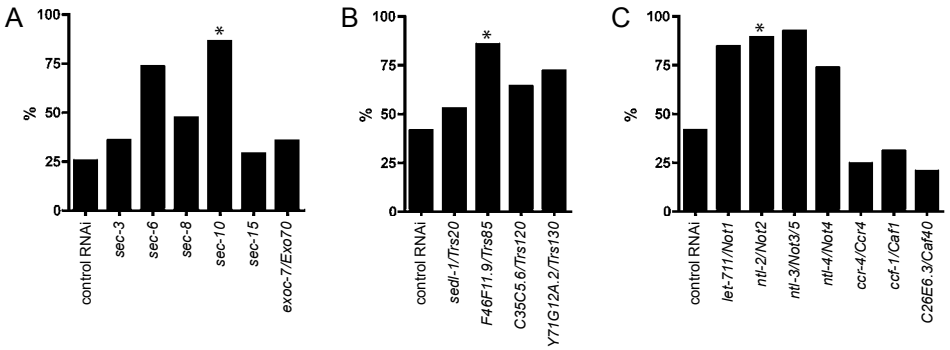

Figure 2 Depletion of several other components of the Exocyst, TRAPP and CCR4-NOT complex also enhances the phenotype of the sensitized mutant background. QL.d migration defect (in %) upon RNAi against several components of the Exocyst complex (A), TRAPP complex (B) and the CCR4-NOT complex (C) in the *vps-29* mutant. The asterisk indicated the gene identified in the sensitized mutant background screens.

shown that the NOT module of the complex may function independently of the CCR4 sub-complex in transcriptional repression (Cui et al., 2008), suggesting that it is the repressor function of the CCR4/NOT complex that is required for QL.d migration.

Interestingly, we also identified genes encoding vitellogenins (*vit-3*, *vit-4* and *vit-5*). Vitellogenins are the major components of *C. elegans* lipoprotein particles, which mediate the transport of yolk from the intestine to developing oocytes (Schneider, 1996). It has been proposed that the diffusion of the hydrophobic Wnt protein is facilitated by the association with lipoprotein particles. This is particularly clear in *Drosophila*, where knock down of the lipoprotein lipophorin leads to a reduction in the Wingless/Wnt morphogen gradient (Panakova et al., 2005). Our results suggest that vitellogenins may be similarly required for EGL-20/Wnt spreading in *C. elegans*.

Genes required for the anterior migration of the QR.d

In our first genome-wide RNAi screen, we identified 3 genes which are required for the anterior migration of the QR.d. One gene is the nuclear hormone receptor *nhr-25*, which is a homolog of *Drosophila* FTZ-F1 and human SF-1 and LHR-1 (Gissendanner and Sluder, 2000). We found that *nhr-25* is a negative regulator of *mab-5*. Thus, *nhr-25* depletion resulted in ectopic expression of *mab-5* in QR and its descendants (Fig 3). Furthermore, the QR.d migration defect was suppressed in the *mab-5* mutant (Table 4), consistent with a function of *nhr-25* upstream

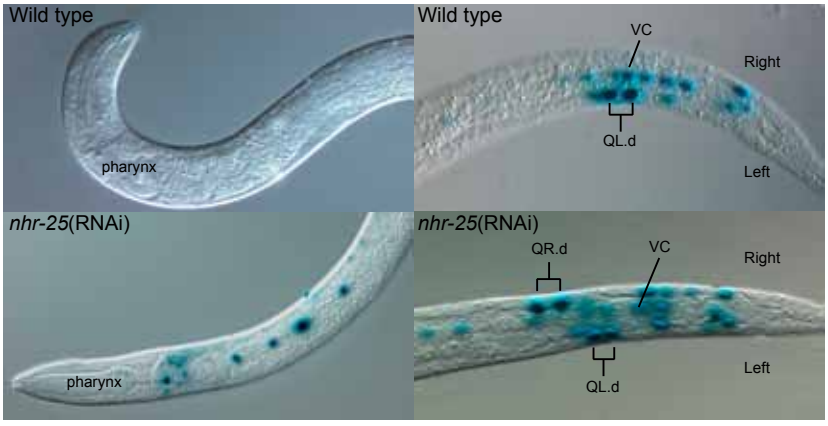

Figure 3 *nhr-25(RNAi)* results in ectopic expression of *mab-5*. As previously described, loss of NHR-25 activates a *mab-5::lacZ* reporter transgene in anterior seam cells (left panels, lateral views). We found that *nhr-25(RNAi)* also results in ectopic expression of *mab-5* in the QR.d (right panels, dorsal views), promoting their posterior migration. Anterior is to the left, posterior to the right; VC: ventral nerve cord.

RNAi	Wildtype	*rff-3(pk1426)*	*egl-20(n585)*	*pry-1(mu38); axl-1(tm1095)*	*bar-1(ga80)*	*pop-1(hu9)*	*mab-5(e1751)*	*rff-3(pf142); mab-5(e1751)*	*mab-5(e1239)*
empty	0/100/0	0/100/0	98/2/0	0/0/100	100/0/0	14/86/0	0/2/98	0/1/99	100/0/00
imp-2	6/86/8	20/74/6	99/1/0	0/0/100	100/0/0	68/32/0	0/0/100	0/0/100	99/1/0
F01F1.13	51/46/3	62/34/4	99/1/0	0/0/100	99/1/0	83/16/1	0/0/100	0/0/100	100/0/0
sqt-3	19/76/5	**	97/2/1	0/0/100	100/0/0	56/44/0	0/1/99	**	100/0/0
vps-35	79/21/0	84/16/0	-	0/0/100	-	96/4/0	0/0/100	0/0/100	-
apb-3	5 <	5 <	-	-	-	23/77/0	-	-	-
nhr-25	0/74/26	*	82/17/1	-	97/3/0	14/85/1	-	-	99/1/0
dab-1	3/97/0	40/60/0	-	0/0/100	-	44/56/0	0/0/100	0/1/99	-
lam-1	0/56/44	Emb/Ste	36/16/48	-	Emb/Ste	7/59/34	-	-	42/17/41
C54D1.5	0/72/28	Emb/Ste	47/11/42	-	Emb/Ste	7/66/27	-	-	71/13/16
F53F4.11	37/63/0	27/73/0	-	**	-	69/31/0	0/0/100	0/1/99	-

Table 4 Epistatic analysis positions the identified proteins at different levels in the Q cell migration pathway. The numbers are percentages presented as follows: % QL.d in the anterior/ % wild type / % QR.d in the posterior. Anterior and posterior are defined in comparison to the vulva position. For each combination, n ≥ 200. In *apb-3(RNAi)*, only small and dumpy animals were scored. *apb-3(RNAi)* results in less than 5% anterior migration of the QL.d. Informative epistatic relations for apb-3 could therefore not be investigated. *nhr-25(RNAi)* occasionally resulted in additional GFP positive cells. Only worms with a normal number of mec-7::gfp expressing cells were scored. * Very strong Lin phenotypes prevented the precise identification of the Q.paa; ** animals were too sick to be scored; Emb: embryonic lethal; Ste: sterile

of *mab-5*. NHR-25 was recently found to physically interact with the *C. elegans* ß-catenins SYS-1 and WRM-1 and depending on the context, to act as a positive or negative modulator of the Wnt/ß-catenin asymmetry pathway (Asahina et al., 2006; Hajduskova et al., 2009). It remains to be established whether NRH-25 also interacts with and modulates the activity of BAR-1/ß-catenin. Alternatively, NHR-25 may negatively regulate *mab-5* activity independently of the canonical Wnt/ß-catenin pathway.

We also found that depletion of the extracellular matrix component laminin interferes with the anterior migration of the QR.d. Thus, knock down of W03F8.10, which encodes the *C. elegans* laminin beta subunit and *lam-2*, which encodes the gamma subunit, both induce a clear defect in anterior QR.d migration. These genes act downstream of *mab-5*, since their depletion also results in posterior localization of the QR.d in *mab-5* loss of function mutants (Table 4). Furthermore, no change in *mab-5* expression was observed upon depletion of these genes (results not shown), indicating that laminin is required for the migration process itself. Interestingly, it was recently shown that laminin gamma2 expression is induced by Wnt5a and is required for the Wnt5a mediated migration and invasion of gastric cancer cells (Yamamoto et al., 2009). These results indicate that laminin is an important prerequisite for Wnt dependent cell migration. Future studies will determine whether laminin is also induced by Wnt signaling in *C. elegans*.

Genes required for the specification of the left right asymmetric Wnt signaling response

Finally, we found 3 genes which induced a random anterior or posterior localization of the QL.d and QR.d. These were the semaphoring 5A related protein encoded by F01F1.13, the intra-membrane protease *imp-2* and the collagen *sqt-3*. The random migration phenotype was suppressed by mutations in *egl-20*/Wnt, *bar-1*/ß-catenin, *pry-1* and *axl-1*/Axin and by loss or gain of function mutations in *mab-5* (Table 4). These results suggest that the 3 genes act upstream of the EGL-20/Wnt pathway in determining the left right asymmetric Wnt signaling response of the Q neuroblasts, which is linked to the initial polarization of the Q neuroblasts (Honigberg and Kenyon, 2000).

CONCLUSIONS

In this study we have taken a systematic knockdown approach to investigate the Wnt dependent migration of the Q neuroblasts in *C. elegans*. In a first screen, we identified 10 novel genes which induce defects in the migration of the QL.d, the QR.d or both. In addition to the retromer subunits *vps-35* (Coudreuse et al., 2006), we found that *dab-1* and *apb-3* induce defects in QL.d migration, as was validated using genetic mutants. Finding *dab-1* was surprising, since it was previously reported to be a negative regulator of Wnt signaling (Hocevar et al., 2003), whereas we find here that it can also promote Wnt signaling. QR.d migration was affected by knock down of laminins, which may be required for the migration process itself and by depletion of the nuclear hormone receptor *nhr-25*, which may regulate *mab-5* expression independently of Wnt signaling. Finally, we found 3 genes which induced both QL.d and QR.d migration defects. Such random migration is observed in mutants which are defective in the initial polarization of the Q neuroblasts, a process that is required for the specification of the left right asymmetry in Wnt signaling response (Honigberg and Kenyon, 2000).

In a second genome-wide RNAi screen, we have used two different sensitized genetic backgrounds to identify new regulators of Q cell migration. Using this approach we found 46 genes. To validate these hits, we have analyzed available genetic mutants and found that out of 10 viable mutants, 5 had a significant QL.d migration defect. Moreover, 3 of these could not have been identified in the first RNAi screen, as their depletion does not induce a defect in wild type animal. The second screen also led to the identification of a number of genes that induce a sterile or lethal null phenotype. Analysis of 5 of these mutants did not reveal defects in Q descendant migration. As these mutations could only be analyzed in the first homozygous generation, maternal contribution of the wild type product may have masked potential functions in Q neuroblast migration.

Taken together, we have identified a set of genes that are required for the highly stereotypic migration of the Q cell descendants. Some of these genes will

be components of the Wnt signaling pathway, as we have demonstrated for the retromer subunits (Coudreuse et al., 2006) and for *snx-3* (as described in Chapter 3). Detailed analysis of the other genes may reveal important new functions in Wnt signaling and cell migration.

MATERIAL AND METHODS

General methods and strains

Strains were maintained at 20°C, according to standard procedures (Lewis and Fleminig, 1995). The different mutants used in this study are the following, sorted according to their linkage group (LG): LGI: *apb-3(ok429)*, *axl-1(tm1095)*, *obr-4(hu189)*, *pop-1(hu9)*, *pry-1(mu38)*, *snx-3(tm1595)*; LGII: *C08H9.2(ok1071)*; *dab-1(hu186)*, *dab-1(gk291)*, *din-1(dh149)*, *ntl-2(ok974)*, *rrf-3(pk1426)*, *vps-35(hu68)*, *wdfy-2(tm3806)*; LGIII: *cup-4(ok837)*, *mab-5(e1239)*, *mab-5(e1751)*, *ogt-1(ok430)*, *vps-29(tm1320)*; LGIV: *bec-1(ok691)*, *egl-20(n585)*, *hrp-1(ok963)*, *imp-2(tm1397)*, *rack-1(tm2262)*, *sec-10(tm3437)*, *vps-26(tm1523)*; LGX: *bar-1(ga80)*. The following transgenes were used: LGII: *muIs32 [mec-7::GFP, lin-15(+)]* (Ch'ng et al., 2003); Unassigned: *huIs71 [Pmig-14::mig-14::gfp]* (Yang et al., 2008), *muIs2 [Pmab-5::lacZ]* (Salser and Kenyon, 1992).

RNAi screen and epistatic analysis

Each 96-well plate of the RNAi library glycerol stocks was inoculated on LB Agar plates containing 50 μg/ml Ampicillin and 12 μg/ml Tetracyclin, and grown overnight at 37°C. The resulting colonies were inoculated in 500 μl of LB containing 50 μg/ml Ampicillin medium, and grown overnight at 37°C in 2.5 ml deep 96-well plates (HJ-Bioanalytik). 100 μl of the obtained cultures were induced for 24 hours on standard 3 cm NGM plates containing 50 μg/ml Ampicillin and 1mM IPTG. Four to eight L4 animals were then added to each plate. The plates were incubated at 15°C for 72 hours and then at 20°C for 24 to 48 hours. Q cell migration was scored using a Leica MZFLIII stereomicroscope equipped with an epifluorescence unit. The migrating Q daughter cells were identified by the expression of a *mec-7::gfp* transgene. A minimum of 100 animals was counted on each plate. *mig-5* (RNAi) and *pry-1* (RNAi) were systematically taken along as controls for each round of screening in the wildtype screen and *vps-35* (RNAi) for the sensitized genetic background screens. Epistatic analysis for the different genes identified in the screen was performed following a similar method.

RNAi vector sequencing

For each plate showing a Q cell migration defect, bacteria from the corresponding address in the library, as well as from the plate itself, were cultured overnight at

37°C in LB medium containing 50 μg/ml Ampicillin. RNAi vectors were purified from these cultures using a QIAGEN miniprep kit, and the identity of the targeted gene confirmed by sequencing, using the L4440 sequencing primers. Alternatively the vectors were directly PCR amplified from the bacteria using the L4440 sequencing primers, which were also used for sequencing. L4440F: 5'-GTTTTCCCAGTCACGACGTT-3', L4440R: 5'- TGGATAACCGTATTACCGCC-3'.

β-Galactosidase assay

Animals carrying the *muIs2[mab-5::lacZ]* transgene were washed in water, placed on microscope slides and dried for 30 minutes in a Savant DNA110 SpeedVac. The dried slides were fixed by two sequential additions of cold acetone. 100 μl of staining solution (30mM NaH_2PO_4, 167 mM Na_2HPO_4, 0.1 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.004% SDS, 0.3% formamide, 0.025% X-gal) was added and the slides were incubated 4 to 8 hours in the dark. β-Galactosidase activity was subsequently scored using a Zeiss M^2Bio stereomicroscope. To more precisely identify the positive cells, stained animals were also mounted on 2% agarose pads and scored by Nomarski optics (Zeiss Axioplan 2).

ACKNOWLEDGEMENTS

We thank Shohei Mitani (National Bioresource Project for the Nematode, Tokyo) for strains. This work was supported by the Boehringer Inhelhein Fondation (MH), the Dutch Cancer Foundation (HCK).

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3 A NOVEL SNX3-DEPENDENT RETROMER PATHWAY MEDIATES RETROGRADE TRANSPORT OF THE WNT SORTING RECEPTOR WNTLESS AND IS REQUIRED FOR WNT SECRETION

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Nature Cell Biology, in press

Wnt proteins are lipid modified glycoproteins that play a central role in development, adult tissue homeostasis and disease. Secretion of Wnt proteins is mediated by the Wnt-binding protein Wntless (Wls), which transports Wnt from the Golgi network to the cell surface for release. It has recently been shown that recycling of Wls through a retromer-dependent endosome-to-Golgi trafficking pathway is required for efficient Wnt secretion, but the mechanism of this retrograde transport pathway is poorly understood. Here, we report that Wls recycling is mediated through a novel retromer pathway that is independent of the retromer sorting nexins SNX1/SNX2 and SNX5/SNX6. We found that the unrelated sorting nexin, SNX3, has an evolutionarily conserved function in Wls recycling and Wnt secretion and show that SNX3 interacts directly with the cargo-selective sub-complex of the retromer to sort Wls into a morphologically distinct retrieval pathway. These results demonstrate that SNX3 is part of an alternative retromer pathway that functionally separates the retrograde transport of Wls from other retromer cargo.

The classical retromer complex consists of a membrane-bound coat formed by the sorting nexins SNX1/SNX2 and SNX5/SNX6 (referred to as SNX-BAR sorting nexins)(Carlton et al., 2004; Carlton et al., 2005; Wassmer et al., 2007; Wassmer et al., 2009) and a cargo-selective sub-complex comprised of the subunits VPS26, VPS29 and VPS35(Attar and Cullen, 2009; Seaman, 2005), which binds to a sorting motif in the cytoplasmic tail of cargo proteins(Seaman, 2007). The SNX-BAR sorting nexins are recruited to cargo containing endosomes through a phosphatidylinositol-3-monophosphate (PI3P) binding Phox homology (PX) domain, and utilize the carboxy-terminal Bin-amphiphysin-Rvs (BAR) domain to drive membrane deformation and to generate membrane tubules. In recruiting the cargo-selective sub-complex to the forming tubules, the SNX-BAR coat complex is thought to traffic cargo into a tubular-based endosomal sorting pathway(Cullen, 2008). One of the principal functions of this pathway is to mediate retrograde transport between endosomes and the *trans*-Golgi network (TGN), as has been established for cargo proteins such as Vps10p in yeast(Seaman et al., 1998) and the cation-independent mannose 6-phosphate receptor (CI-MPR) in mammals(Arighi et al., 2004; Seaman, 2004, 2007). It has recently been shown that Wls (also known as Evi or Sprinter)(Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006) is also a retromer cargo(Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008). Wls binds to the cargo-selective sub-complex and in mutants of the cargo-selective subunits, Wls is missorted and degraded in lysosomes, leading to a strong defect in Wnt secretion and downstream signaling(Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008).

To further examine the function of the retromer complex in the Wnt secretion pathway, we studied the function of the SNX-BAR coat components in Wls recycling. We made the surprising discovery that the SNX-BAR sorting nexins, which are required for the retromer-dependent trafficking of all retromer cargo proteins that have been studied to date(Attar and Cullen, 2009; Carlton et al., 2004; Hettema et al., 2003; Mari et al., 2008; Nothwehr and Hindes, 1997), are fully dispensable for Wls recycling and Wnt secretion. We show that the unrelated sorting nexin, SNX3, has an evolutionarily conserved function in the Wnt secretion pathway. SNX3 directly interacts with the cargo-selective subunits of the retromer in a complex that does not contain the SNX-BAR coat components. Furthermore, we show that the SNX3 retromer pathway sorts Wls into a retrieval pathway that is morphologically distinct from the SNX-BAR retromer pathway. Our results demonstrate that Wls recycling is mediated by a novel retromer pathway that separates the recycling of Wls from cargo proteins that take the classical SNX-BAR dependent retromer pathway. We propose that such uncoupling may be essential to achieve the tight regulation of Wnt secretion that is necessary for normal development and adult tissue homeostasis.

RESULTS

The SNX-BAR retromer sorting nexins are dispensable for Wls recycling and Wnt signaling in *C. elegans* and *Drosophila*

In *C. elegans*, mutation of the cargo-selective sub-complex induces defects in several Wnt dependent processes(Coudreuse et al., 2006; Prasad and Clark, 2006), including the EGL-20/Wnt dependent posterior migration of the left Q neuroblast descendants (QL.d). Unexpectedly, we found that mutation of the single SNX1/SNX2 ortholog *snx-1*(Coudreuse et al., 2006) and the single SNX5/SNX6 ortholog *snx-6*(Chen et al., 2010) did not induce defects in QL.d positioning (Fig. 1A). A comprehensive analysis of other Wnt dependent processes did not reveal defects either (Fig. S2, Table 1), indicating that *snx-1* and *snx-6* are not required for Wnt signaling in *C. elegans*. Consistently, we found that *snx-1* and *snx-6* are dispensable for the retromer-dependent recycling of the *C. elegans* Wls ortholog MIG-14 (Fig. 1D). In contrast, recycling of the retromer cargo protein CED-1 was fully dependent on *snx-1* and *snx-6* (Fig. S3)(Chen et al., 2010).

To extend these observations, we knocked down the single SNX5/SNX6 ortholog *Dsnx6* in the posterior compartment of the *Drosophila* wing imaginal disc by transgene mediated RNAi. The wing pouch is patterned along the dorsoventral axis by the Wnt protein Wingless (Wg)(Zecca et al., 1996), which is expressed by cells that are located at the dorsoventral boundary of the disc (Fig. 2A). In the absence of Dvps35, Wg secretion is strongly reduced, resulting in accumulation of Wg in the producing cells and a loss of expression of the Wg target gene *senseless*(Belenkaya et al., 2008; Franch-Marro et al., 2008; Port et al., 2008). We found that knock down of *Dsnx6* did not induce accumulation of Wg (Fig. 2F) and that it also did not reduce the expression of *senseless* (Fig. 2D). Furthermore, knock down of *Dsnx6* did not affect the levels of endogenous Wls (Fig. 2H), whereas in the absence of Dvps35, Wls levels are strongly reduced in Wg producing cells(Belenkaya et al., 2008; Franch-Marro et al., 2008; Port et al., 2008). Taken together, we conclude that the *C. elegans* and *Drosophila* SNX-BAR orthologs are dispensable for Wls trafficking and Wnt signaling. To our knowledge this is the first example of the cargo-selective sub-complex of the retromer functioning independently of the SNX-BAR retromer sorting nexins.

The PX domain-only sorting nexin SNX3 is required for Wnt signaling

In a genome-wide RNAi screen in *C. elegans*, we found that the PX domain-only sorting nexin, *snx-3*, which is closely related to yeast Grd19p, *Drosophila* DSnx3 and vertebrate SNX3 and SNX12 (Fig. S1B)(Cullen, 2008), is required for the EGL-20/Wnt dependent posterior migration of the QL.d, a result that we confirmed using the predicted *snx-3* null allele *tm1595* (Fig. 1A, S1C). EGL-20/Wnt induces posterior migration of the QL.d by activating the target gene

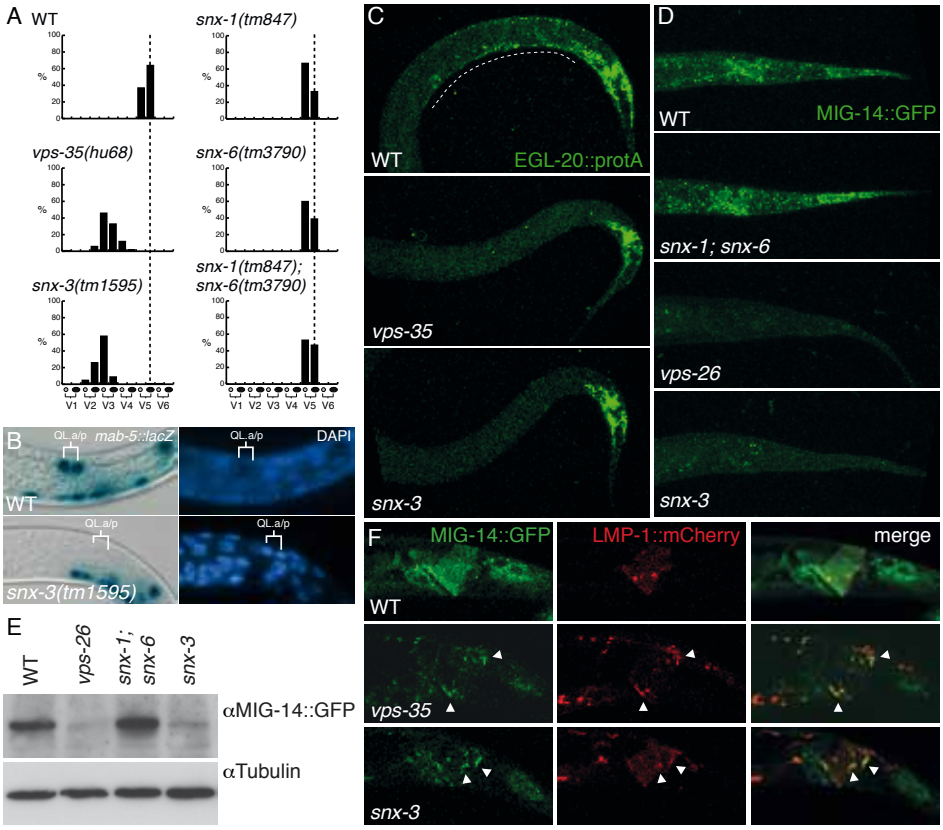

Fig. 1. SNX-3 is required for EGL-20/Wnt signaling and MIG-14/Wls recycling in *C. elegans*. (**A**) The final positions of the QL.paa and QL.pap cells relative to the invariant positions of the seam cells V1 to V6 (n>100). Both *snx-1(tm847)* and *snx-6(tm3790)* are viable as single or double mutants and could be propagated as homozygous strains, excluding a contribution of maternally provided protein in our assays. (**B**) Expression of the EGL-20/Wnt target gene *mab-5* in the QL descendants QL.a and QL.p. Cell nuclei are shown by DAPI staining. (**C**) Staining of EGL-20::proteinA with rabbit anti-goat-Cy5(Coudreuse et al., 2006) in wild type, *vps-35(hu68)* and *snx-3(tm1595)*. Expression is visible within the *egl-20* expressing cells (closed line) and as a punctate posterior to anterior gradient (dotted line). In all images, anterior is to the left and dorsal is up. (**D**) Confocal images of MIG-14::GFP (*huSi2*) at identical exposure settings in wild type and in *snx-1(tm847); snx-6(tm3790)*, *vps-26(tm1523)* and *snx-3(tm1595)*. (**E**) Western blot quantification of MIG-14::GFP (*huSi2*) protein levels. (**F**) Confocal images of MIG-14::GFP (*huIs71*) and LMP-1::mCherry in wild type, *vps-35(hu68)* and *snx-3(tm1595)*. Arrowheads indicate examples of co-localization between MIG-14::GFP and LMP-1::mCherry.

mab-5(Harris et al., 1996; Salser and Kenyon, 1992). In *snx-3(tm1595)* mutants, *mab-5* expression was lost in the QL lineage (Fig. 1B), consistent with the notion that *snx-3* is required for the EGL-20/Wnt dependent activation of *mab-5*. This

Table 1. Wnt signaling phenotypes of retromer and sorting nexin mutants

	WT	*vps-35(hu68)*	*snx-3(tm1595)*	*snx-1(tm847); snx-6(tm3790)*
QL.d migration	0	100	100	0
QR.d migration	1	98	74	2
HSN	1	97	92	7
ALM polarity	0	21	53	0
PLM polarity	0	29	57	0
P12 to P11	0	6	0	0
T polarity	1	12	2	1
V5 polarity	0	32	6	0
Embryonic lethal	2	n.d.	1	4

Numbers indicate percentage defective (n>100). The final positions of QL.paa (PVM) and QR.paa (AVM) was scored using Nomarski optics and the polarity of the ALM and PLM mechanosensory neurons was scored using *a mec-7::gfp (muls32 or muls35)* expressing transgene(Ch'ng et al., 2003). The final position of the HSN neurons, the polarity of the V5 division and P12 to P11 fate transformation were scored using Nomarski microscopy at the appropriate developmental stage. T cell polarity was scored by DiO filling of the T derived phasmid structure in young adults.

conclusion is supported by the rescue of the QL.d migration defect of *snx-3* by EGL-20/Wnt independent activation of *mab-5*. Thus, the QL.d localize at their normal posterior positions in double mutants between *snx-3* and the *mab-5* gain of function allele *e1751*(Salser and Kenyon, 1992) and in double mutants with the Axin ortholog *pry-1* (Table 2)(Korswagen et al., 2002; Maloof et al., 1999). In addition to the defect in QL.d migration, *snx-3(tm1595)* showed a range of other Wnt related phenotypes. Thus, the final position of the QR.d and the HSN neurons was shifted towards the posterior and the polarity of the ALM and PLM mechanosensory neurons lost or reversed (Table 1, Fig. S2). Taken together, these results show that *snx-3* is required for several Wnt dependent processes and that the range and penetrance of these phenotypes is similar to mutations in components of the cargo-selective retromer complex(Coudreuse et al., 2006; Prasad and Clark, 2006).

To investigate whether SNX3 is required for Wnt signaling in *Drosophila*, we knocked down the single SNX3 ortholog *Dsnx3* in the posterior compartment of the wing imaginal disc. As shown in Fig. 2D', there was a strong reduction in expression of the high-threshold Wg target gene *senseless*. Furthermore, knock down of *Dsnx3* in the posterior compartment or in clones frequently resulted in typical *wg* loss of function defects in the adult wing, such as notches and a loss of sensory bristles at the wing periphery (Fig. 2E). These results establish that SNX-3 has an evolutionarily conserved function in Wnt signaling.

SNX3 is required in Wnt producing cells for Wnt secretion

We found that *snx-3* is ubiquitously expressed in *C. elegans*, with prominent expression in coelomocytes, the pharynx and in rectal epithelial cells, which include the cells that produce and secrete EGL-20/Wnt (Fig. S1D). We have previously shown that the cargo-selective sub-complex of the retromer is specifically required in Wnt producing cells(Coudreuse et al., 2006). To investigate whether *snx-3* has a similar site of action, we tested whether specific expression of wild type *snx-3* in the EGL-20/Wnt producing cells of *snx-3(tm1595)* mutants restores the EGL-20/Wnt dependent posterior migration of the QL.d. We found that *egl-20* promoter directed expression of *snx-3* significantly rescued QL.d migration (Table 2). In contrast, expression of *snx-1* did not restore QL.d migration. We conclude that the presence of *snx-3* in Wnt producing cells is necessary and sufficient for its function in Wnt signaling. Furthermore, these results show that *snx-3* and the cargo-selective retromer sub-complex do not only produce similar mutant phenotypes, but also share a common site of action.

The requirement of *snx-3* in Wnt producing cells suggests a function of SNX-3 in Wnt secretion. To investigate this possibility, we tested whether the secretion of EGL-20/Wnt is affected in *snx-3* mutants. EGL-20/Wnt (visualized using the immunoglobulin binding domain of protein A as a tag) forms a punctate concentration gradient that ranges from the *egl-20* expressing cells in the tail to the mid-body region (Fig. 1C)(Coudreuse et al., 2006). In *vps-35* mutants, this gradient is strongly reduced or absent. We found that the EGL-20/Wnt gradient was similarly reduced in *snx-3(tm1595)* (Fig. 1C), indicating that *snx-3* is necessary for EGL-20/Wnt secretion. To determine whether DSnx3 is also required for Wnt secretion and gradient formation in *Drosophila*, we depleted *Dsnx3* in the posterior compartment of the wing imaginal disc and stained for endogenous

Table 2. Rescue of the *snx-3(tm1595)* QL.d migration defect

	% QL.d in posterior	n
WT	100	112
snx-3(tm1595)	0	>200
mab-5(e1751)gf	100	110
snx-3(tm1595); mab-5(e1751)gf	100	114
pry-1(mu38)	100	103
snx-3(tm1595); pry-1(mu38)	78	105
snx-3(tm1595); Pegl-20::snx-3::gfp	54	263
snx-3(tm1595); Pegl-20::snx-1::gfp	0	141
snx-3(tm1595); Pmig-14::mig-14::gfp	100	50

The final position of QL.paa (PVM) was scored as anterior or posterior to the vulva in young adult hermaphrodites using a *mec-7::gfp (muIs32)* reporter transgene(Ch'ng et al., 2003) or using Nomarski optics.

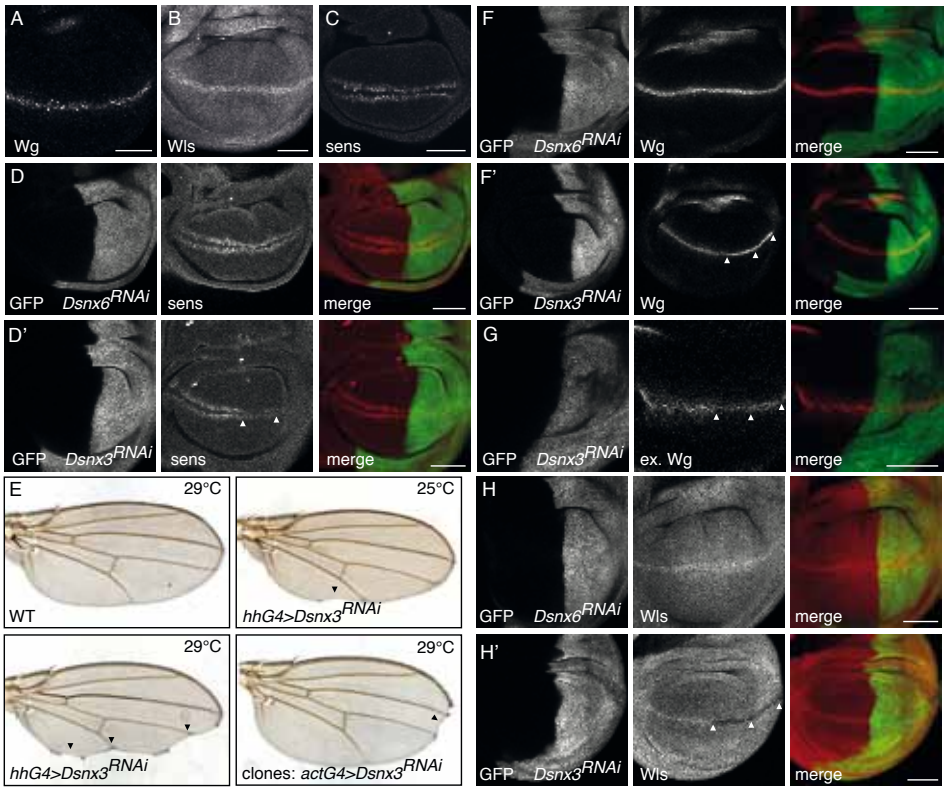

Fig. 2. DSnx3 is required for Wg secretion and Wls recycling in the *Drosophila* wing imaginal disc. (**A, B, C**) Immunostaining of Wg, Wls and Senseless in wild type wing disc. (**D, D', F, F', G, H, H'**) Expression of *Dsnx6* or *Dsnx3* RNAi transgenes was induced in the posterior compartment of the wing disc (marked by GFP) using a *hhGal4* driver (see Fig. S4A, B for quantification of knock down efficiency). (**D, D'**) Immunostaining of Senseless. Arrowheads indicate loss of *senseless* expression in the *Dsnx3* RNAi expressing posterior compartment. (**E**) *Dsnx3* RNAi was induced in the posterior compartment using *hhGal4* or in clones using an *actinGal4* driver. Arrowheads indicate notches and loss of sensory bristles. (**F, F'**) Immunostaining of total Wg. Arrowheads indicate Wg accumulation in the *Dsnx3* RNAi expressing posterior compartment. (**G**) Immunostaining of extracellular Wg. Arrowheads indicate loss of extracellular Wg staining. (**H, H'**) Immunostaining of Wls. Arrowheads indicate loss of Wls in *wg* expressing cells in the *Dsnx3* RNAi expressing posterior compartment. Scale bars, 50 μm.

Wg. As shown in Fig. 2F' and S4, knock down or mutation of *Dsnx3* resulted in a strong accumulation of Wg within the stripe of *wg* expressing cells along the dorsoventral boundary, indicating that Wg secretion is strongly reduced. Indeed, staining of extracellular Wg showed that there was a strong reduction in the level of Wg outside of the *wg* expressing cells (Fig. 2G). Taken together, these results are consistent with a conserved function of SNX3 in Wnt secretion.

To address the specificity of SNX3 for Wnt secretion, we tested whether knock down of *Dsnx3* affects the secretion of two other morphogens in the wing disc: the lipid-modified Hedgehog (Hh) protein(Hidalgo and Ingham, 1990) and the *Drosophila* BMP ortholog decapentaplegic (Dpp)(Entchev et al., 2000). Depletion of *Dsnx3* in the dorsal compartment or in large clones spanning the *hh* expressing domain of the wing imaginal disc did not interfere with Hh secretion, as determined by monitoring endogenous Hh protein (Fig. 3A). To examine effects on Dpp secretion, Dpp signaling activity was measured by staining of phosphorylated Mad protein (pMad), a downstream effector in the Dpp pathway(Teleman and Cohen, 2000), but again no effects were observed when *Dsnx3* was depleted (Fig. 3B). Although these experiments assay only a limited set of secreted proteins, these results clearly indicate that DSnx3 is not

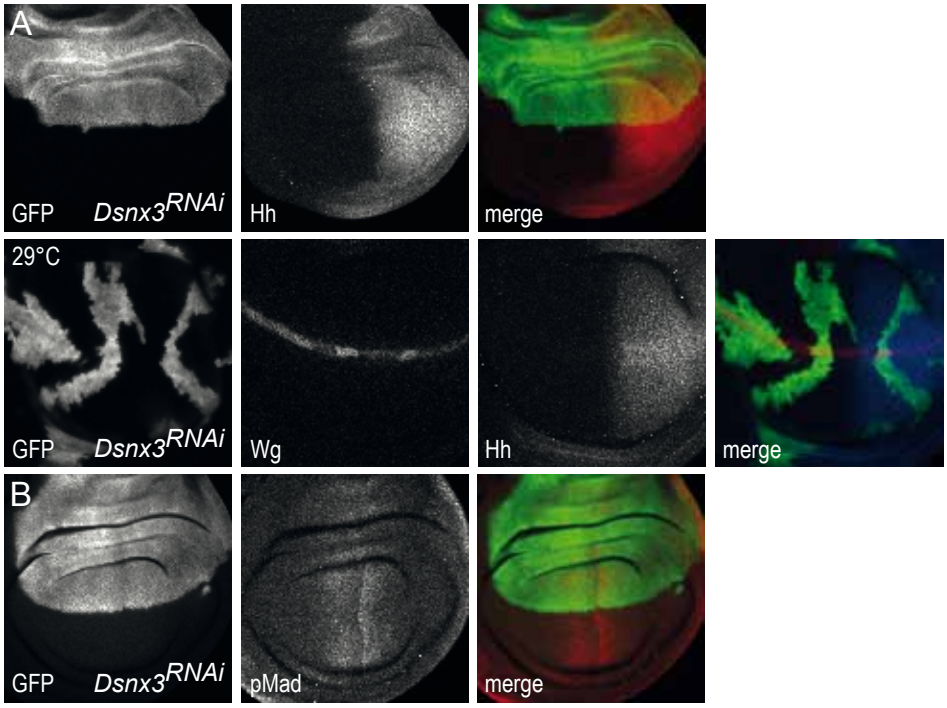

Fig. 3. Reduction of *Dsnx3* expression does not interfere with Hh secretion or Dpp signaling. (**A**) *Dsnx3* was knocked-down by expressing the corresponding UAS-hairpin with *apGal4* in the dorsal compartment of the wing imaginal disc. The expression domain is marked by a *UAS-CD8GFP* transgene. Hh protein levels are similar in the dorsal and ventral compartments (upper panel). *Dsnx3^{RNAi}* was expressed in clones of cells using an *hsp-FLP;;act>y>Gal4* driver line and appropriate heat shock conditions. *Dsnx3^{RNAi}* clones accumulate Wg in producing cells, but leave Hh protein levels unaffected (lower panel). (**B**) Dpp dependent phosphorylation of Mad is not altered when *Dsnx3* is knocked-down by expression with *apGal4*.

generally required for protein secretion. This conclusion is further supported by the *snx-3* mutant phenotype in *C. elegans*: apart from a defect in Wnt signaling, *snx-3* mutants have no other obvious developmental defects (data not shown).

SNX-3 has a conserved function in regulating MIG-14/Wls stability

To examine the function of SNX-3 in the Wnt secretion pathway, we tested whether depletion of *snx-3* has an effect on the subcellular localization or stability of MIG-14/Wls(Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). As shown in Fig. 1D and E, there was a striking decrease in MIG-14/Wls protein levels in *snx-3(tm1595)*, which was similar to the reduction observed in the cargo-selective sub-complex mutant *vps-26*. In contrast, steady state levels of the retromer cargo protein CED-1 were not significantly changed in the absence of *snx-3* (Fig. S3). Also in *Drosophila*, depletion of *Dsnx3* resulted in a loss of endogenous Wls protein (Fig. 2H', S4C). Interestingly, Wls levels were only reduced within the *wg* expressing cells, an effect that was also observed upon knock down of *Dvps35*(Port et al., 2008). We conclude that SNX3 has a conserved function in maintaining MIG-14/Wls protein levels.

To determine whether the reduction in MIG-14/Wls protein levels is the result of lysosomal degradation, we performed co-localization studies between MIG-14/Wls and the late endosomal and lysosomal marker LMP-1::mCherry in *C. elegans* (Yang et al., 2008). In wild type animals, no significant co-localization between MIG-14::GFP and LMP-1::mCherry could be observed in *egl-20/*Wnt expressing cells (Fig. 1F). However, in *snx-3(tm1595)* mutants, the remaining MIG-14::GFP was mostly localized to LMP-1::mCherry positive structures. These results indicate that MIG-14/Wls is missorted into the lysosomal degradation pathway in *snx-3* mutants, consistent with a function of SNX-3 in the retromer dependent endosome-to-TGN transport of MIG-14/Wls.

To investigate whether MIG-14/Wls becomes limiting for Wnt signaling in *snx-3* mutants, we tested whether *mig-14* overexpression can rescue EGL-20/Wnt signaling. Overexpression of a functional MIG-14::GFP fusion protein from a multi-copy transgene fully rescued the QL.d migration defect of *snx-3(tm1595)* (Table 2), supporting the hypothesis that the Wnt secretion defect of *snx-3* mutants is caused by a decrease in MIG-14/Wls protein level.

Human SNX3 co-localizes with Wls and VPS26 on endosomes and facilitates membrane association of the cargo-selective retromer sub-complex

To investigate how SNX-3 and the cargo-selective sub-complex functionally interact in Wls recycling, we examined human SNX3 in HeLa cells, which express SNX3 but lack detectable expression of the related sorting nexin SNX12 (Fig. S5A, B). Using a lentivirally expressed GFP-SNX3 fusion protein, we found that SNX3 co-localizes with the cargo-selective retromer subunit VPS26 on endosomes (Fig. 4A),

as was confirmed by immuno-electron microscopy (EM) (Fig. 4B). Furthermore, we found that GFP-SNX3 co-localizes with Wls-mCherry (Fig. 5A, and Fig. S1E for co-localization in *C. elegans*). Knock down of SNX3 led to a significant reduction in endogenous human Wls protein levels (Fig. 5C), consistent with the conserved function of SNX3 in maintaining Wls protein levels. SNX3 depletion also induced a striking reduction in co-localization between Wls-mCherry and endogenous VPS26 (Fig. 5B'). As the VPS26 staining pattern appeared more cytoplasmic than in control siRNA treated cells, we tested whether SNX3 is required for membrane association of VPS26. Similar to knock down of Rab7, which has been shown to mediate membrane recruitment of the cargo-selective sub-complex to late endosomes(Rojas et al., 2008; Seaman et al., 2009), depletion of SNX3 induced a decrease in membrane associated VPS26 and a corresponding increase in cytoplasmically localized VPS26 (Fig. 5D). These results are consistent with a function of SNX3 in aiding the association of the cargo-selective subunits to Wls containing endosomes. Whether this function is independent of other retromer recruitment mechanisms, such as mediated by Rab7(Rojas et al., 2008; Seaman et al., 2009) and Hrs(Popoff et al., 2009), remains to be established.

SNX3 physically interacts with the cargo-selective retromer subunits in a complex that does not contain the SNX-BAR sorting nexins

To investigate whether SNX3 and the retromer physically interact, we performed co-immunoprecipitation (CoIP) experiments with GFP-SNX3. As shown in Fig. 4C, there was significant CoIP between SNX3 and both endogenous VPS35 and VPS26, indicating that SNX3 and the cargo-selective retromer sub-complex can associate *in vivo*. This interaction is direct, as recombinant full length SNX3 co-precipitated with purified VPS26-VPS29-VPS35 trimeric complex (Fig. 4C' and S5C). Next, we compared IPs of SNX3 with IPs of the SNX-BAR sorting nexins SNX1 and SNX5 (Fig. 4C). Although in both cases the cargo-selective subunits were co-precipitated (note however that the interaction with SNX1/SNX5 is weaker than with SNX3), we failed to detect endogenous SNX1 and SNX5 in the SNX3 IP and endogenous SNX3 in the SNX1 and SNX5 IP. Based on these results, we conclude that there are two distinct retromer complexes: a SNX-BAR containing retromer complex and a novel complex in which SNX3 interacts with the cargo-selective retromer sub-complex.

SNX3 localizes to early endosomes and segregates Wls into a retrieval pathway that is morphologically distinct from the SNX-BAR retromer pathway

To examine the spatial distribution of SNX3 and the SNX-BAR sorting nexins along the endosomal maturation pathway, we expressed GFP-SNX3 and GFP-SNX1 in HeLa cells. Using markers for early (EEA1 and Rab5) and late endosomes and lysosomes (LAMP1 and Rab7), we found that GFP-SNX3 primarily localizes to

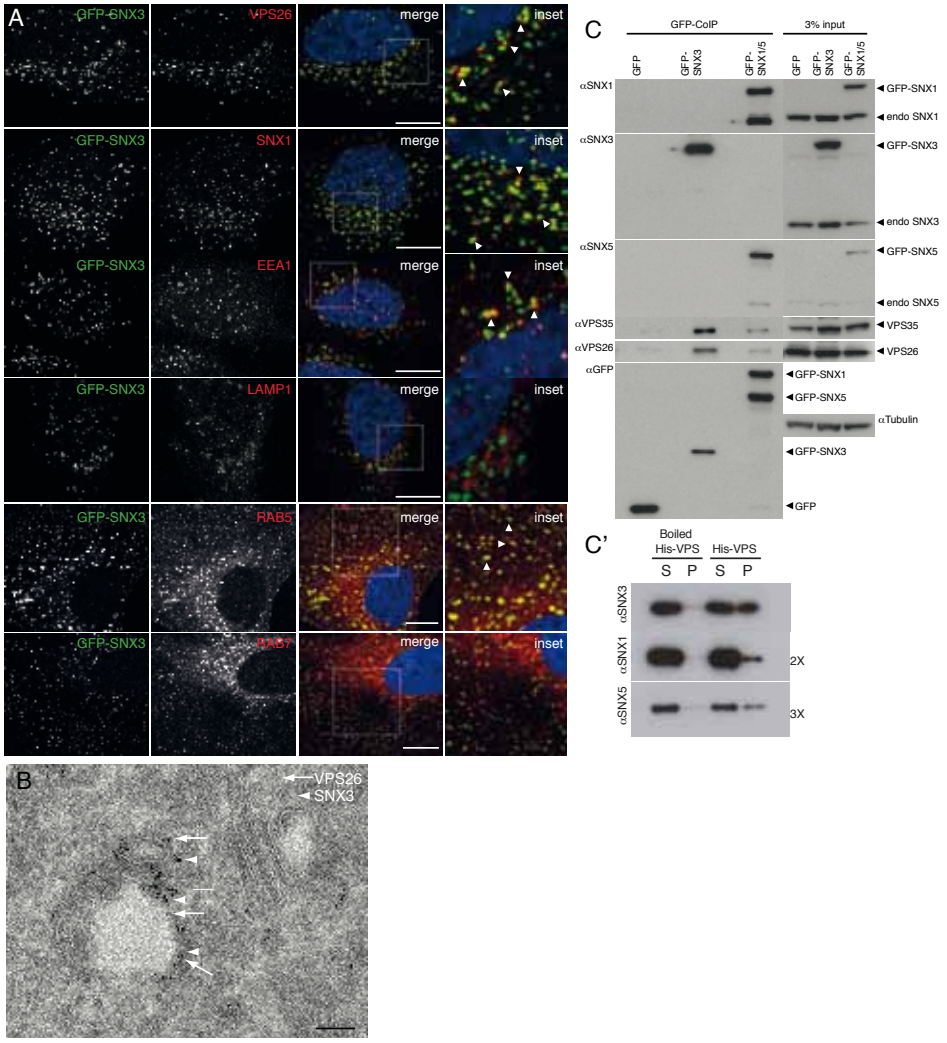

Fig. 4. Co-localization and physical interaction of SNX3 with the cargo-selective sub-complex of the retromer (**A**) SNX3 partially co-localizes with VPS26-positive early endosomes. HeLa cells lentivirally transduced to express GFP-SNX3 were fixed and stained for VPS26, SNX1, EEA1 or LAMP1. Co-localization between GFP-SNX3 and VPS26, SNX1, EEA1, LAMP1, Rab5 and Rab7 was quantified as 0.43 ± 0.05, 0.55 ± 0.04, 0.38 ± 0.02 and 0.07 ± 0.04, 0.61 ± 0.02, and 0.34 ± 0.02, respectively (Pearson's coefficient, mean ± SD, n=3 with 30 cells per condition, for Rab5 and Rab7, n=20 cells). Scale bar, 11 μm. (**B**) At the ultrastructural level, SNX3 and VPS26 localize to common vesicular endosomal profiles. GFP-SNX3 is labeled with 10 nm gold and mCherry-VPS26 with 6 nm gold. The image is representative of that observed from the analysis of 5 other endosomal vacuoles. Scale bar, 100 nm. (**C, C'**) SNX3 interacts with the cargo-selective sub-complex of the retromer. Cell extracts derived from HeLa cells lentivirally transduced with GFP, GFP-SNX3 or both GFP-SNX1 and GFP-SNX5 (GFP-SNX1/5), were subjected to GFP-nanotrap. The ▶

early endosomes (Fig. 4A), in agreement with the previously reported localization of SNX3 in A431 cells(Xu et al., 2001). In contrast, the classical SNX-BAR retromer sorting nexins are most abundant on endosomes that are at the early-to-late endosomal transition point(Mari et al., 2008) (J.R.T. van Weering and P.J. Cullen, unpublished). Consistent with this distinct but overlapping distribution, co-localization between SNX1 and SNX3 was only partial (Fig. 4A), supporting the notion that SNX3 and SNX1 display an element of spatial segregation along the endosomal maturation pathway (see Fig. S8).

The SNX-BAR retromer sorting nexins contain a membrane curvature sensing BAR domain which drives membrane deformation to segregate cargo into a tubular-based endosomal trafficking pathway(Cullen, 2008). SNX3 lacks a BAR domain, indicating that it may direct Wls and the cargo-selective retromer sub-complex into a morphologically distinct sorting pathway. Using live cell confocal imaging to define the dynamic relationship between SNX3, the SNX-BAR sorting nexins and Wls, we found that Wls is not sorted into SNX-BAR labeled tubular endosomal profiles. Thus, in cells expressing GFP-SNX1 and Wls-mCherry, Wls was not enriched in SNX1 decorated tubules emerging from endosomes labeled for both proteins (Fig. 6A, B, S5D) (18/22 tubules were negative for Wls, while 4/22 were weakly positive). Instead, we observed the emergence of small GFP-SNX3 and Wls-mCherry labeled transport vesicles from endosomes co-labeled for both proteins (Fig. 6C). These results are consistent with our observation that the SNX-BAR sorting nexins are dispensable for Wls trafficking and indicate that Wls exits SNX3-labelled early endosomes through vesicular carriers rather than through SNX-BAR decorated tubular profiles. This conclusion is further supported by the observation that SNX3 interacts with clathrin(Skanland et al., 2009), a result that we have confirmed here through endogenous CoIP (Fig. S6A). Although SNX3 has been suggested to contain an inverted clathrin box(Skanland et al., 2009), the interaction appears indirect, as recombinant SNX3 did not associate with recombinant clathrin (residues 1-579) either in the absence or presence of PI3P-containing liposomes (Fig. S6B, C). Immunostaining of endogenous clathrin revealed that a small population of SNX3 decorated endosomes co-localizes with clathrin (Fig. S6D). At the resolution of immuno-EM, these appear as clathrin coated, small 50-75 nm vesicles (Fig. S6E) that are morphologically distinct from the previously characterized retromer decorated endosome-to-TGN transport carriers (ETC's), which appear as clathrin-negative, non-branched tubules

▶ classical retromer SNX-BARs form heterodimeric complexes leading to the presence of both endogenous SNX1 and SNX5 in the GFP-SNX1/5 IPs(Wassmer et al., 2007). **(C')** 3xFLAG-VPS26-VPS29-VPS35-His_6 complex (His-VPS) was isolated from BL21 *E. coli* onto TALON resin and incubated with 2 μM of recombinant SNX3, SNX1 or SNX5 for 2 hours at 4°C. Supernatant (S) and TALON containing resin (P) were isolated. SNX3 directly associates with His-VPS as do SNX1 and SNX5 although this is less well pronounced (longer exposures are shown). Control: boiled His-VPS resin.

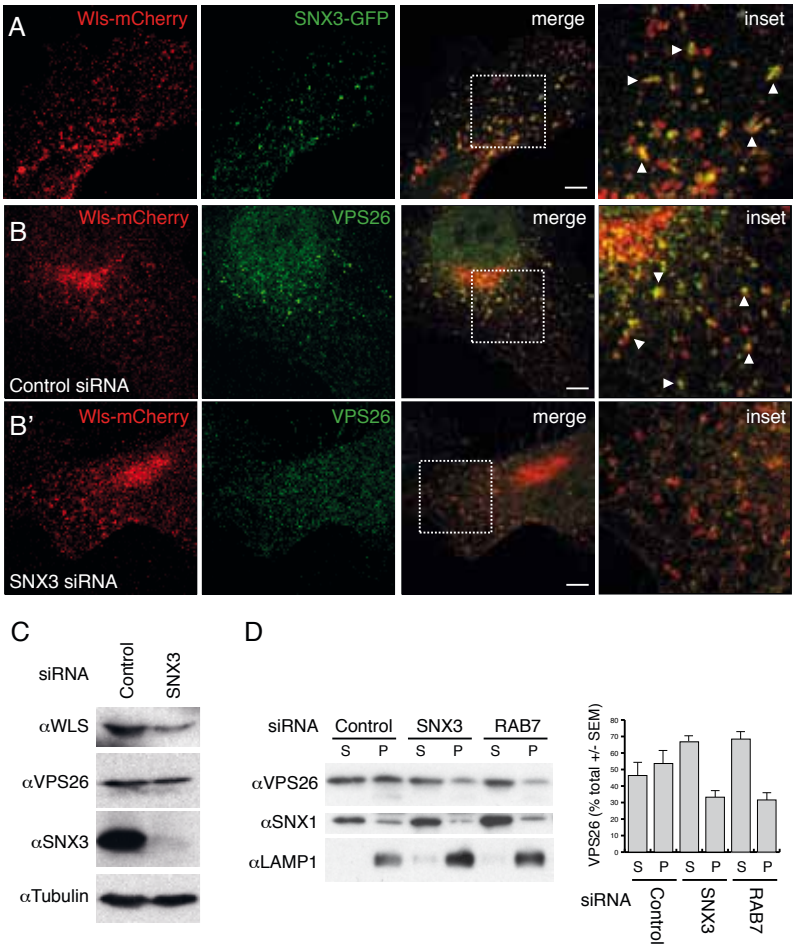

Fig. 5. SNX3 co-localizes with Wls and facilitates membrane association of the cargo-selective sub-complex of the retromer. (**A**) Co-localization between SNX3-GFP and Wls-mCherry in HeLa cells was quantified as 0.25 ± 0.02% (Pearson's coefficient; mean ± SEM, n=2 with 23 and 11 cells). Arrowheads show examples of co-localization. Scale bar, 10 μm. (**B, B'**) Co-localization between Wls-mCherry and endogenous VPS26 in HeLa cells treated with control or SNX3 siRNA was quantified as 0.19 ± 0.02% and 0.08 ± 0.02%, respectively (Pearson's coefficient; mean ± SEM, n=4 with 7-10 cells each). Arrowheads show examples of co-localization. (**C**) HeLa cells were transfected with control or SNX3 siRNA and assayed for endogenous Wls, VPS26, SNX3 and tubulin protein levels. (**D**) HeLa cells treated with control, SNX3 or RAB7 siRNA were separated into a supernatant (S) fraction containing cytosol and a pellet fraction (P) containing membranes(Seaman et al., 2009) and were stained for endogenous VPS26, SNX1 and LAMP1. The amount of VPS26 in the supernatant and pellet fractions was quantified using densitometry and is shown as percentage of the total. Data are presented as mean ± SEM and represent three independent experiments. There was no significant change in SNX1 membrane association upon SNX3 knock down (17.8 ± 3.8% versus 22 ± 8.1%). Knock down of RAB7 was included as a positive control(Seaman et al., 2009).

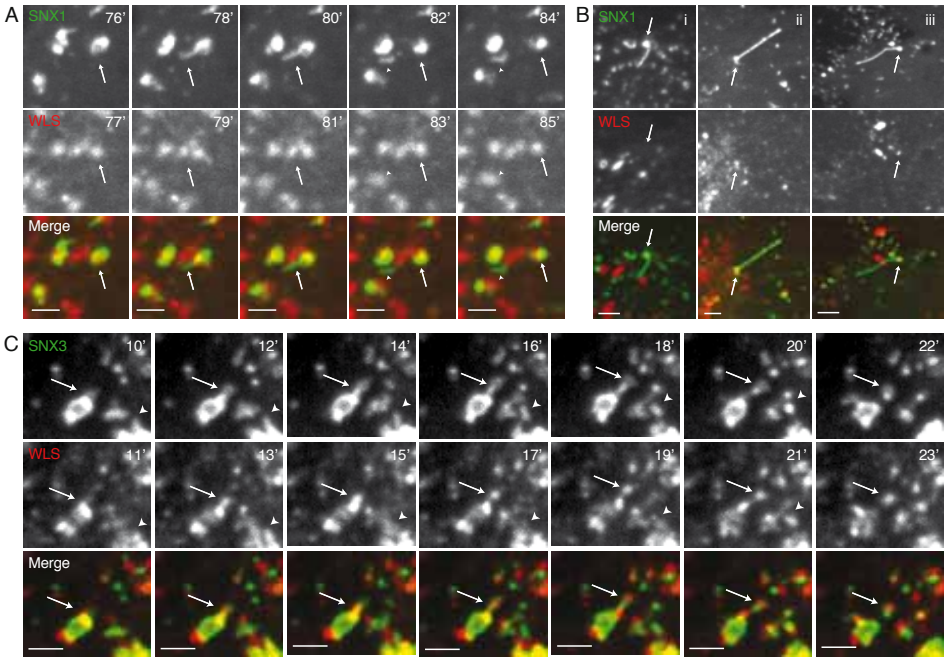

Fig. 6. Wls is contained within SNX3 positive vesicular carriers but is absent from SNX1 retromer decorated tubular carriers. **(A)** RPE-1 cells were transiently co-transfected with pEGFP-SNX1 and Wls-mCherry and cells were subsequently imaged live after a 16 hour incubation period. Frames depicting the formation and scission of a GFP-SNX1 tubule from a vesicle positive for both SNX1 and Wls are shown (arrows indicate the dual expressing vesicle, while arrowheads indicate the carrier post scission) (see Supplementary Information, Movie 1). Scale bars represent 6 µm. Of the 100 SNX1 decorated tubulating endosomes that were analyzed, 22 were positive for Wls; 18/22 tubules emanating from these endosomes were negative for Wls, while 4/22 were weakly positive. Quantification of Wls-mCherry and GFP-SNX1 levels in an endosome and corresponding tubule is shown in Fig. S5D. **(B)** Further examples of SNX1 retromer tubules negative for Wls. (i) An example of a SNX1 retromer positive endosome and tubule both of which are negative for Wls. (ii and iii) Further examples of SNX1-labelled endosomes positive for Wls, but with tubules negative for Wls. Scale bars represent 6 µm. **(C)** RPE-1 cells were transiently co-transfected with pEGFP-SNX3 and Wls-mCherry and cells were subsequently imaged live after a 16 hour incubation period. Frames depicting the formation and scission of GFP-SNX3 labeled buds from vesicles positive for both SNX3 and Wls are shown. Note the 1 second delay between acquisitions for a given image pair. Arrows and arrowheads show two examples of buds positive for both Wls and SNX3 (see Supplementary Information, Movie 2). Scale bars represent 6 µm.

(average length 170-230 nm and diameter 20-50 nm)(Mari et al., 2008). These results suggest that SNX3 may direct the Wls-retromer complex into a clathrin-dependent vesicular transport pathway. Future studies will examine how Wls trafficking is mediated by the SNX3 retromer complex.

DISCUSSION

The identification of the Wnt sorting receptor Wls has shown that the secretion of Wnt proteins is mediated by a specialized trafficking pathway that provides an important layer of regulation to Wnt signaling(Lorenowicz and Korswagen, 2009; Port and Basler, 2010). A key step in this pathway is the retromer-dependent transport of Wls from endosomes to the TGN(Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008). Here, we report that Wls retrieval is mediated by a novel retromer pathway that functions independently of the SNX-BAR retromer coat components.

The retromer consists of a cargo-selective sub-complex that interacts with sorting nexins of the SNX-BAR family to segregate cargo into a tubular endosomal sorting pathway(Cullen, 2008). Our results show that the cargo-selective sub-complex also interacts with SNX3 as part of an alternative retromer pathway that mediates the recycling of Wls. Three lines of evidence suggest that these are functionally distinct retromer pathways. First, genetic analysis in *C. elegans* showed that *snx-3* and the SNX-BAR sorting nexins function in parallel pathways. Thus, retrieval of the phagocytic receptor CED-1(Chen et al., 2010) is dependent on the SNX-BAR sorting nexins but independent of SNX3, whereas Wls recycling requires SNX3 but not the SNX-BAR sorting nexins. Second, CoIP experiments showed that the interaction of the cargo-selective sub-complex of the retromer with the SNX-BAR sorting nexins and SNX3 is mutually exclusive. Finally, live cell imaging revealed that the SNX3 retromer pathway sorts Wls into a vesicular retrieval pathway that is morphologically distinct from the SNX-BAR dependent tubular endosomal sorting pathway(Cullen, 2008). Based on these results we conclude that the SNX-BAR and SNX3 pathways are independent and mechanistically distinct retromer pathways.

Studies in yeast have shown that the SNX3 ortholog Grd19p also functions in retromer-dependent endosome to Golgi retrieval(Hettema et al., 2003; Nothwehr et al., 2000; Strochlic et al., 2007; Voos and Stevens, 1998), but in contrast to SNX3, Grd19p functions together with the SNX-BAR sorting nexins Vps5p and Vps17p in the retrieval of cargo proteins such as the iron transporter Fet3p-Ftr1p. Grd19p physically interacts with a sorting sequence in the cytoplasmic tail of Ftr1p and with the SNX-BAR retromer complex, which led to the hypothesis that Grd19p acts as a cargo-specific adaptor that links Ftr1p to the SNX-BAR retromer complex(Strochlic et al., 2007). We did not observe an interaction between SNX3 and Wls in CoIP experiments (data not shown) and also did not find co-precipitation of SNX3 with the SNX-BAR sorting nexins. Furthermore, we found that mutation of the SNX-BAR sorting nexins did not affect the SNX3 dependent retrieval of Wls, indicating that the function of SNX3 is fundamentally different from Grd19p in yeast.

How do the two distinct SNX3- and SNX-BAR-retromer complexes regulate sorting of different endosomal cargo? One simple model to answer this question

relies on the spatial segregation of SNX3 and the SNX-BAR sorting nexins along the endosomal maturation pathway. While there is significant co-localization between these sorting nexins, SNX3 is predominantly localized to early endosomes by its high affinity interaction with PI3P(Yu and Lemmon, 2001), while the SNX-BAR retromer sorting nexins reside at the interface between early and late endosomes(Mari et al., 2008). Endocytosed Wls therefore initially enters SNX3-labeled early endosomes where it engages the VPS26-VPS29-VPS35 trimeric complex, recruited to this compartment by the interaction with SNX3. Through a vesicular pathway, possibly dependent upon indirect binding to clathrin as well as additional membrane re-modeling proteins, the SNX3 retromer complex sorts Wls for retrieval back to the TGN. In the absence of SNX3, Wls can be missorted into intraluminal vesicles and hence lysosomal degradation, or can be recycled through SNX-BAR retromer back to the TGN. The relative flux through these two distinct pathways therefore determines the steady-state level of Wls. As the level of Wls is severely reduced upon loss of SNX3, the flux into the lysosomal degradative pathway appears to be dominant. Thus, while a proportion of Wls may undergo SNX-BAR retromer-mediated recycling in the absence of SNX3, this is insufficient to maintain the required level of Wls for Wnt gradient formation during iterative rounds of Wnt secretion and Wls retrieval from the cell surface.

Interestingly, the steady-state trafficking of the classical SNX-BAR retromer cargo CI-MPR is primarily defined by intracellular cycling between the TGN and late endosomes with retrieval back to the TGN via the SNX-BAR retromer. The spatial segregation model therefore suggests that the lack of effect of SNX3 suppression on steady-state CI-MPR distribution arises from CI-MPR entering the endosomal network at a point downstream of SNX3(Wassmer et al., 2007). That said, the complexity of CI-MPR trafficking - a proportion of this receptor traffics to the plasma membrane before undergoing endocytosis and retrograde transport to the TGN(Duncan and Kornfeld, 1988; Jin et al., 1989; Lin et al., 2004) - suggests that such a simple spatial segregation model may be an oversimplification. We therefore speculate that alongside spatial segregation, cargo binding to the VPS26-VPS29-VPS35 complex may be an important element in selecting the sorting nexin coat that specifies the subsequent retrograde trafficking route. Thus, binding of VPS26-VPS29-VPS35 to Wls may favor association with SNX3, while engagement with CI-MPR favors binding to the SNX-BAR coat complex.

ACKNOWLEDGEMENTS

We thank Dr. Catherine Rabouille for critically reading the manuscript, Dr Matthew Seaman for invaluable advice, Dr. Ding Xue (University of Colorado, Boulder) for *smIs34*, Dr Mitsuaki Tabuchi (Kawasaki Medical School, Okayama, Japan) for the bacterial construct expressing 3xFLAG-tagged retromer complex, Dr. Shohei Mitani (National Bioresource Project for the Nematode, Tokyo, Japan) for deletion

mutants, Dr. Andrew Fire for expression vectors and the *Caenorhabditis* Genetic Center (University of Minnesota, Minneapolis) for strains. This work was funded by the Dutch Cancer Society (HUBR 2008-4114), the EU FP6 Program Cells into Organs and NWO VIDI (016.076.317) (H.C.K.), an NWO VENI fellowship (M.J.L), a Boehringer Ingelheim Foundation fellowship (M.H.), the Swiss National Science Foundation and the Forschungskredit of the University of Zürich (F.P. and K.B.) and the Wellcome Trust (089928/Z/09/Z and 085743) (P.J.C.). I.J.M. is a Wellcome Trust-funded PhD student (086777/Z/08/Z).

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SUPPORTING ONLINE MATERIAL - METHODS

C. elegans strains and culturing

General methods for culture, manipulation and genetics of *C. elegans* were as described(Lewis and Fleming, 1995). Strains were cultured at 20ºC. Mutations and transgenes used in this study were: LGI, *pry-1(mu38)*(Korswagen et al., 2002; Maloof et al., 1999) and *snx-3(tm1595)*; LGII, *mab-5(e1751)*(Salser and Kenyon, 1992), *vps-35(hu68)*(Coudreuse et al., 2006), *huSi2[Pmig-14;;mig-14::gfp]* and *muIs32[Pmec-7::gfp]*(Ch'ng et al., 2003); LGIII, *vps-29(tm1320)*(Coudreuse et al., 2006); LGIV, *snx-6(tm3790)*(Chen et al., 2010) and *vps-26(tm1523)*(Coudreuse et al., 2006); LGV, *muIs35[Pmec-7::gfp]*(Ch'ng et al., 2003); LGX, *snx-1(tm847)* (Coudreuse et al., 2006), and unassigned, *huIs71[Pmig-14::mig-14::gfp]*(Yang et al., 2008), *muIs2[Pmab-5::LacZ]*(Salser and Kenyon, 1992), *huIs60[Pegl-20::egl-20::protA]*(Coudreuse et al., 2006), *smIs34[Pced-1::ced-1::gfp]*(Chen et al., 2010), *huIs110[Psnx-3::snx-3::gfp]* and the extra-chromosomal line *huEx149[Pegl-20::lmp-1::mcherry]*(Yang et al., 2008).

C. elegans phenotypes, expression constructs and transgenesis

The final positions of the HSN and Q descendants and the polarity of the V5 seam cell division were scored in L1 larvae(Harris et al., 1996; Whangbo et al., 2000). The polarity of the ALM and PLM neurons, dye filling, P12 to P11 fate transformation and *Pmab-5::lacZ* reporter gene expression were analyzed as described(Herman and Horvitz, 1994; Prasad and Clark, 2006; Salser and Kenyon, 1992). To construct *Psnx-3::snx-3::gfp*, the *snx-3* genomic sequence and 2 kb of its promoter region were PCR amplified and cloned in frame into the *gfp* expression vector pPD95.75. A 4.4 kb upstream region of *egl-20* that specifically drives expression in the *egl-20* producing cells(Coudreuse et al., 2006) was used to generate *Pegl-20::snx-3::gfp* and *Pegl-20::snx-3::mcherry*. Extra-chromosomal arrays were integrated as described(Mello and Fire, 1995).

RNAi screen in *C. elegans*

Glycerol stocks of the Ahringer RNAi library(Kamath et al., 2003) were inoculated in 500 μl of LB medium containing 50 μg/ml Ampicillin and grown overnight at 37°C in 2.5 ml deep 96-well plates (HJ-Bioanalytik). 100 μl of bacterial culture was seeded on 3 cm NGM agar plates containing 50 μg/ml Ampicillin and 1 mM IPTG and incubated for 24 hours at room temperature to induce double stranded RNA expression. Four to eight L4 stage wild type, *pop-1(hu9)* or *vps-29(tm1320)* animals expressing *mec-7::gfp* (*muIs32*) were added to each plate and grown at 15°C for 72 hours and then at 20°C for 24 to 48 hours. The final position of the Q descendants AVM and PVM was scored using a Leica MZFLIII stereomicroscope equipped with an epifluorescence unit. A minimum of 100 animals was counted on each plate. RNAi clones inducing a defect in wild type

or an enhancement in one of the sensitized genetic backgrounds were retained and repeated in triplicate.

Drosophila stocks

The following RNAi lines were obtained from the Vienna *Drosophila* RNAi Center (http://www.vdrc.at/): UAS-snx3^{RNAi} (104494), UAS-snx6^{RNAi} (24275) and UAS-snx6^{RNAi} (24276). Note that UAS-snx3^{RNAi} (34166) gives a similar but weaker phenotype than UAS-snx3^{RNAi} (104494) (data not shown). The Gal4-driver lines used are indicated in the figure legend and are available from the Bloomington *Drosophila* Stock Center (BDSC). The P-element line yw;;P{EPgy2}CG6359^{EY05688} (stock nr. 16668) was obtained from the BDSC and the P-element was recombined with ywhsp-Flp;sp/cyo;FRT82/Tm6b to generate ywhsp-Flp;sp/cyo;FRT82 CG6359^{EY05688}/Tmb6. Clones were generated by crossing flies to ywhsp-Flp;;FRT82 ubiGFP and the F1 progeny was heat shocked 42 hours after egg laying for 45 minutes at 37°C.

Drosophila immuno-staining

Immuno-staining was performed using standard protocols. Briefly, 3rd 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 minutes at room temperature (RT). Discs were washed in PBS containing 0.05% Triton X-100 (PBT) for 1 hour 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 hour at RT and subsequently incubated in secondary antibody solution for 2 hour at RT. After a final wash for 1 hour 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. The extracellular Wg staining was performed as described(Baeg et al., 2001). Images were collected on a Zeiss LSM710 or Leica SP5 confocal microscope using the sequential scanning mode. Images were analyzed using ImageJ (NIH).

Cell culture, transfection, immuno-fluorescence and Western blot analysis

HeLa cells were maintained in RPMI 1640 medium (GIBCO) containing 10% heat-inactivated FCS (GIBCO), 2 mM gluta-MAX, 100 U/ml penicillin, and 100 µg/ml streptomycin. HeLa cells were transfected with control siRNA (Dharmacon), RAB7 siRNA(Kawakami et al., 2008) or SMART pooled siRNAs against SNX3 (M-011521-01-0010) or SNX12 (M-013648-00) (Dharmacon) using Oligofectamine (Invitrogen). Cells were transfected two times with a 48 hour interval between transfections and were analyzed by immuno-fluorescence or Western Blotting 24 hours after the last transfection. Plasmid containing human Wls-mCherry (1 µg) and plasmids containing human SNX3-GFP and SNX12-GFP (1 µg) were

transfected using FuGENE Transfection Reagent (Roche) 24 hours and 8 hours prior to further analyses, respectively. Human Wls cDNA was cloned in frame with mCherry into the pcDNA3.1zeo vector (Invitrogen). Human SNX3 and SNX12 cDNA were cloned into the pEGFP-N3, the pEGFP-C1 or the pEGFP-C2 vector (Clonetech). GFP-SNX3 was cloned into the vector XLG3 for lenti-viral expression.

For immuno-fluorescence analysis, HeLa cells were plated on glass cover slips, fixed in 0.1 M Phosphate Buffer containing 4% paraformaldehyde for 10 minutes on ice and permeabilized with 0.1% Triton X-100 for 5 minutes, except for LAMP1 localization, for which 0.1% (w/v) saponin was used for permeabilization. Thereafter, cells were incubated with 0.5% BSA for 30 minutes 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 488 (Molecular Probes). For nuclear staining DAPI was used. Images were recorded on a Leica SPE or a Leica AOBS-SP2 confocal microscope. Co-localization analysis was performed using the ICA plug-in from ImageJ (NIH), or by using Volocity co-localization software (Perkin Elmer). Volume integration of voxel intensity was calculated using the Pearson's coefficient (PC), measuring the protein of interest relative to a marker. Thresholds were set independently for each channel.

For 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. Cell fractionation samples were prepared as described(Seaman et al., 2009). To quantify MIG-14::GFP protein levels, synchronized L1 larvae were lysed in 4 volumes of 25 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM $CaCl_2$ and 1% TX-114 supplemented with protease inhibitors (Roche). Densitometric analysis was performed on scanned images using ImageJ (NIH).

GFP-nanotrap

Culture dishes (15 cm) with HeLa cells at approximately 95% confluency were washed twice with ice-cold PBS and lysed by the addition of lysis buffer containing 0.1 M MES-NaOH pH 6.5, 1 mM Mg-acetate, 0.5 mM EGTA, 200 μM sodium vanadate, 1% (w/v) digitonin and protease inhibitors. After scraping, lysates were cleared by centrifugation at 13,000g for 10 minutes at 4°C. Cell lysates were added to 30 μl of equilibrated GFP-Trap beads (ChromoTek) followed by incubation with gentle end-over-end mixing for 1 hour at 4°C. Beads were then spun down at 2000g for 2 minutes at 4°C and washed three times in detergent-free lysis buffer. Complexes were eluted from the GFP-Trap beads by boiling in NuPAGE LDS sample buffer (Invitrogen) and subjected to gel electrophoresis and western blotting.

Electron Microscopy

Cryo-immuno-electron microscopy was performed as described(Mari et al., 2008). HeLa cells expressing GFP-SNX3 and mCherry-VPS26 were fixed in 4%

(w/v) paraformaldehyde, 0.05% (w/v) glutaraldehyde in 0.1 M phosphate buffer. The fixed cells were scraped off the dish in 1% gelatine in phosphate buffer and spun down in 10% gelatine. After 1 hour of solidification on ice, pellets were cut into small blocks and infiltrated with 2.3 M sucrose at 4° C overnight. The blocks were mounted on aluminum pins and frozen in liquid nitrogen for ultrathin cryo-sectioning. Sections (70 nm) were collected at –120°C in 1% methyl-cellulose in 1.2 M sucrose on formvar/carbon-coated copper mesh grids. The sections were labeled with polyclonal goat anti-GFP (Rockland Immunochemicals Inc.) and polyclonal rabbit anti-RFP (Molecular Probes Invitrogen) antibodies and secondary antibodies anti-goat 10 nm gold and anti-rabbit 6 nm gold (Aurion). The sections were counterstained with 0.3% (w/v) uranyl acetate in 1.8% (w/v) methyl-cellulose and imaged on a FEI Tecnai 12 Biotwin transmission electron microscope equipped with a bottom mount.

Antibodies

The following antibodies were used in this study: polyclonal antibody against *Drosophila* Wls(Port et al., 2008); polyclonal chicken anti-Wls (Abcam); polyclonal rabbit anti-SNX3 (Abcam); polyclonal goat antibody recognizing both SNX3 as well as SNX12 (C16, Santa Cruz); polyclonal goat anti-SNX5 (D18, Santa Cruz); polyclonal goat anti-EEA1 (N19, Santa Cruz); monoclonal mouse anti-EEA1 (BD Transduction Laboratories); monoclonal mouse anti-tubulin (clone DM1A, Sigma-Aldrich); monoclonal mouse anti-GFP (mix of clones 7.1 and 13.1, Roche); anti-GFP (Clonetech); monoclonal mouse anti-SNX1 (clone 51,BD Transduction Laboratories); monoclonal mouse anti-Wg (4D4; DSHB); chicken-anti-mouse-Ig or chicken-anti-rabbit-Ig antibody labeled with Alexa 488 or goat anti-mouse Alexa 594 (Molecular Probes), rabbit anti-goat and goat anti-rabbit Cy5 (Jackson Immunoresearch Laboratories); mouse monoclonal anti-LAMP1 (DSHB). The following antibodies were kindly provided by other researchers: polyclonal rabbit anti-LAMP1 (270C) from Dr. Ash Toye; polyclonal rabbit anti-hVps26 and anti-hVps35 from Dr. M. Seaman; polyclonal guinea pig anti-Sens (GP55) from Dr. H. Bellen; polyclonal rabbit anti-Hh from Dr. S. Eaton and polyclonal rabbit anti-pMad from Dr. G. Morata.

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SUPPORTING ONLINE MATERIAL - FIGURES

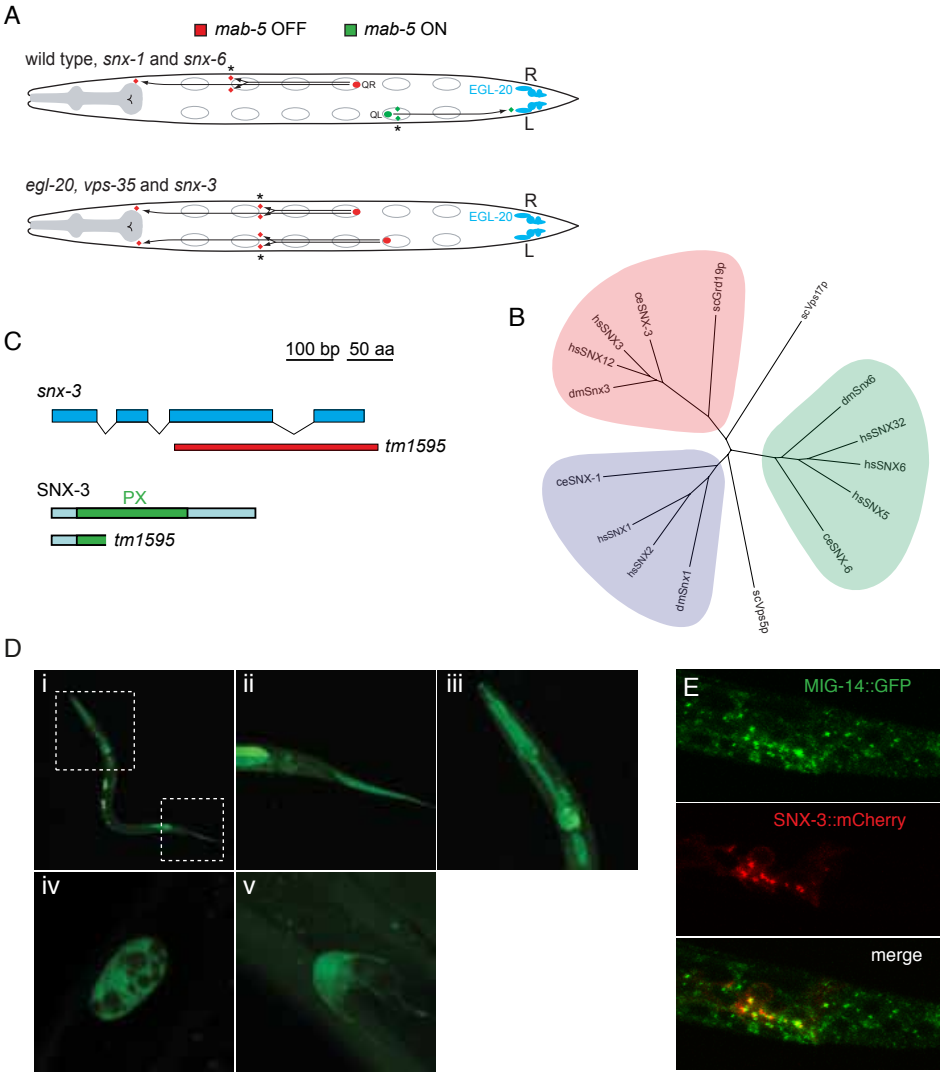


Figure S1. (**A**) Schematic representation of Q.d migration. Dorsal view, anterior is to the left. Cells are in green or red when *mab-5* expression is activated or absent, respectively. The *egl-20* expressing cells are indicated in blue. Grey circles indicate the position of the seam cells V1 to V6. The final positions of the Q.paa and Q.pap cells in wild type and *egl-20*, *vps-35* and *snx-3* mutants are indicated by an asterisk. (**B**) Phylogenetic tree of yeast (sc), *C. elegans* (ce), *Drosophila* (dm) and human (hs) SNX-BAR (green and blue) and SNX3 (pink) related sorting nexins. Phylogenetic analysis was performed using CLUSTALW (default program parameters). (**C**) Schematic representation of the deleted region in *snx-3(tm1595)*. The Phox-homology (PX) domain is shown in green. *snx-3(tm1595)* is a ▶

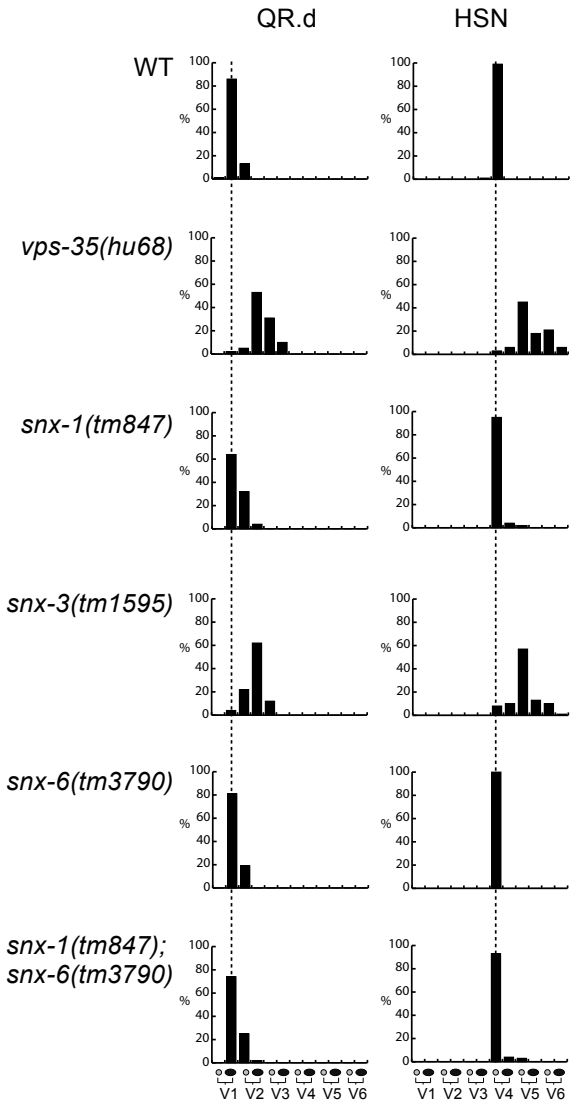


Figure S2. *snx-3*, but not *snx-1* and *snx-6* are required for the EGL-20/Wnt dependent anterior migration of the QR.d and the HSN neurons. The final positions of QR.paa and QR.pap and the HSN neurons are indicated relative to the invariant positions of the seam cells V1 to V6 (n>50).

▶ combined insertion and deletion allele that removes 420 base pairs and inserts 14 base pairs (inserted sequence is TTCTCCGAAAAATC). (**D**) Expression pattern of a fusion of the *snx-3* promoter and coding sequence to *gfp* (*huIs110*). *snx-3* is ubiquitously expressed (**i**), with most prominent expression in the tail hypodermis and rectal epithelial cells (including the EGL-20/Wnt producing cells) (**ii**), the pharynx (**iii**), coelomocytes (**iv**) and the distal tip cells (**v**). (**E**) Co-localization of MIG-14::GFP and SNX-3::mCherry in EGL-20/Wnt producing cells in *C. elegans*. Confocal images of the *Pmig-14::mig-14::gfp* expressing transgene *huSi2* combined with the *Pegl-20::snx-3::mcherry* expressing transgene *huEx221*. Arrowheads indicate examples of co-localization. Images are projections of several confocal sections.

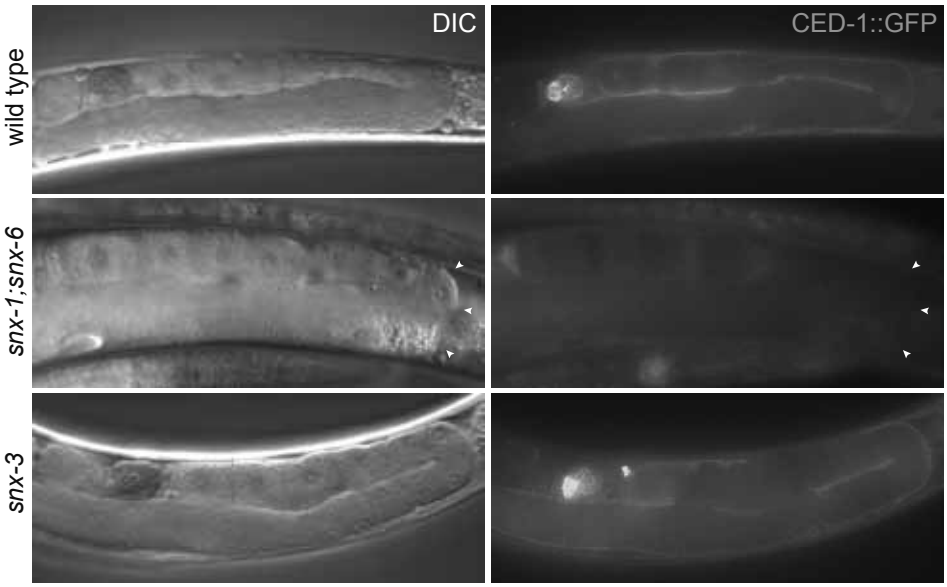


Figure S3. CED-1::GFP levels in the gonadal sheath cell are strongly reduced in *snx-1; snx-6* double mutants. CED-1::GFP was expressed using the *smIs34* transgene(Chen et al., 2010). CED-1::GFP levels are not reduced in *snx-3* mutants. Consistently, *snx-3* RNAi does not induce defects in cell corpse engulfment(Chen et al., 2010).

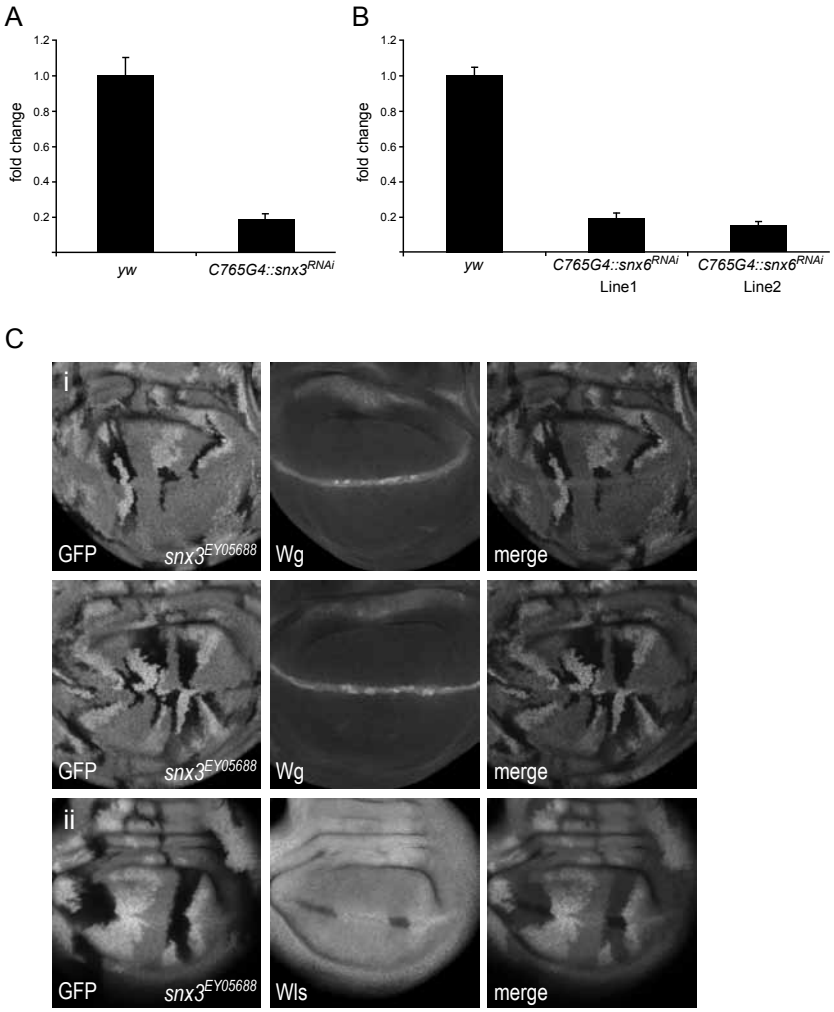


Figure S4. (**A**) Efficient knock-down of *Dsnx3* and *Dsnx6* transcript upon RNAi. Imaginal discs from third instar larvae were analyzed by qt-RT-PCR. Total RNA was extracted from 16 wing imaginal discs from each genotype using the Nucleospin RNA II kit (Macherey-Nagel). Quantitative PCR reactions were performed in triplicates and monitored using the Applied Biosystems SYBR Green kit and the ABI Prism 7900HT System (Applied Biosystems). All results were simultaneously normalized to Actin5C, tubulin-1a and TBP mRNA levels and the expression levels calculated using the DDCt method (Applied Biosystems user bulletin #2, updated version 04/2001). Relative change of mRNA abundance relative to wild type imaginal discs (yw) is shown. Error bars represent standard deviation. RNAi Lines from VDRC Vienna are as follows: *snx3* CG6359 TF 104494; *snx6* Line1 CG8282 TF 24275; *snx6* Line2 CG8282 TF 24276. (**B**) Homozygous clones of $Dsnx3^{EY05688}$ accumulate Wg and loose Wls in Wnt producing cells. (**i**) Wg levels are elevated in homozygous $Dsnx3^{EY05688}$ clones, which are marked by the absence of GFP expression. (**ii**) $Dsnx3^{EY05688}$ clones loose Wls protein specifically in Wg producing cells. Clones are marked by the absence of GFP.

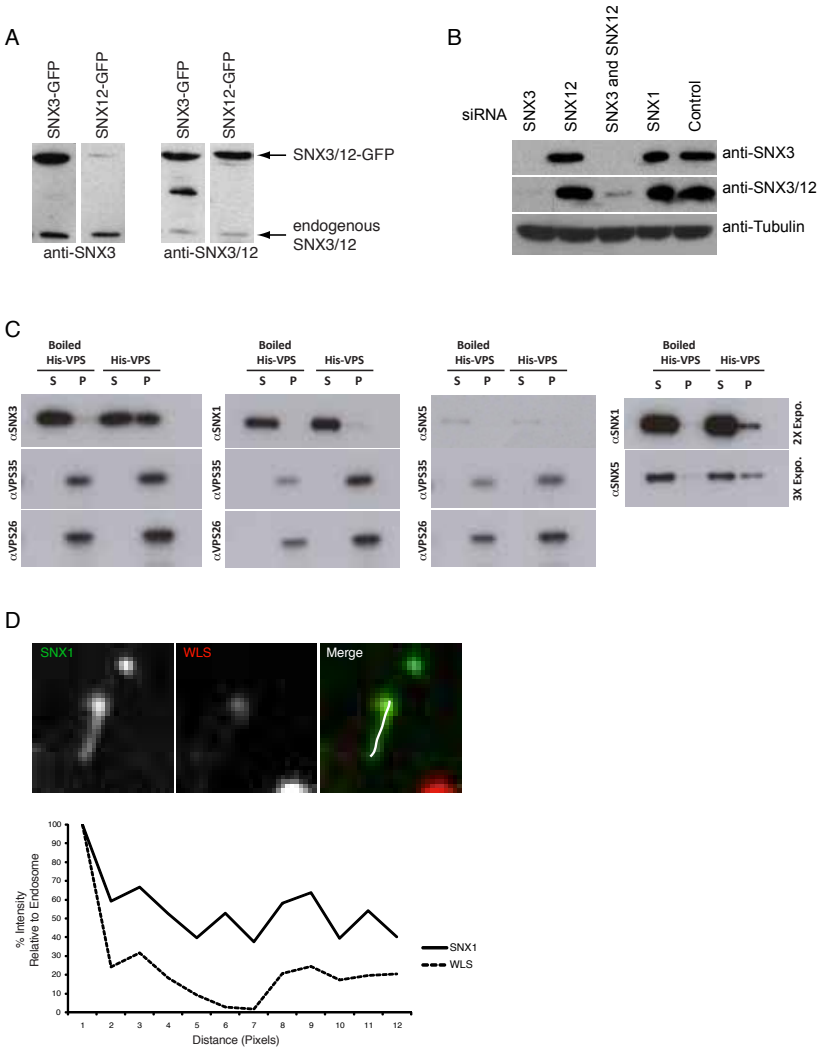
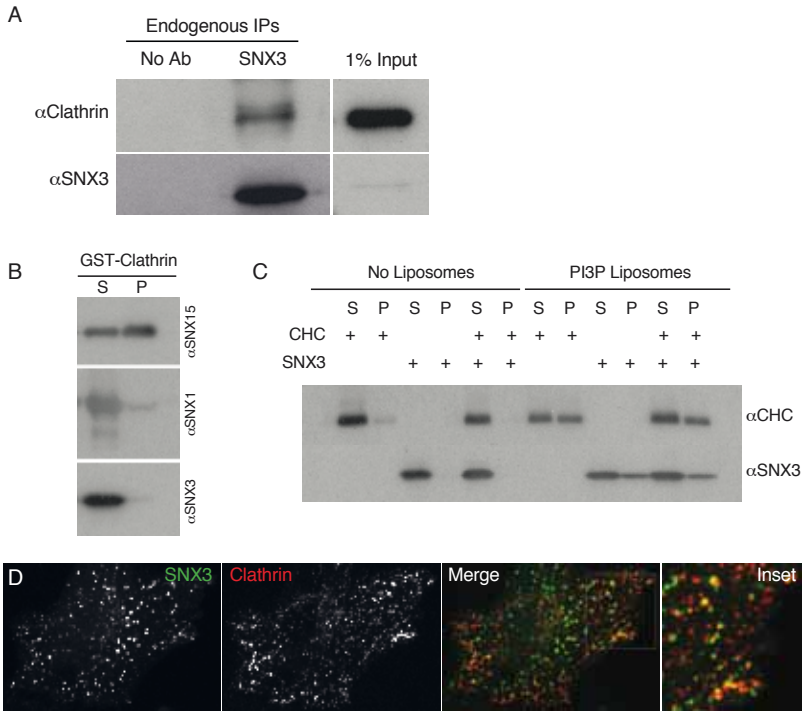


Figure S5. Western blot detection of SNX3 and SNX12 in HeLa cells. (**A**) Cells were transfected with SNX3-GFP or SNX12-GFP and Western blots were stained with a polyclonal rabbit anti-SNX3 antibody (Abcam) or a polyclonal goat antibody recognizing both SNX3 and SNX12 (C16, Santa Cruz). (**B**) Cells were treated with control, SNX3, SNX12 or SNX1 siRNA and SNX3 and SNX12 were detected using the SNX3 specific antibody or the antibody that recognizes both SNX3 and SNX12. Note that SNX3 and SNX12 are of similar size and are therefore not separated. (**C**) SNX3 directly associates with the cargo-selective sub-complex of the retromer. 3xFLAG-VPS26-VPS29-VPS35-His_6 trimeric complex (His-VPS) was isolated from BL21 *E. coli* onto TALON resin and incubated with 2 μM of either recombinant SNX3, SNX1 or SNX5 for 2 hours at 4°C. Supernatant (S) and TALON containing resin (P) were isolated prior to Western analysis. SNX3 directly associates with His-VPS, as do SNX1 and SNX5, although this is less well pronounced (longer exposures are shown). Control: boiled His-VPS resin. (**D**) Quantification of GFP-SNX1 and Wls-mCherry in an endosome and the tubule that is projecting from it.



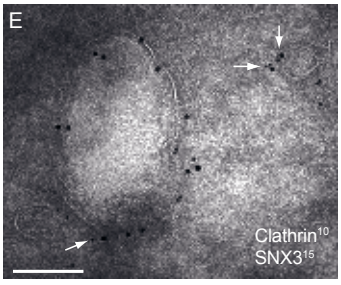


Figure S6. SNX3 associates indirectly with clathrin. (**A**) Immunoprecipitation of endogenous SNX3 reveals an association with clathrin heavy chain. Cell extracts from HeLa cells were incubated with anti-SNX3 antibody prior to western analysis. Control, no antibody. (**B**) Purified recombinant GST-clathrin (residues 1-579) was isolated from BL21 *E. coli* onto glutathione resin and incubated with 5 μM of purified recombinant SNX3, SNX1 or as a positive control SNX15 (a sorting nexin that directly associates with clathrin, C. Danson, and P.J. Cullen, unpublished). After centrifugation, supernatant (S) and glutathione resin containing pellet (P) were resolved prior to western analysis with anti-SNX3, anti-SNX1 or anti-SNX15 antibodies. (**C**) To determine whether PI(3)P-mediated membrane association is required for the direct binding of SNX3 with clathrin, the interaction of 20 μM clathrin heavy chain (cleaved from purified recombinant GST-clathrin (residues 1-579) with 5 μM recombinant SNX3 was performed on artificial liposomes supplemented with 1 molar percent PI(3)P (control - no liposomes to verify that association is lipid dependent). After separation of supernatant (S) from pellet (P), western blotting revealed that the binding of SNX3 to PI(3)P-containing liposomes did not enhance, above basal level, the liposome association of clathrin. This is consistent with clathrin not binding directly to SNX3 even after engagement with PI(3)P-containing liposomes. (**D**) Partial co-localization between GFP-SNX3 and dsRed-clathrin light chain in HeLa cells. Imaging was performed in live cells with a single selected frame being shown (Pearson's correlation is 0.10 ± 0.03; data are represented as mean ± SD, n=30 cells). Scale bar, 20 μm. (**E**) Immuno-electron microscopy of GFP-SNX3 (15 nm gold) and endogenous clathrin heavy chain (10 nm gold) in HeLa cells. Scale bar, 200 nm.

4

WNT SIGNALING REQUIRES MTM-6 AND MTM-9 MYOTUBULARIN LIPID-PHOSPHATASE FUNCTION IN WNT PRODUCING CELLS

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EMBO journal (2010) 29, 4094-4105

ABSTRACT

Wnt proteins are lipid modified glycoproteins that play 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. Here, we report the identification of MTM-6 and MTM-9 as novel regulators of MIG-14/Wls trafficking in *C. 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 ortholog *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.

INTRODUCTION

During the development of multi-cellular organisms, cells communicate with each other to coordinate complex processes such as cell fate determination and morphogenesis. A family of secreted signaling proteins that 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 (Takada et al, 2006; Willert et al, 2003), raising questions on how these hydrophobic proteins are secreted and released from 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 (reviewed in Eaton, 2006; Kornberg & 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 trans-membrane 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, since defects in Wnt signaling are also observed in mutants of mouse Wls (Fu et al, 2009) and the *C. elegans* Wls ortholog *mig-14* (Banziger et al, 2006; Harris et al, 1996; Thorpe et al, 1997), 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*, where mutants of the cargo-selective subunits of the retromer (encoded by *vps-35*, *vps-26* and *vps-29*) display defects in Wnt signaling (Coudreuse et al, 2006; Prasad & Clark, 2006). This function is evolutionarily conserved, as loss of retromer function also disrupts Wnt signaling in *Drosophila*, *Xenopus* and mammalian cells (Belenkaya et al, 2008; Coudreuse et al, 2006; Franch-Marro et al, 2008; Kim et al, 2009; Port et al, 2008). 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 signaling 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 signaling 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 analyzed 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 signaling 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 better understand the mechanism of Wnt secretion, 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 & 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 signaling 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) that 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, while the Q.d on the left side migrate to the posterior of the animal (Fig. 1A). Posterior migration is regulated by the Wnt protein EGL-20,

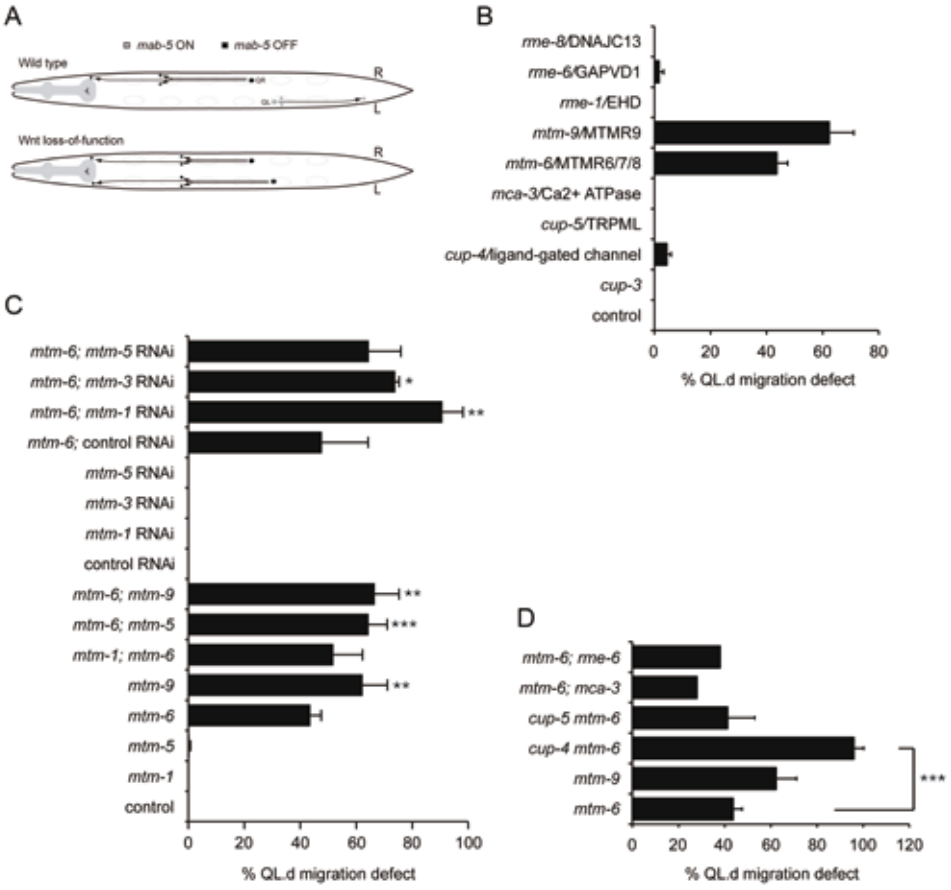

Figure 1. The myotubularin-related genes *mtm-6* and *mtm-9* are required for correct QL descendant migration. (**A**) Schematic representation of the migration of Q neuroblast descendants in wild type L1 larvae and in animals with impaired EGL-20/Wnt signaling. In wild type animals, the QR.d migrate to the anterior, while the QL.d activate the Wnt target gene *mab-5* and migrate to the posterior. Loss of Wnt signaling 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 *muls32* transgene expressing *mec-7::gfp* (Ch'ng et al, 2003). All error bars represent standard deviations of at least three independent assays. Full genotypes and total numbers of animals scored are shown in Table S1. * $P < 0.02$, ** $P < 0.005$, *** $P < 0.001$, unpaired t-test (compared to *mtm-6* or *mtm-6*; control RNAi)

which induces expression of the homeotic gene *mab-5* in QL and its descendants (Salser & 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 (Coudreuse et al, 2006; Harris et al, 1996; Prasad & Clark, 2006; Salser & Kenyon, 1992). Among nine genes tested, we found two Cup mutants with a significant proportion of anteriorly misplaced QL.d, *mtm-6* and *mtm-9* (Fig. 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, where MTM-6 is an active phosphatase, while 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 (Fig. 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 (Fig. S1A) (Dang et al, 2004). The lower penetrance of the *mtm-6(ok330)* QL.d migration phenotype compared to *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 which removes the FYVE domain, and *ar515*, a missense mutation in the catalytic site (Dang et al, 2004; Fares & Greenwald, 2001). 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 to 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 (Fig. 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 play a minor role in QL.d migration by acting partially redundantly with *mtm-6* and *mtm-9*. In most of the following work we focused on *mtm-6* as the enzymatically active part of the complex.

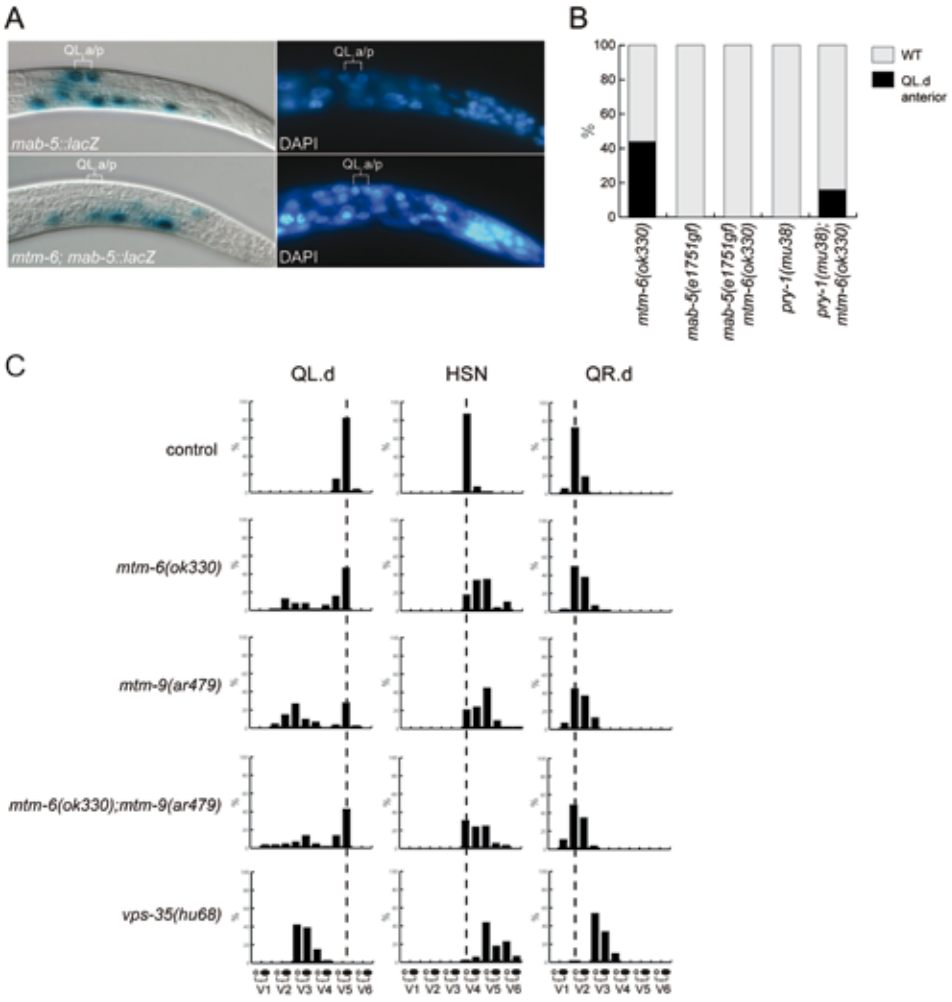

Figure 2. Characterization of the Wnt-related phenotypes of *mtm-6* mutants. (**A**) Activation of a *mab-5::lacZ* transcriptional reporter in the QL.a/p daughter cells. *mab-5* expression is not activated in *mtm-6* mutants, even though QL.a/p are present. (**B**) Suppression of the QL.d migration phenotype 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.

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* enhanced the phenotype of *mtm-6* (Fig. 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* (Harris et al, 1996; Salser & Kenyon, 1992). To investigate whether the defect in QL.d migration is caused by a loss of *mab-5* expression in the QL lineage, we analyzed the expression of a *mab-5::lacZ* reporter that has been shown to closely mimic the expression of the endogenous *mab-5* gene (Cowing & 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 (Fig. 2A). Importantly, the EGL-20-independent expression of *mab-5* in other cells, such as the posterior ventral nerve cord neurons (Salser & 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* (Fig. 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 (Fig. 2B), a negative regulator of the canonical Wnt/β-catenin pathway that is triggered by EGL-20/Wnt in the QL lineage (Korswagen et al, 2002; Maloof et al, 1999).

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, 2006; Prasad & 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 (Fig. 2C). Furthermore, there was a weak but reproducible defect in the polarity of the ALM neurons (Table 1), 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.

Table 1. 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.

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 signaling. *mtm-6* is part of a large operon (Fig. S1B) which complicates the determination of its expression pattern. The promoter element used by Xue and co-workers 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*, since 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 muscle, intestine, hypodermis and neurons (Fig. S2 and data not shown). *mtm-9* was also expressed in the rectal epithelial cells, which are the major source of EGL-20/Wnt (Whangbo & Kenyon, 1999) (Fig. 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 (Fig. 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

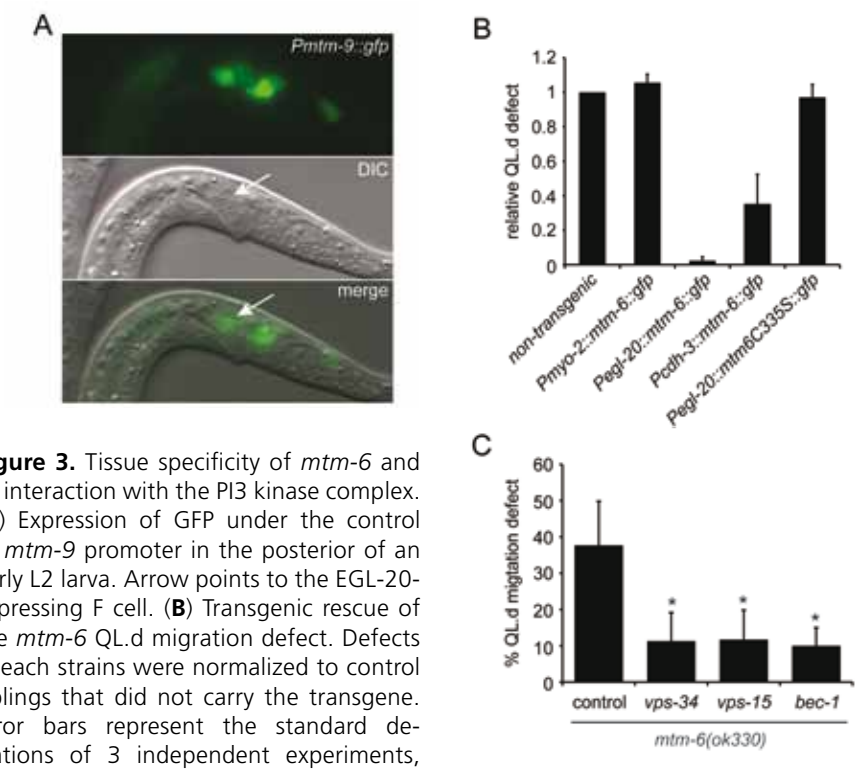

Figure 3. Tissue specificity of *mtm-6* and its interaction with the PI3 kinase complex. (**A**) Expression of GFP under the control of *mtm-9* promoter in the posterior of an early L2 larva. Arrow points to the EGL-20-expressing F cell. (**B**) Transgenic rescue of the *mtm-6* QL.d migration defect.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

results demonstrate that MTM-6 is required in Wnt producing cells and that the phosphatase activity is indispensable for its function.

Knock-down of the Vps34 PI3 kinase complex restores EGL-20/Wnt signaling 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 signaling, we examined the function of *vps-34* in QL.d migration. As *vps-34* mutants are embryonic lethal (Xue et al, 2003), we employed RNAi to partially knock down *vps-34* expression. Knock down 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 (Fig. 3C). A similar effect was achieved with RNAi against *ZK930.1* and *bec-1*, genes encoding two subunits of the Vps34

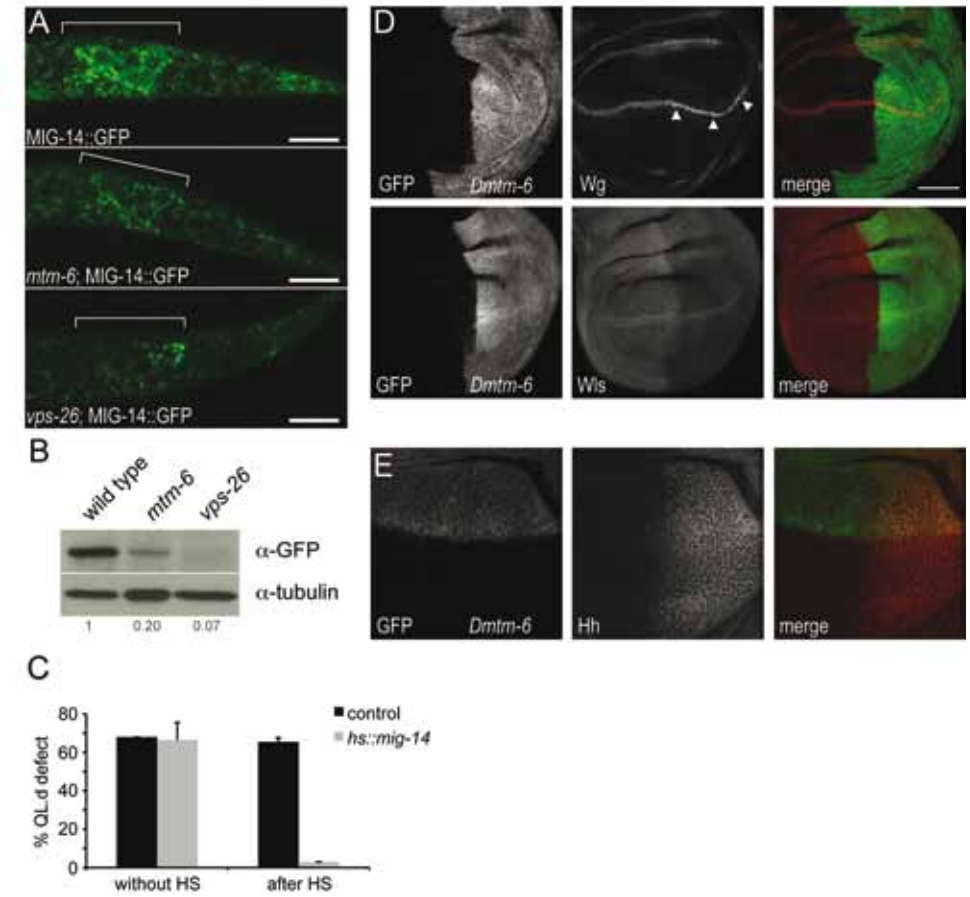

Figure 4. *mtm-6* regulates MIG-14/Wls protein levels in *C. elegans* and *Drosophila*. (**A**) Levels of MIG-14::GFP expressed from its own promoter from the single copy transgene *huSi2* are reduced in *mtm-6* and *vps-26* backgrounds compared to wild type. Images are maximum projections of 10 confocal 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 standard deviations (3 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 Fig. S4.

kinase complex, Vps15 and Vps30/Beclin1, respectively (Fig. 3C) (Kihara et al, 2001; Stack et al, 1993). These results support the notion that the primary cause of the Wnt signaling 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 signaling.

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

Since *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). Since these mutants show a fully penetrant defect in QL.d migration, we focused on the Wnt-dependent polarization of the ALM and PLM neurons. While *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 1). 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 signaling.

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 Fig. 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 signaling than *mtm-6* mutants.

To investigate whether MIG-14/Wls becomes limiting for Wnt signaling in *mtm-6* mutants, we tested whether *mig-14* overexpression can rescue EGL-20/Wnt signaling. 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 (Fig. 4C). The rescue of Wnt signaling by *mig-14* overexpression is consistent with a function of *mtm-6* in MIG-14/Wls trafficking and suggests that the defect in Wnt signaling 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 signaling, 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* ortholog *Dmtm6* in the posterior compartment of the disc by transgene-mediated RNAi (Fig. S3), we observed accumulation of the 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 (Fig. 4D, S4). Importantly, *Dmtm6* knock-down did not affect secretion of Hedgehog, another lipid-modified morphogen (Fig. 4E, S4) 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 signaling, *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 Fig. 4D and S4, 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 plays 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 (reviewed in Di Paolo & De Camilli, 2006). Among these are the sorting nexins (SNX), a family of proteins containing a PI3P-binding Phox-homology (PX) domain (Cullen, 2008), that 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

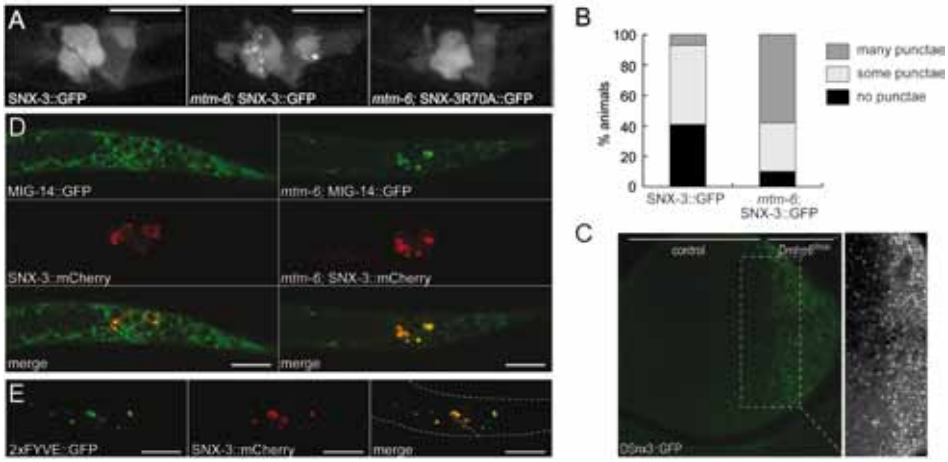

Figure 5. MTM-6 controls the endosomal localization of SNX-3. (**A**) Localization of SNX-3::GFP in the EGL-20-expressing cells of a wild type and *mtm-6* L1 larva. *mtm-6* mutants display more punctuate localization of SNX-3 that is lost upon introduction of the R70A mutation in the PI3P binding domain of SNX-3. Images are maximum projections of 10 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 (1-5 distinct punctae) many punctae (>5 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* promoter. 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 Fig. 4A). Scale bar is 10 μm and images are maximum projections of 4 confocal sections taken at 0.2 μm z-steps (**E**) Co-localization between SNX-3 and the endosomal marker 2xFYVE::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.

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 (Chapter 3) 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, while in *mtm-6* mutants the punctate localization became much more prominent (Fig. 5A, B). A similar phenotype was observed in the *Drosophila* wing imaginal disc, where DSnx3 localized much more frequently to punctate structures in posterior cells expressing a D*mtm6* RNAi transgene compared to cells in the anterior where the RNAi transgene was not expressed (Fig. 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 (Fig. 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 on intracellular punctae, with most of the SNX-3 positive vesicles also containing MIG-14::GFP (Fig. 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 (Fig. 5D, 4A), 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 2xFYVE::GFP (Roggo et al, 2002). As shown in Fig. 5E, SNX-3 and 2xFYVE::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 (Fig. S5). Disruption of this regulation induces a defect in MIG-14/Wls trafficking that 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 signaling 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.

Here, 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 (Schaletzky et al, 2003; Taylor et al, 2000; Walker et al, 2001), two important phosphoinositide species involved in regulation of membrane trafficking (reviewed in Vicinanza et al, 2008). Since several myotubularins act on the same substrate, they may either act redundantly or they 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 signaling, suggesting that individual myotubularins have distinct roles. Indeed, minor functions of the other myotubularins in Wnt signaling 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 (Cao et al, 2007; Lorenzo et al, 2006), while others have reported diffuse cytoplasmic localization (Blondeau et al, 2000; Laporte et al, 2002; Taylor et al, 2000). An intriguing example of differential localization has been described for human MTM1 and MTMR2, where MTM1 localizes to Rab5-positive early endosomes and only partially to Rab7-positive late endosomes (Cao et al, 2007), while 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 knock-down 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 (Dang et al, 2004; Mochizuki & Majerus, 2003), 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 regulation of Ca^{2+} signaling during T cell activation (Srivastava et al, 2006). Although *C. elegans* MTM-6 interacts with the $K_{Ca}3.1$ ortholog KCNL-2 in a yeast two-hybrid assay (Srivastava et al, 2005), the physiological relevance of this interaction in *C. elegans* is not clear. Knock down 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, submitted). 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 (Fig. S5). 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 signaling in *mtm-6* mutants can be restored by MIG-14/Wls overexpression.

While mutation of *mig-14*/Wls affects all Wnt dependent processes in *C. elegans* (Pan et al, 2008; Thorpe et al, 1997; 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 & 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 & Clark, 2006). The Wnt signaling 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 signaling defect of *mtm-6* can be strongly enhanced by partial knock down 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, knock-down 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 plays 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 while 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 signaling pathways. Furthermore, we found that the secretion of Hh is not affected by *Dmtm6* knock down in *Drosophila*. As loss of retromer function also mainly affects Wnt signaling (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 signaling suggests that other developmentally important signaling 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 (Azzedine et al, 2003; Bolino et al, 2000; Senderek et al, 2003) and azoospermia (Firestein et al, 2002). A common mechanism underlying these pathologies is defective membrane transport and homeostasis (reviewed in (Nicot & Laporte, 2008). However, the exact mechanism through which these myotubularin family members cause the different pathologies is not understood. We provide a novel link between myotubularin phosphatases and the Wnt signaling pathway, raising the interesting possibility that some of the pathologies associated with myotubularin mutations may be caused by defects in this essential signaling pathway.

MATERIALS AND METHODS

C. elegans strains and culture

General methods for culture, manipulation and genetics of *C. elegans* were performed as described (Lewis & Fleming, 1995). Strains, mutations and integrated arrays used in this study were Bristol N2 and LGI: *arIs37[Pmyo-3::ssgfp; dpy-20(+)]* (Fares & Greenwald, 2001), *mtm-1(op309)*, *pry-1(mu38)* (Maloof et al, 1999), *rme-8(b1023)* (Fares & Greenwald, 2001); LGII: *cup-3(ar498)* (Fares & Greenwald, 2001), *mig-14(mu71)* (Harris et al, 1996), *mig-14(ga62)* (Eisenmann & 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 & Greenwald, 2001), *cup-5(ar465)* (Fares & Greenwald, 2001), *mtm-6(ar513)* (Fares & Greenwald, 2001), *mtm-6(ar515)* (Dang et al, 2004), *mtm-6(ok330)*; LGIV: *mab-5(e1751)* (Salser & Kenyon, 1992), *mca-3(ok2048)*, *muIs2[Pmab-5::lacZ; unc-31(+)]* (Salser & 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 & Hirsh, 1999); LGX: *mtm-5(ok469)*, *rme-6(b1014)* (Grant & 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::2xFYVE fragment was released from the 2xT10G3.5(FYVE) construct described in (Roggo et al, 2002) (a kind gift of Dr. Fritz Müller, University of Fribourg, Switzerland) and cloned behind 4.4kb 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 & 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°C 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 hour 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 (Fire et al, 1990). Briefly, synchronized L1 larvae were collected 4-5 hours after hatching in M9 buffer, washed with distilled 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 counter stained 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 hours. Larvae were then collected in TX-114 buffer (25mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5mM $CaCl_2$, 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 x g at 4°C for 30 minutes, mixed with Laemmli sample buffer, separated on 8% SDS-PAGE and transferred onto PVDF membrane (BioRad). 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 1-5 distinguishable vesicles or having more than 5 clearly visible vesicles.

Drosophila stocks and immuno-staining

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. Immuno-staining was performed using standard protocols. Briefly, 3rd 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 minutes at room temperature (RT). Discs were washed in PBS containing 0.05% Triton X-100 (PBT) for 1 hour 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 hour at RT and subsequently incubated in secondary antibody solution for 2 hour at RT. After a final wash for 1 hour 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. Snx3GFP 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 analyzed using ImageJ (NIH).

ACKNOWLEDGEMENTS

We thank Dr. Shohei Mitani (National Bioresource Project for the Nematode, Tokyo, Japan) for deletion mutants, Dr. Hanna Fares for *mtm-6(ar513)* and *mtm-6(ar515)*, Dr. Michael Hengartner for *mtm-1(op309)*, Dr. Andrew Fire for expression vectors, Dr. Fritz Müller (University of Fribourg, Switzerland) for the 2xFYVE construct and the *Caenorhabditis* Genetic Center (University of Minnesota, Minneapolis) for strains. This work was funded by the Dutch Cancer Society (HUBR 2008-4114),

the EU FP6 Program Cells into Organs and NWO VIDI (016.076.317) (H.C.K.), a Boehringer Ingelheim Foundation fellowship (M.H.), a grant from the Czech Science foundation (P305/10/0507) (M.S.) and the Swiss National Science Foundation and the Forschungskredit of the University of Zürich (F.P. and K.B.).

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SUPPLEMENTARY ONLINE MATERIAL

Table S1. Genotypes and n values of strains described in Fig. 1C and D.

Strain	**n**
muIs32	360
cup-3(ar498); arIs37; muIs32	360
cup-4(ok837); muIs32	600
cup-5(ar465); arIs37; muIs32	360
mca-3(ok2048); muIs32	360
mtm-6(ok330); muIs32	600
mtm-9(ar479); arIs37; muIs32	700
rme-1(b1045); muIs32	240
rme-6(b1014); muIs32	360
rme-8(b1023); muIs32	240
cup-4(ok837) mtm-6(ok330); muIs32	240
cup-5(ar465) mtm-6(ok330); muIs32	460
mtm-6(ok330); rme-6(b1014); muIs32	120
mtm-6(ok330); mca-3(ok2048); muIs32	120
muIs32	360
mtm-1(op309); muIs32	240
mtm-5(ok469); muIs32	480
mtm-1(op309); mtm-6(ok330); muIs32	700
mtm-6(ok330); mtm-5(ok469); muIs32	600
mtm-6(ok330); mtm-9(ar479); arIs37; muIs32	360
control RNAi	120
mtm-1 RNAi	120
mtm-3 RNAi	120
mtm-5 RNAi	120
mtm-6(ok330); control RNAi	480
mtm-6(ok330); mtm-1 RNAi	360
mtm-6(ok330); mtm-3 RNAi	360
mtm-6(ok330); mtm-5 RNAi	360

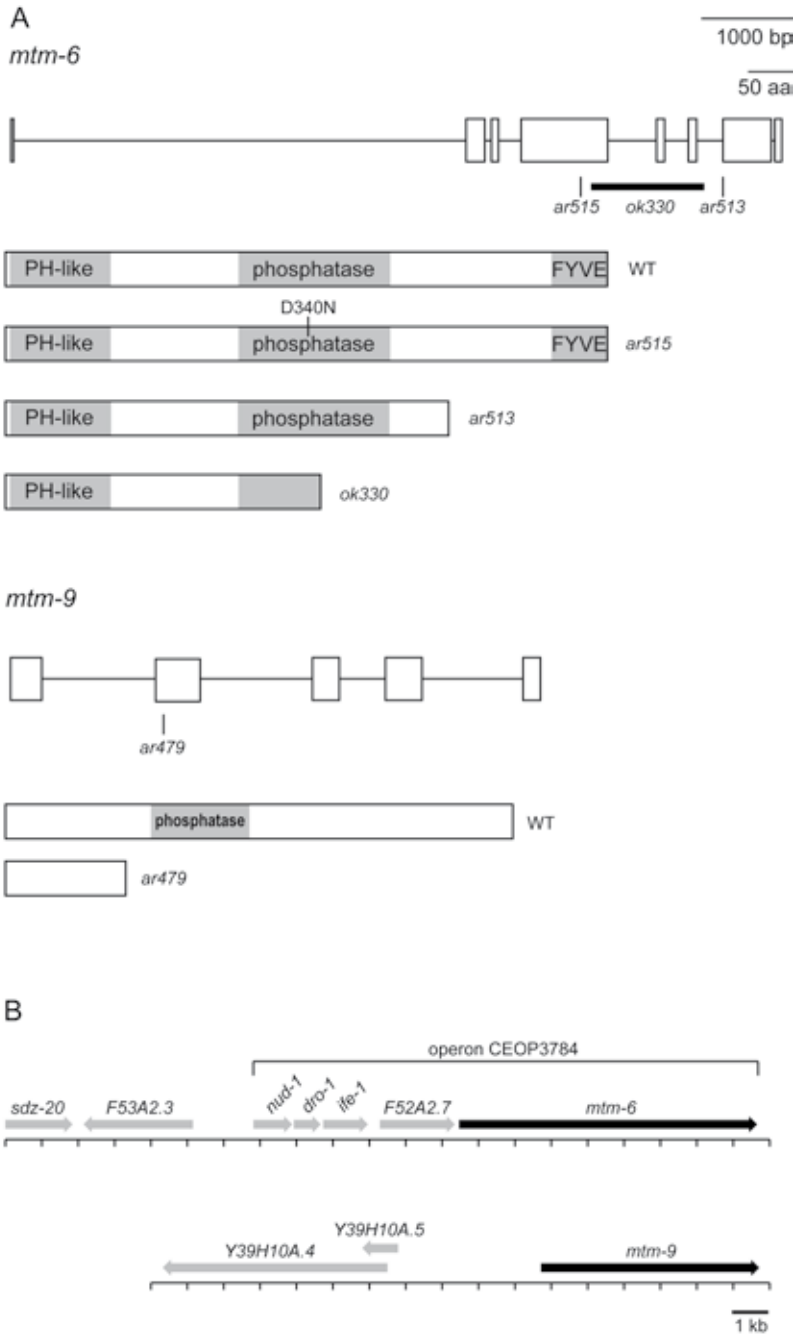

Figure S1. (A) Structure of the *mtm-6* and *mtm-9* genes, position of mutations and predicted protein products. (**B**) Schematic representation of the genomic context of the *mtm-6* and *mtm-9* genes. The extent of the *mtm-6* operon is indicated. Based on Wormbase release WS218 (www.wormbase.org).

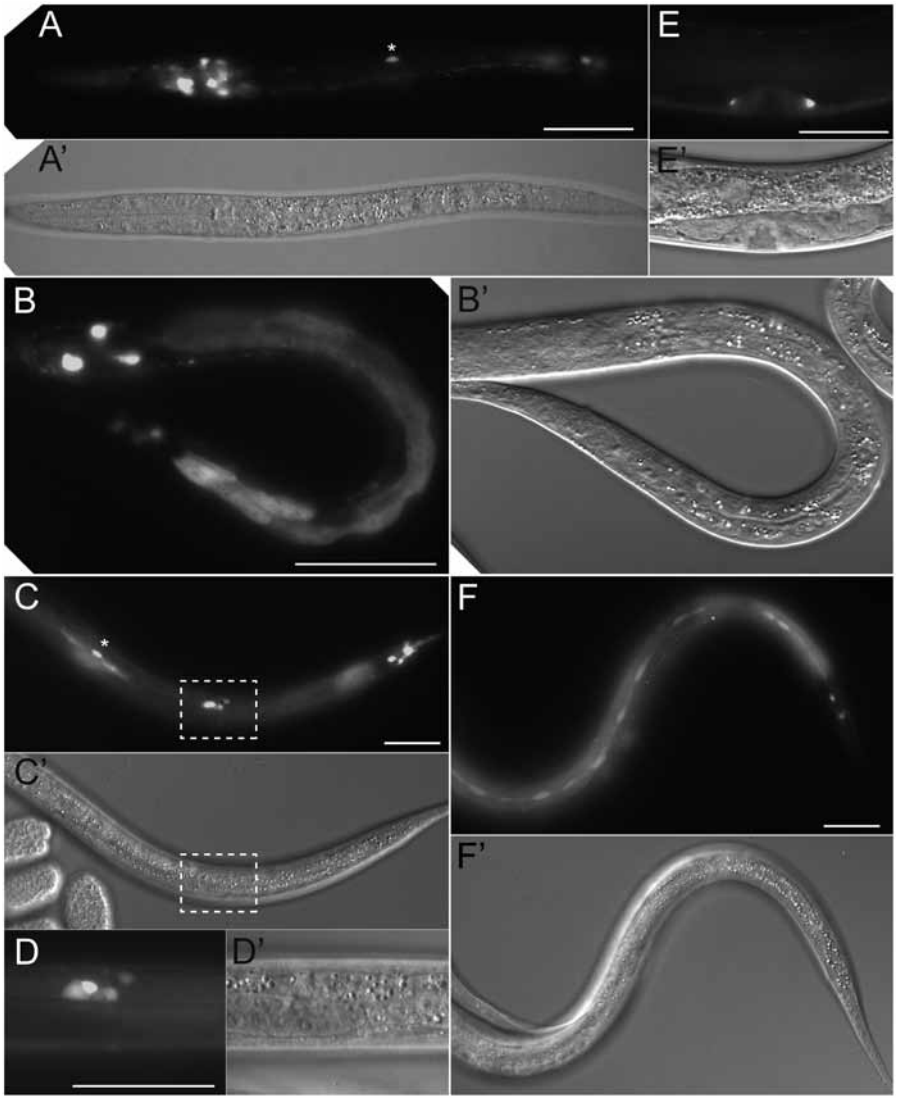

Figure S2. *mtm-9* expression pattern. Expression of an extrachromosomal transcriptional *mtm-9::gfp* reporter in (**A**) head and tail neurons of an L1 larva. Note also the expression in the CAN neuron in the mid body region (indicated by an asterisk). (**B**) Expression in head neurons and the intestine. (**C**) Expression in the postdeirid of an L2 larva. The boxed area is shown in D. (**E**) Expression in the developing vulva. (**F**) Expression in lateral hypodermal (seam) cells and the hypodermal syncytium (hyp7) of an L3 stage animal. Scale bar is 20 μm.

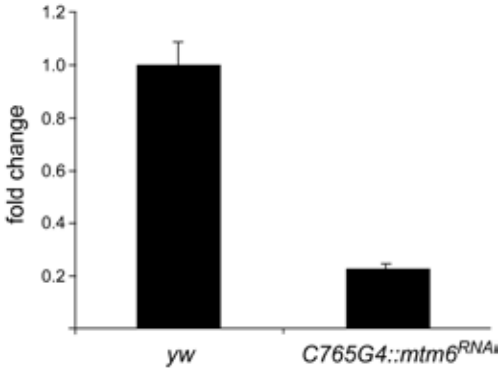

Figure S3. Efficient knock-down of *Dmtm6* transcript upon RNAi. Imaginal discs from third instar larvae were analyzed by qt-RT-PCR. Total RNA was extracted from 20 wing imaginal discs from each genotype using the Nucleospin RNA II kit (Macherey-Nagel). Quantitative PCR reactions were performed in triplicates and monitored using the Applied Biosystems SYBR Green kit and the ABI Prism 7900HT System (Applied Biosystems). All results were simultaneously normalized to the Actin5C, tubulin-1a and TBP mRNA levels and the expression levels calculated using the DDCt method (Applied Biosystems user bulletin #2, updated version 04/2001). Relative change of mRNA abundance relative to wild type imaginal discs (yw) is shown. Error bars represent standard deviation. RNAi line CG3530 26216 from the Vienna Drosophila RNAi Center was used.

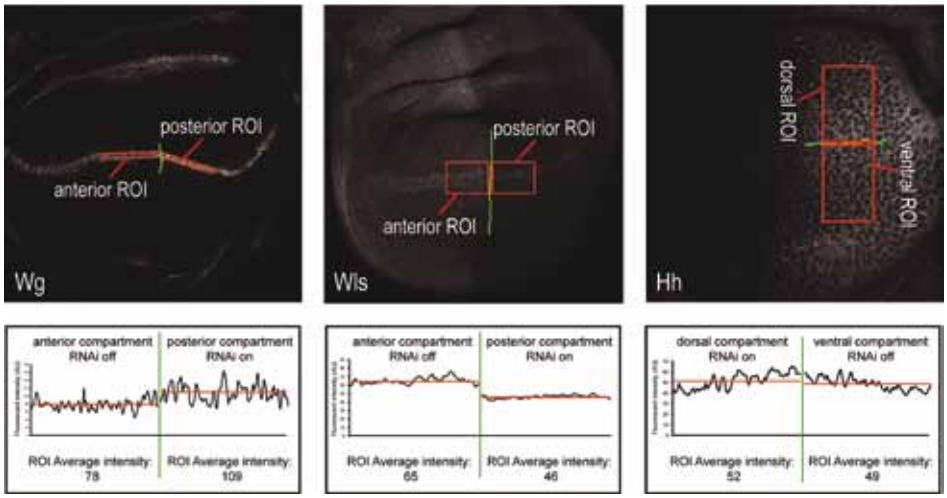

Figure S4. Quantification of immunofluorescent stainings shown in Figure 4D and E. Regions of interest (ROI) were selected and average fluorescent intensity was calculated using the "measure" function of ImageJ. Average intensities are displayed as red lines in the histograms and stated below the graphs. Histograms were produced using the "plot profile" function of ImageJ and run from anterior to posterior in case of anti-Wg and anti-Wls staining, and from dorsal to ventral in case of anti-Hh staining.

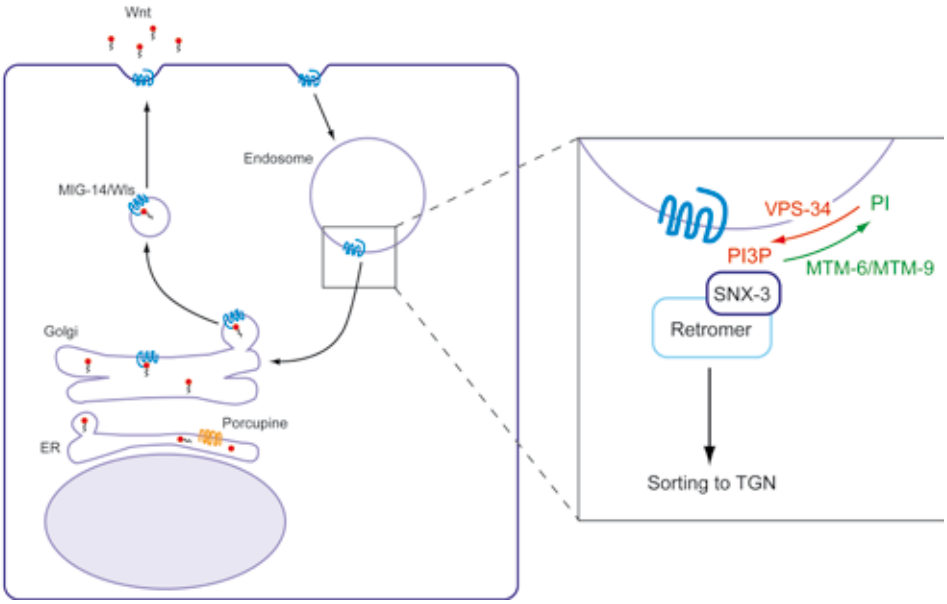

Figure S5. Model of MTM-6/MTM-9 function in Wnt secretion. Wnt binds MIG-14/Wls in the Golgi and together they are transported to the plasma membrane. After Wnt is released, MIG-14/Wls is endocytosed. In the endosome, Wls is sorted for transport to the *trans*-Golgi network by the concerted action of SNX-3 and the retromer complex (Chaper 3). Regulation of local PI3P levels by the MTM-6/9 myotubularin complex controls the recruitment of SNX-3 to endosomal membranes and the efficiency of the retromer-dependent recycling of MIG-14/Wls.

5 NEUROBLAST MIGRATION ALONG THE ANTEROPOSTERIOR AXIS OF *C. ELEGANS* IS CONTROLLED BY OPPOSING GRADIENTS OF WNTS AND A SECRETED FRIZZLED RELATED PROTEIN

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Development, in press

SUMMARY

The migration of neuroblasts along the anteroposterior body axis of *C. elegans* is controlled by multiple Wnts that act partially redundantly to guide cells to their precisely defined final destinations. How positional information is specified by this system is however still largely unknown. Here, we used a novel fluorescent *in situ* hybridization method to generate a quantitative spatio-temporal expression map of the *C. elegans* Wnt genes. We found that the five Wnt genes are expressed in a series of partially overlapping domains along the anteroposterior axis, with a predominant expression in the posterior half of the body. Furthermore, we show that a secreted Frizzled related protein is expressed at the anterior end of the body axis, where it inhibits Wnt signaling to control neuroblast migration. Our findings reveal that a system of regionalized Wnt gene expression and anterior Wnt inhibition guides the highly stereotypic migration of neuroblasts in *C. elegans*. Opposing expression of Wnts and Wnt inhibitors has been observed in basal metazoans and in the vertebrate neurectoderm. Our results in *C. elegans* support the notion that a system of posterior Wnt signaling and anterior Wnt inhibition is an evolutionarily conserved principle of primary body axis specification.

INTRODUCTION

Wnt proteins control many aspects of metazoan development, with prominent functions in cell fate determination, cell proliferation and cell migration (van Amerongen and Nusse, 2009). The activity of Wnt proteins is counteracted by a range of inhibitors, including secreted proteins such as the secreted Frizzled related proteins (SFRPs) (Bovolenta et al., 2008; Leyns et al., 1997) and Dickkopf (Niehrs, 2006), and intracellular inhibitors such as the TCF/Lef transcription factor Tcf3 (Kim et al., 2000). During neurectoderm development in vertebrates, these inhibitors are expressed in the anterior and counteract the activity of posteriorly expressed Wnts to enable the formation of anterior brain structures and the eyes (Kiecker and Niehrs, 2001; Leyns et al., 1997; Tendeng and Houart, 2006). Opposing expression of Wnts and Wnt inhibitors has also been observed in basal metazoan organisms such as the cnidarians *Hydra* and *Nematostella vectensis* (Guder et al., 2006b; Hobmayer et al., 2000; Kusserow et al., 2005; Lee et al., 2006) and in the planarian *Schmidtea mediterranea* (Petersen and Reddien, 2008), which led to the hypothesis that a system of posterior Wnt signaling and anterior Wnt inhibition is an ancient mechanism that may be used across animal phyla to pattern the primary body axis (Petersen and Reddien, 2009).

The nematode *Caenorhabditis elegans* expresses five different Wnt proteins that control many aspects of development, including cell fate specification, cell polarity and the highly stereotypic migration of neuroblasts along the anteroposterior body axis (Korswagen, 2002; Silhankova and Korswagen, 2007). Neuroblasts that migrate in a Wnt dependent manner include the HSN neurons, which migrate from the posterior to the mid-body region (Hedgecock et al., 1987; Pan et al., 2006; Sulston et al., 1983), the ALM and CAN neurons, which migrate from the anterior to positions in the mid-body region (Hedgecock et al., 1987; Sulston et al., 1983; Zinovyeva and Forrester, 2005) and the Q neuroblast descendants, which migrate in opposite directions on the left and right lateral sides (Harris et al., 1996; Sulston and Horvitz, 1977). With the exception of the HSN neurons and the left Q cell descendants, the migration of these neuroblasts is controlled through multiple, partially redundantly acting Wnt proteins (Zinovyeva et al., 2008), indicating that positional information from different Wnt gradients is used to guide the cells to their precisely defined final positions. The expression patterns of the *C. elegans* Wnt genes have been analyzed using transgenic reporter constructs. These studies revealed a predominantly posterior expression for the Wnt genes *lin-44*, *egl-20* and *cwn-1* (Herman et al., 1995; Pan et al., 2006; Whangbo and Kenyon, 1999), whereas *mom-2* and *cwn-2* were reported to be generally expressed along the anteroposterior axis (Gleason et al., 2006), with a more prominent expression of *cwn-2* in the pharynx and anterior muscle cells (Kennerdell et al., 2009; Song et al., 2010). Although there is a large degree of overlap between the reported expression patterns, there are also important differences in the extent of expression along the anteroposterior axis

and in the specific cell types that are involved, complicating the analysis of Wnt gene function in neuroblast migration and other aspects of development.

Here, we used single molecule mRNA fluorescent *in situ* hybridization (smFISH) to quantitatively determine the spatio-temporal expression patterns of the five *C. elegans* Wnt genes. Our results show that the different Wnt genes are expressed in a series of partially overlapping expression domains, with a predominant expression in the posterior body half and a single Wnt gene with an anterior expression domain. Furthermore, we show that the *C. elegans* genome contains a single SFRP ortholog that is specifically expressed at the anterior end of the body axis. SFRP-1 functions as an inhibitor of Wnt signaling that represses the most anteriorly expressed Wnts to control the migration of neuroblasts in the anterior body region. Our results demonstrate that opposing Wnt and Wnt inhibitory activities are also key to anteroposterior patterning in *C. elegans* and provide further support for the evolutionary conservation of this system in primary body axis specification.

MATERIALS AND METHODS

C. elegans strains and culturing

General methods for culture, manipulation and genetics of *C. elegans* were as described (Lewis and Fleming, 1995). Strains were cultured at 20ºC. Mutations and transgenes used in this study were: LGI, *lin-44(n1792)* (Herman et al., 1995), *mom-5(gk812)*, *pry-1(mu38)* (Korswagen et al., 2002; Maloof et al., 1999), *ccIs4251[Pmyo-3::gfp]* (Fire et al., 1998); LGII, *cwn-1(ok546)* (Zinovyeva and Forrester, 2005), *mab-5(*gk670), *mig-14(mu71)* (Bänziger et al., 2006), *vps-35(hu68)* (Coudreuse et al., 2006), *muIs32[Pmec-7::gfp]* (Ch'ng et al., 2003); LGIV, *sfrp-1(gk554)*, *cwn-2(ok895)* (Zinovyeva and Forrester, 2005), *egl-20(hu105)* (Coudreuse et al., 2006); *otIs33* (*Pkal-1::GFP*) (Bulow et al., 2002); *ayIs7[Phlh-8::gfp]* (Harfe et al., 1998); LGV, *mom-2(or309)* (Zinovyeva and Forrester, 2005), *muIs35[Pmec-7::gfp]* (Ch'ng et al., 2003); *heIs63[Pwrt-2::ph::gfp]* (Wildwater et al., in preparation); and unassigned, *huIs120[Phsp::sfrp-1]*.

Single molecule mRNA FISH

Probe design and hybridization to perform FISH for single transcript measurement in *C. elegans* larvae was performed as previously described (Raj et al., 2008) (see also www.singlemoleculefish.com). Animals were collected by washing plates with M9 and were fixed in 4% formaldehyde in 1X PBS for 45 minutes. Fixed animals were permeabilized in 70% ethanol overnight. All probes for hybridization were coupled to either Cy5 (GE Amersham), Alexa594 (Invitrogen) or tetramethylrhodamine (TMR) (Invitrogen) depending on the desired gene combinations for image acquisition. The type of coupled fluorophore did not

affect any quantitative results in this study. Images were taken in z-stacks using a Nikon TE2000 epi-fluorescence microscope with a Princeton Instruments CCD camera and appropriate optical filters for DAPI, Cy5, Alexa594 and TMR. All experiments were performed using either wild-type (N2) or wild type animals expressing cell type specific GFP markers. Three dimensional positions of bright fluorescent spots in each animal were detected with the aid of a custom program written in MATLAB, as described (Raj et al., 2008). Nuclei were visualized with DAPI. We used body length to gauge the developmental age of individual animals. We obtained body lengths of each animal by measuring the distance between the nuclei of hyp4 (the anterior-most cell) and hyp10 (the posterior-most cell) along the antero-posterior (AP) axis. The identity of transcript containing cells was determined using specific GFP markers (body wall muscle cells, seam cells and the undifferentiated M cell descendants), or by determining nuclear positions by DAPI staining (Long et al., 2009; Sulston and Horvitz, 1977).

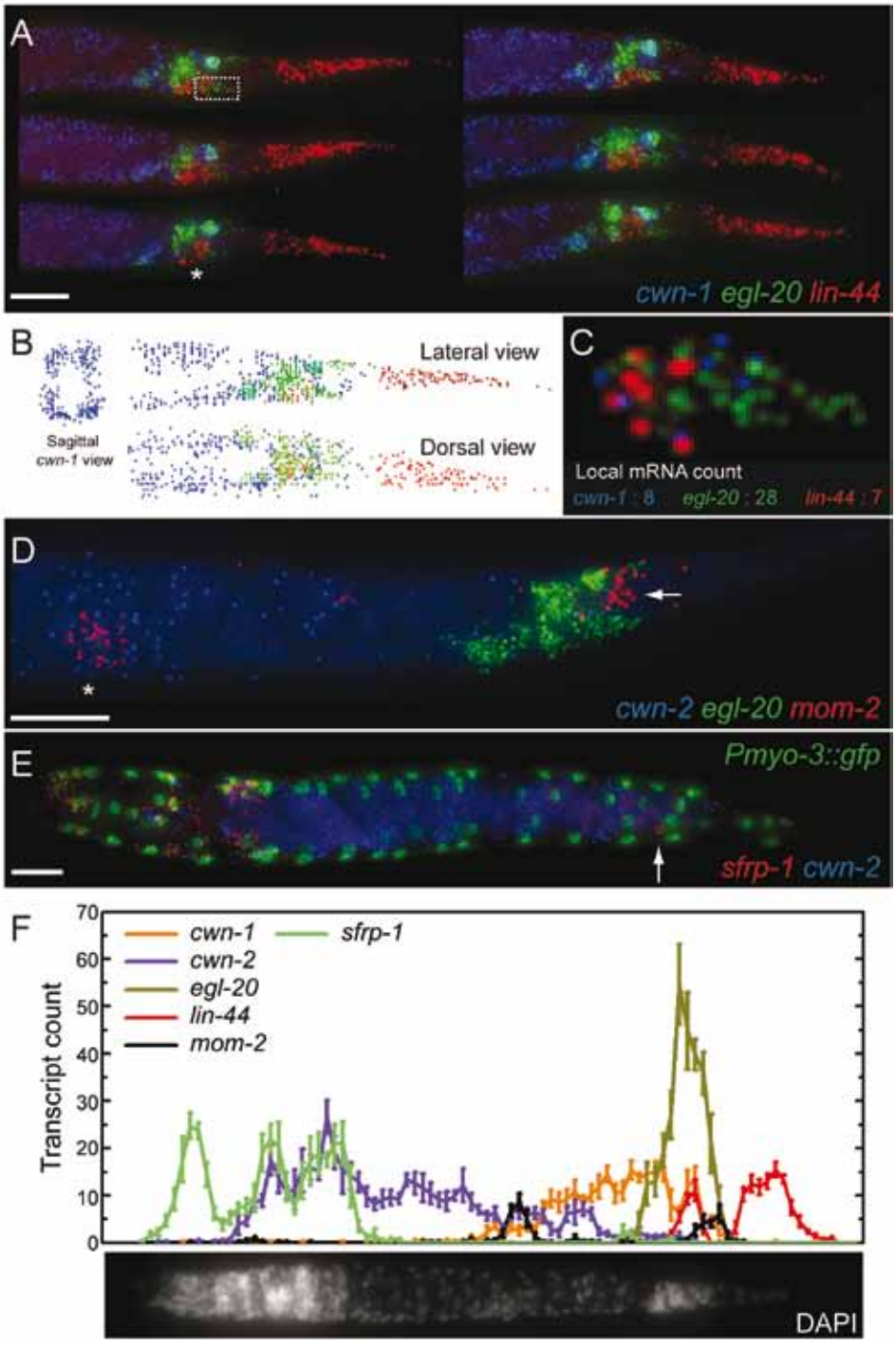
C. elegans phenotypes, expression constructs and transgenesis

The final positions of the CAN, ALM, HSN and Q descendants and the polarity of the V5 seam cell division were scored in L1 larvae by Nomarski microscopy (Harris et al., 1996; Whangbo et al., 2000). The polarity of the ALM and PLM neurons, dye filling and P12 to P11 fate transformation were analyzed as described (Herman and Horvitz, 1994; Prasad and Clark, 2006). To generate a heat shock inducible *sfpr-1* expression plasmid, the *sfrp-1* cDNA was cloned into the pPD49.78 vector. The plasmid was injected with a *Pmyo-2::Tomato* co-injection marker and integrated as described (Mello and Fire, 1995).

RESULTS

The five *C. elegans* Wnt genes are expressed in a series of partially overlapping domains along the anteroposterior axis

To quantitatively determine the spatio-temporal expression patterns of the five *C. elegans* Wnt genes, we performed single molecule mRNA FISH (smFISH) to measure endogenous transcript levels in staged L1 larvae (Raj et al., 2008). Using this technique, we were able to simultaneously label and visualize individual transcripts of up to three Wnt genes as bright diffraction-limited fluorescent spots in animals with preserved shape (Fig. 1A). Counting of these spots using a custom program written in MATLAB allowed us to quantify the number of transcripts in any three-dimensional region of the animal (Fig. 1B, C, F). These measurements revealed that the expression patterns of the five Wnt genes are strikingly reproducible in wild type animals of the same developmental stage (Fig. 1A, F). In general, the overall transcript expression profile of the different Wnt genes agreed with previous expression patterns obtained with transgenes


A
cwn-1 egl-20 lin-44
B
Lateral view
Sagittal
cwn-1 view
Dorsal view
C
Local mRNA count
cwn-1 : 8
egl-20 : 28
lin-44 : 7
D
cwn-2 egl-20 mom-2
E
Pmyo-3::gfp
sfrp-1 cwn-2
F
cwn-1
cwn-2
egl-20
lin-44
mom-2
sfrp-1
Transcript count
70
60
50
40
30
20
10
0
DAPI

expressing transcriptional or translational reporter constructs, but as detailed below, there were a number of important differences.

We found that of the five Wnt transcripts, three (*lin-44*, *egl-20* and *cwn-1*) were mostly localized to the posterior half of the body (Fig. 1A, F). *lin-44* transcripts were present in the tail hypodermal cells hyp8, hyp9, hyp10 and hyp11 and at later larval stages in the phasmid socket cells PHso1 and PHso2 (Fig. S1A), as previously reported (Herman et al., 1995). In addition, we found that *lin-44* is expressed in the rectal epithelial cells B and Y, demonstrating that *lin-44* has a more anterior expression domain than has been observed using reporter transgenes. *egl-20* was expressed in the rectal epithelial cells K, F, U and B, in the anal depressor muscle and in P11/12, which is in agreement with previous reporter studies (Whangbo and Kenyon, 1999). However, we found that in L1 larvae, *egl-20* is also expressed in the posterior ventral body wall muscle quadrants VL23 and VR24 and the rectal epithelial cell Y. *cwn-1* was mainly expressed in posterior body wall muscle cells (Fig. 1A, B) and in the M cell descendants that give rise to body wall muscle cells and the vulva and uterine muscle cells (Fig. 1F, S1A). In addition, several cells were found to co-express *cwn-1* and *egl-20*, including the anal depressor muscle, the body wall muscle quadrants VL23 and VR24 and P11/12. Interestingly, we observed that the two lateral canal associated neurons (CANs) simultaneously induce *cwn-1* expression during late L1 (Fig. S1A, B), an expression that persists throughout larval development.

mom-2 was previously reported to be widely expressed along the anteroposterior axis in body wall muscle cells, ventral cord neurons, intestinal cells and seam cells (Gleason et al., 2006). In contrast, we found that *mom-2* shows a restricted expression pattern, with *mom-2* transcripts only localizing to the germ cell precursors Z2 and Z3 and their descendants and a few unidentified cells in the tail (Fig. 1D). *mom-2* expression in the germ cells continued throughout larval development while the tail expression reached a maximum at the mid L1 stage

◀ **Figure 1.** Single molecule mRNA FISH analyses of the *C. elegans* Wnt genes and *sfrp-1*. (**A**) Detection of *cwn-1*, *egl-20* and *lin-44* transcripts in the L1 larval tail. The asterisk indicates the position of the *lin-44* expressing B and Y rectal epithelial cells. Scale bar is 10 µm. (**B**) Transcript identification using a custom program written in MATLAB. A sagittal view of the *cwn-1* transcripts shows predominant expression in the four body wall muscle quadrants. (**C**) Magnification of the area indicated in panel A. Transcript counts of *cwn-1*, *egl-20* and *mom-2* are indicated. (**D**) Expression of *cwn-2*, *egl-20* and *mom-2* in the posterior half of the animal. The asterisk indicates the Z2 and Z3 germline precursor cells, the arrow the position of the tail cells that transiently express *mom-2*. Scale bar is 10 µm. (**E**) Expression of *sfrp-1* and *cwn-2* in an L1 larva. The nuclei of body wall muscle cells are highlighted by nuclear GFP. The posterior ventral nerve cord neuron expressing *sfrp-1* was identified as DA7 (indicated by arrow). Scale bar is 10 µm. (**F**) Quantification of Wnt and *sfrp-1* transcripts along the anteroposterior axis of early L1 stage larvae. A DAPI stained animal is included for orientation. For all images anterior is to the left and posterior to the right.

and disappears before the L1 to L2 molt (Fig. S1D). Also, one or two *mom-2* transcripts were occasionally detected in posterior seam cells in early L1 larvae.

The expression of *cwn-2* has been described using different reporter transgenes, showing either a general expression in body wall muscle cells and ventral nerve cord neurons along the whole body axis (Gleason et al., 2006), or a more restricted expression in the pharynx, anterior muscle cells and the intestine (Kennerdell et al., 2009; Song et al., 2010). We found that *cwn-2* transcripts mainly localized to head neurons, anterior body wall muscle cells, anterior P.n cells and the intestine (Fig. 1E, F, S1A). The highest *cwn-2* transcript count was observed around the terminal bulb of the pharynx, with a gradual decline in expression levels in more posterior cells.

Quantification of Wnt transcripts along the anteroposterior axis revealed that the five Wnt genes are expressed in a series of partially overlapping expression domains (Fig. 1F). At the posterior end of the animal, only *lin-44* is expressed. Around the rectum, the most abundantly expressed Wnt gene is *egl-20*. In the posterior region between the gonad premordium and the rectum, *cwn-1* is the dominant Wnt, while the anterior half of the animal is the domain of *cwn-2* expression. This overall anteroposterior expression profile remained essentially unchanged during the remainder of L1 larval development, although quantification of total Wnt transcript numbers revealed changes in the expression levels of the five Wnt genes (Fig. S1A, C). Thus, whereas *mom-2* expression remained mostly unchanged during early larval development and there was only a gradual increase in the expression of *lin-44* and *egl-20*, there was a sharp increase in the expression of *cwn-1* and *cwn-2*.

The anteriorly expressed secreted Frizzled related protein gene *sfrp-1* controls neuronal migration along the anteroposterior axis

The activity of Wnt proteins is modulated by secreted Wnt binding proteins such as members of the secreted Frizzled related proteins (SFRPs), an ancient family of Wnt regulators that are present in organism ranging from sponges to vertebrates (Bovolenta et al., 2008) (Fig. S2A). Sequence similarity searches revealed that the *C. elegans* genome contains a single SFRP ortholog encoded by the predicted gene Y73B6BL.21, which we renamed *sfrp-1*. Similar to other SFRP family members, SFRP-1 contains a cysteine rich Frizzled related domain (CRD) and a netrin-related (NTR) domain (Fig. S2B), which is characterized by positively charged residues and six conserved cysteines (Chong et al., 2002).

To determine the expression pattern of *sfrp-1*, we analyzed *sfrp-1* mRNA localization using smFISH. As shown in Fig. 1E, *sfrp-1* is expressed in four stripes of cells in the head region. Using a muscle specific marker, these cells were identified as head body wall muscle cells. In addition, we found that *sfrp-1* is expressed at low levels in a single posterior ventral nerve cord neuron and occasionally in one or more cells around the rectum (Fig. 1E and S1E). The predominantly anterior

expression of *sfrp-1* indicates that SFRP-1 and the posteriorly expressed Wnts form opposing gradients. Although we have not been able to directly visualize such an SFRP-1 concentration gradient, the genetic analysis of *sfrp-1* function described below demonstrates that SFRP-1 has both short and long-range functions in modulating Wnt activity.

To investigate the function of *sfrp-1*, we used the deletion allele *gk554*, which truncates the *sfrp-1* gene upstream of the CRD and NTR domains and likely represents the null phenotype (Fig. S2B). *sfrp-1(gk554)* is viable and does not induce obvious morphological defects. However, *sfrp-1* mutants show clear alterations in the Wnt dependent anteroposterior positioning of migrating neuroblasts.

One group of neuroblasts that migrates along the anteroposterior axis are the Q neuroblast descendants (Hedgecock et al., 1987; Sulston and Horvitz, 1977). At the end of embryogenesis, two Q neuroblasts are generated at equivalent positions on the left (QL) and right (QR) lateral side of the animal (Fig. 2A). During the first stage of larval development, the two Q neuroblasts each generate three descendants that migrate in opposite directions: on the left side,

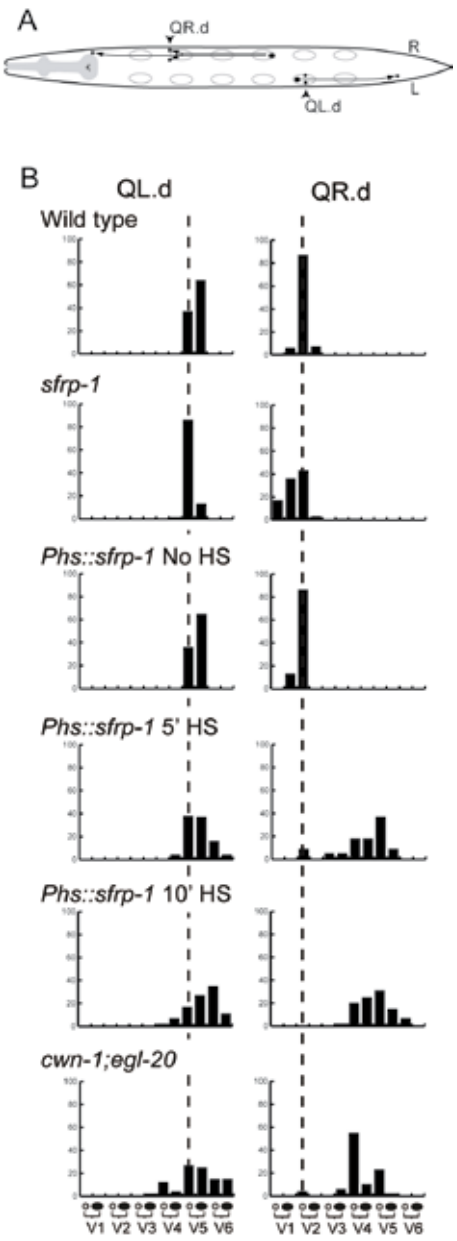


Figure 2. *sfrp-1* is required for the migration of the QR descendants. (**A**) Schematic representation of Q neuroblast descendant migration. Arrowheads indicate the final positions of the Q.paa and Q.pap cells. (**B**) The final positions of the left and right Q.paa and Q.pap cells are indicated relative to the invariant positions of the seam cells V1 to V6 (n>100 for wild type and *sfrp-1*; for the other conditions, n>20). The dashed line indicates the wild type position.

the QL descendants (QL.d) migrate towards the posterior, whereas on the right side, the QR.d migrate towards the anterior. Both anterior and posterior migration is controlled by Wnt signaling. The posterior migration of the QL.d is mediated by EGL-20, which triggers a canonical Wnt/β-catenin pathway to induce expression of the target gene *mab-5* and to direct migration towards the posterior (Harris et al., 1996; Maloof et al., 1999; Whangbo and Kenyon, 1999). The anterior migration of the QR.d is also dependent on EGL-20, but here EGL-20 functions together with CWN-1 to activate a non-canonical Wnt signaling pathway that is required for anterior directed migration (Zinovyeva et al., 2008). Although the mechanism remains to be established, current models suggest that a difference in response threshold to EGL-20 determines which pathway is activated (Whangbo and Kenyon, 1999). Thus, QL is primed to activate canonical Wnt/β-catenin signaling in response to EGL-20, whereas QR will only activate this pathway when EGL-20 is overexpressed. At intermediate levels, overexpression of EGL-20 induces overmigration of the QR.d, indicating that Wnt signaling activity not only specifies the direction of migration, but also influences the position at which the cells terminate their migration (Whangbo and Kenyon, 1999). To investigate whether *sfrp-1* regulates the Wnt dependent migration of the Q descendants, we determined the final positions of the Q descendants Q.paa and Q.pap relative to the hypodermal seam cells V1 to V6. We found that the QL.d localized at their normal positions in *sfrp-1* mutants (Fig. 2B). There was, however, a clear change in the final position of the QR.d, with the QR.d migrating significantly further into the anterior than in wild type animals. As this phenotype is similar to the extended migration induced by EGL-20 overexpression, these data are consistent with a negative regulatory role for *sfrp-1* in QR.d migration. Loss of this negative regulatory activity is however insufficient to trigger canonical Wnt/β-catenin signaling and *mab-5* expression in QR.

In addition to the defect in the anterior migration of the QR.d, we found that *sfrp-1* mutants show misplacement of the ALM and CAN neurons. Both neurons migrate during the end of embryogenesis from the anterior to final positions in the mid-body region (Hedgecock et al., 1987; Sulston et al., 1983) (Fig. 5A, S5A). In both cases, posterior migration depends on the combined activity of CWN-1 and CWN-2 (Zinovyeva et al., 2008). In *sfrp-1* mutants, the posterior migration of the ALM neurons was significantly truncated (Fig. 5). Also in case of the CAN neurons, mutation of *sfrp-1* induced undermigration, although this effect was less pronounced as observed with the ALM neurons (Fig. S5).

Mutation of *sfrp-1* did not significantly affect other Wnt dependent processes (Table 1). Thus, there were no defects in the anterior migration of the HSN neurons (Pan et al., 2006), the polarization of the mechanosensory neurons ALM and PLM (Prasad and Clark, 2006), the polarization of the division of the hypodermal seam cells V5 and T (Herman et al., 1995; Whangbo et al., 2000), the positioning of the nerve ring (Kennerdell et al., 2009), or the specification of P12 fate (Jiang and Sternberg, 1998). With the exception of nerve ring positioning, all of these

processes control cells in the posterior or mid-body region, whereas the migration of the QR.d, ALM and CAN neurons takes place in the anterior. These results are therefore consistent with the anterior specific expression of *sfrp-1* and a function of SFRP-1 in modulating Wnt activity in the anterior body region.

SFRP-1 is a global inhibitor of Wnt signaling

One of the main functions of SFRPs is to negatively regulate Wnt signaling (Kawano and Kypta, 2003). There are, however, also examples of SFRPs functioning as facilitators or direct mediators of Wnt signaling, for example by assisting the spreading of Wnt in the tissue or by directly interacting with the Wnt receptor Frizzled (Mii and Taira, 2009; Rodriguez et al., 2005). To investigate potential Wnt inhibitory or stimulatory functions of *sfrp-1*, we overexpressed *sfrp-1* using a heat-shock inducible promoter (Fig. 2B). A short induction of *sfrp-1* expression before the Wnt dependent migration of the Q descendants resulted in an almost complete loss of the anterior migration of the QR.d, a phenotype that is also observed in double mutants of *egl-20* and *cwn-1* or mutants in which all five Wnt genes have been deleted (Zinovyeva et al., 2008). Also the effect of *sfrp-1* overexpression on the QL.d was similar to *egl-20; cwn-1* double mutants, with a more variable and posterior localization of the cells (Fig. 2B). Taken together, these results show that the migration phenotype induced by overexpression of *sfrp-1* closely resembles that of mutants defective in multiple Wnts, consistent with a negative regulatory role for SFRP-1 in the Wnt dependent control of Q.d migration. We found that *sfrp-1* overexpression similarly inhibited the EGL-20 dependent anterior migration of the HSN neurons as well as other Wnt dependent processes, such as the polarized division of the seam cell V5 (data not shown), indicating that SFRP-1 can inhibit the activity of most if not all of the Wnt proteins of *C. elegans*. Importantly, no phenotypes were observed that suggest a stimulatory function of SFRP-1 in Wnt signaling.

To further investigate the function of *sfrp-1* in modulating Wnt activity, we analyzed double mutants between *sfrp-1* and mutants in which Wnt secretion is reduced: a hypomorphic allele (*mu71*) of the Wnt sorting receptor *mig-14*/Wls (Bänziger et al., 2006; Yang et al., 2008) and a null allele of the retromer subunit gene *vps-35* (Coudreuse et al., 2006). By assaying whether specific Wnt phenotypes are suppressed or enhanced, these mutants provide a sensitive assay to test negative or positive effects of *sfrp-1* on Wnt signaling. In *mig-14* and *vps-35* single mutants, a reduction in EGL-20 levels leads to a loss of *mab-5* expression in QL (Harris et al., 1996) and anterior migration of the QL.d (Fig. 3). We found that in double mutants with *sfrp-1*, posterior migration was almost fully restored in *mig-14(mu71)* and significantly rescued in *vps-35*. This is most likely the result of reactivation of *mab-5*, as mutation of *sfrp-1* failed to restore posterior QL.d localization in a *mab-5* mutant background (Fig. 3). These results suggest that the reduction in EGL-20 signaling can be overcome by removal of SFRP-1, consistent

with a negative regulatory role of SFRP-1 in the EGL-20 dependent activation of canonical Wnt/β-catenin signaling in QL. We observed a similar inhibitory role for *sfrp-1* in QR.d migration. Thus, whereas the QR.d showed reduced anterior migration in *mig-14(mu71)* and *vps-35* mutants, the QR.d localized at their correct positions in double mutants with *sfrp-1* (Fig. S3). A comprehensive analysis of other Wnt phenotypes in *mig-14(mu71)* and *vps-35* mutants showed that loss of *sfrp-1* also suppressed defects in the migration of the ALM neurons and the polarity of the seam cells V5 and T (Fig. 5 and Table 1). Importantly, there were no instances in which the *vps-35* or *mig-14(mu71)* phenotype was enhanced by loss of *sfrp-1*. Taken together with the strong Wnt inhibitory activity of *sfrp-1* overexpression, these results support the conclusion that SFRP-1 functions as a global inhibitor of Wnt signaling in *C. elegans*.

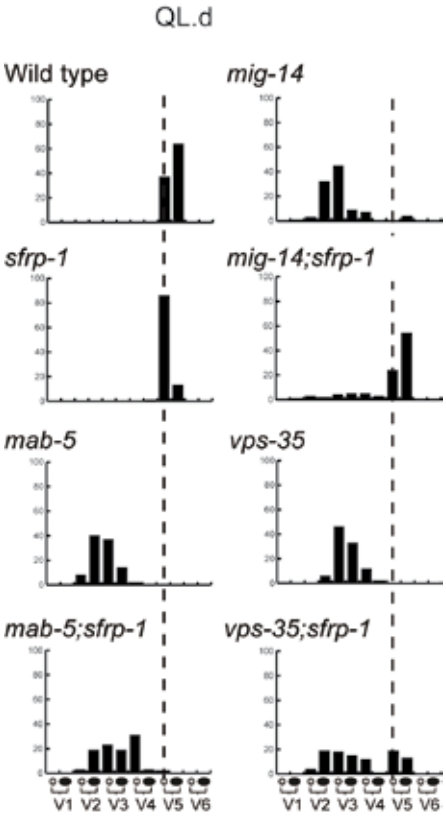


Figure 3. *sfrp-1* rescues QL.d migration in hypomorphic Wnt secretion mutants. The final positions of QL.paa and QL.pap cells are indicated relative to the seam cells V1 to V6 (n>50). The dashed line indicates the wild type position.

The *sfrp-1* induced overmigration of the QR descendants is suppressed by mutation of *cwn-2*

The overmigration of the QR.d in *sfrp-1* mutants is similar to the extended migration induced by ubiquitous EGL-20 expression (Whangbo and Kenyon, 1999), indicating that the overmigration is a result of a gain in Wnt signaling activity. To investigate which Wnts mediate the *sfrp-1* induced overmigration, we constructed double mutants between *sfrp-1* and null mutants of the different Wnt genes. We found that mutation of *lin-44* or *mom-2* did not suppress the *sfrp-1* induced overmigration (Fig. 4). In double mutants between *sfrp-1* and *egl-20*, there was a partial suppression of the overmigration, but also a clear undermigration of the QR.d, an effect that was even more pronounced in double mutants with *cwn-1*. EGL-20 and CWN-1 function partially redundantly in

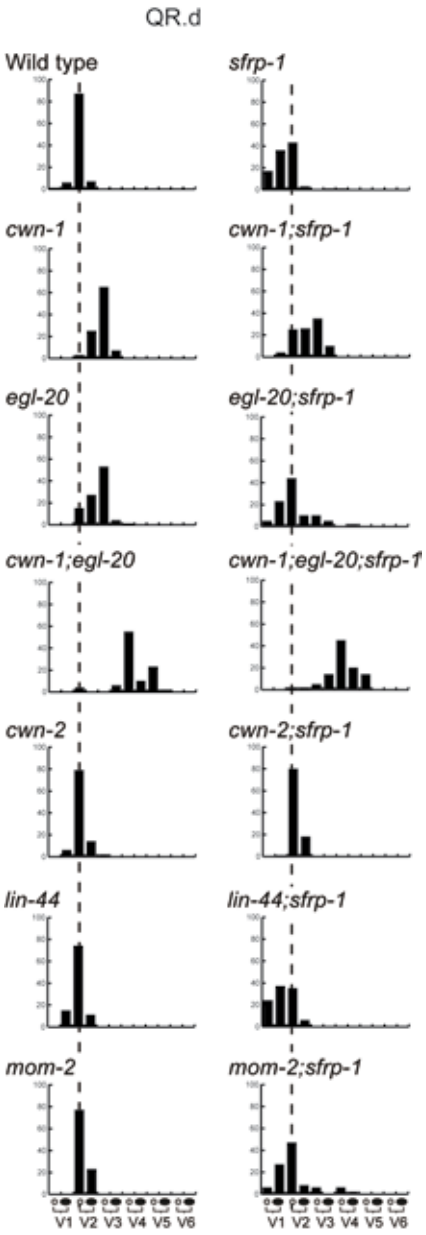


Figure 4. The *sfrp-1* induced overmigration of the QR.d is suppressed by mutation of *cwn-2*. The final positions of QR.paa and QR.pap cells are indicated relative to the seam cells V1 to V6 (n>50). The dashed line indicates the wild type position.

specifying anterior QR.d migration and loss of either *egl-20* or *cwn-1* results in a distinct undermigration of the QR.d (Zinovyeva et al., 2008). The intermediate phenotype of the *sfrp-1; egl-20* and *sfrp-1; cwn-1* double mutants therefore suggests that *egl-20* and *cwn-1* either function in parallel to *sfrp-1* or only play a minor role in the *sfrp-1* induced overmigration of the QR.d. In contrast, we found that the *sfrp-1* induced overmigration was fully rescued by a null mutation in *cwn-2* (Fig. 4). Thus, whereas *cwn-2* has no effect on QR.d positioning on its own, the QR.d localized at their wild type position in *sfrp-1; cwn-2* double mutants. Taken together, these results indicate that derepression of CWN-2 signaling is primarily responsible for the QR.d overmigration phenotype of *sfrp-1* mutants.

The *sfrp-1* induced undermigration of the ALM and CAN neurons is suppressed by mutation of *cwn-1* or *cwn-2*

The posterior migration of the ALM neurons is dependent on the redundant activity of CWN-1 and CWN-2, with a strong inhibition of migration in *cwn-1; cwn-2* double mutants (Zinovyeva et al., 2008). A similar undermigration is observed in *sfrp-1* mutants, indicating that Wnt overactivity also interferes with the correct posterior migration of the ALM neurons. This conclusion is supported by the observation that the ALM undermigration phenotype of *sfrp-1* is suppressed by reducing Wnt secretion through mutation of the Wnt sorting receptor *mig-14*/Wls

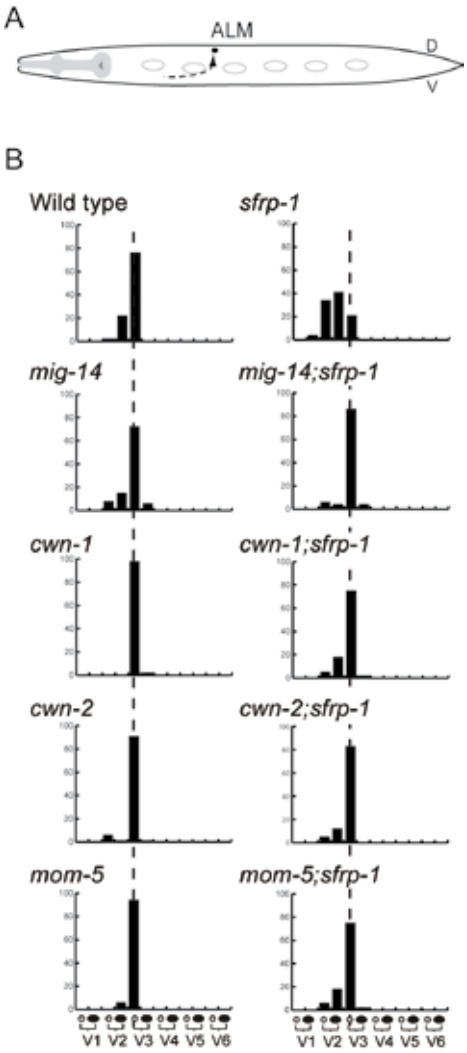


Figure 5. The *sfrp-1* induced undermigration of the ALM neurons is suppressed by mutation of *cwn-1* or *cwn-2*. (**A**) Schematic representation of the ALM migration. Note that the migration takes place at the end of embryogenesis. (**B**) The final positions of the ALML and ALMR neurons are indicated relative to the seam cells V1 to V6 (n>50). The dashed line indicates the wild type position.

(Fig. 5B). To investigate which Wnts are required for the *sfrp-1* induced undermigration of the ALM neurons, we analyzed double mutants with null alleles of each of the five different Wnt genes and tested which combination could suppress the ALM undermigration phenotype. Whereas *lin-44*, *egl-20* and *mom-2* did not affect the *sfrp-1* induced ALM undermigration, mutation of *cwn-1* or *cwn-2* fully restored the migration of the ALM neurons to their wild type positions (Fig. 5 and Fig. S4). These results suggest that in the absence of SFRP-1, overactivity of CWN-1 and CWN-2 interferes with the correct positioning of the ALM neurons, and that normal migration can be restored by removing either of the two Wnt genes. Furthermore, the *sfrp-1* induced undermigration of the ALM neurons was fully suppressed in double mutants between *sfrp-1* and the Frizzled *mom-5* (Fig. 5B), indicating that CWN-1 and CWN-2 control ALM positioning through the MOM-5/ Frizzled receptor.

Similar results were obtained for the *sfrp-1* induced undermigration of the CAN neurons, which was also dependent on CWN-1 and CWN-2. Thus, CAN undermigration was not suppressed in *lin-44*, *egl-20* or *mom-2* mutants, but was rescued in either *cwn-1* or *cwn-2* mutants (Fig. S5).

The function of SFRP-1 in suppressing CWN-1 and CWN-2 activity is consistent with the anterior expression of *sfrp-1* and is

in agreement with a role for SFRP-1 in modulating Wnt activity in the anterior body region. Our results show that this inhibitory activity is particularly important for controlling the Wnt dependent migration of neuroblasts along the primary body axis of *C. elegans*.

DISCUSSION

During *C. elegans* development, the migration of neuroblasts along the anteroposterior axis is controlled through a complex network of partially redundantly acting Wnt proteins. Here, we used smFISH to quantitatively map the spatio-temporal expression pattern of the *C. elegans* Wnt genes. We show that the five Wnt genes are expressed in partially overlapping expression domains along the anteroposterior axis, with the most prominent Wnt expression in the posterior body region. Furthermore, we demonstrate that Wnt signaling in the anterior body region is repressed by the secreted Frizzled related protein SFRP-1. These results show that the anteroposterior positioning of neuroblasts is controlled by opposing Wnt and Wnt inhibitory activities and provide further evidence for the evolutionary conservation of this system in patterning of the primary body axis.

A quantitative gene expression map of the *C. elegans* Wnt family

Conventional methods for gene expression analysis in *C. elegans* are mostly based on transgenic reporter constructs (Mello et al., 1991). In this study we used for the first time smFISH (Raj et al., 2008) to determine the spatio-temporal expression pattern of a gene family in *C. elegans*. By visualizing single transcripts as bright diffraction-limited spots, we could directly measure endogenous gene expression *in vivo* by counting the number of transcripts in a three dimensional area of interest, such as a specific cell or tissue. Importantly, this method also allowed us to quantify dynamic changes in gene expression, such as the transient expression of *mom-2* in cells of the tail and the activation of *cwn-1* expression in the CAN neurons during the first stage of larval development. We found that the expression level and spatial distribution of transcripts detected by smFISH was highly reproducible between staged animals, indicating that this methods accurately visualizes endogenous gene expression patterns. We conclude that smFISH can be used to produce quantitative spatio-temporal maps of endogenous gene expression patterns. It should be noted, however, that the expression pattern of the protein product may be influenced by post-transcriptional regulation.

Our smFISH analysis of the Wnt gene family showed similarities, but also important differences with expression patterns based on transgenic reporter constructs. We found that *cwn-2* is mainly expressed in head neurons and anterior body wall muscle cells, resolving a conflict in the previously reported expression patterns for *cwn-2* (Gleason et al., 2006; Kennerdell et al., 2009; Song

et al., 2010). Furthermore, we observed that *mom-2* is not generally expressed along the anteroposterior body axis (Gleason et al., 2006), but is restricted to the germline precursor cells and transiently to a group of cells in the tail. Another important difference is the expression of *lin-44* outside of the tail hypodermal cells (Herman et al., 1995). The more anterior expression of *lin-44* in the B and Y rectal epithelial cells is particularly interesting for the function of LIN-44 as a directional signal in T cell polarity (Goldstein et al., 2006) and for the inhibition of presynaptic assemblies in the DA9 neuron (Klassen and Shen, 2007).

The smFISH analysis revealed that the five Wnt genes are expressed in a series of partially overlapping expression domains, with expression of three of the five Wnt genes in the posterior and one in the anterior half of the body. The expression of the different Wnt genes in serial domains correlates with their function in controlling the migration of neuroblasts along the anteroposterior axis. Thus, the posteriorly expressed Wnt EGL-20 controls migration in the posterior and mid-body region, while CWN-1 and CWN-2 are particularly important for the migration of the QR descendants and the CAN and ALM neurons in the anterior half of the animal (Harris et al., 1996; Pan et al., 2006; Zinovyeva et al., 2008). We propose that the staggered series of Wnt expression domains provides a system for positional information along the anteroposterior body axis of *C. elegans*.

The secreted Frizzled related protein SFRP-1 is an inhibitor of Wnt signaling

SFRP proteins are characterized by an amino-terminal cysteine-rich domain (CRD) that is similar to the Wnt binding CRD domain of Frizzled (Bovolenta et al., 2008). SFRPs are secreted proteins that have been shown to act as inhibitors of Wnt signaling, most likely by competing with Wnt receptors for Wnt binding. However, SFRPs have also been reported to promote Wnt signaling, for example by facilitating the spreading of Wnt in the tissue (Mii and Taira, 2009) or by directly interacting with Frizzleds to stimulate signaling in a Wnt independent manner (Rodriguez et al., 2005). Phylogenetic analysis has shown that the SFRP family appeared very early in metazoan evolution, as clear SFRP orthologs are already present in the cnidarians *Hydra* and *Nematostella vectensis* (Guder et al., 2006a). Their function has, however, not been studied in any of the genetically tractable invertebrate model systems. The *Drosophila* genome does not contain SFRP orthologs, indicating that this gene family may have been lost in insects (but not in all arthropods, as the genome of the tick *Ixodes scapularis* contains an SFRP ortholog). In this study, we show that the *C. elegans* genome contains a single SFRP ortholog, *sfrp-1*, which enabled us to study potential Wnt inhibitory or stimulatory functions of SFRPs in a well defined model system. We found that SFRP-1 functions exclusively as an inhibitor of Wnt signaling: First, overexpression of *sfrp-1* induced a strong defect in Wnt signaling, similar to the phenotype observed in mutants in which all five Wnt genes have been mutated (Zinovyeva et

al., 2008). Second, loss of *sfrp-1* suppressed the Wnt signaling defect of mutants that induce a reduction in Wnt secretion and finally, all the phenotypes observed in *sfrp-1* mutants could be suppressed by removing specific Wnts, indicating that mutation of *sfrp-1* leads to derepression of Wnt signaling. These results suggest that the stimulatory function of SFRPs in Wnt signaling has either been lost in the nematode lineage, or is a more recent invention of higher organisms. Studies on the cnidarian SFRPs may shed light on this question.

An anterior SFRP-1 inhibitory gradient controls the positioning of neuroblasts in the anterior body region

The predominant anterior expression of *sfrp-1* suggests that it counteracts the more posteriorly expressed Wnts. Loss of this inhibitory activity leads to defects in the migration of neuroblasts in the anterior body region. Thus, the QR.d migrate too far into the anterior, whereas the extent of the posterior migration of the CAN and ALM neurons is reduced. In each of these cases, the final position of the cells is shifted anteriorly, indicating that SFRP-1 counteracts a Wnt activity that promotes anterior localization. We found that the *sfrp-1* induced anterior displacement of the QR.d could be suppressed by mutation of *cwn-2*, whereas ALM and CAN migration could be restored by deletion of either *cwn-2* or *cwn-1*. These results are consistent with a local inhibitory function of SFRP-1 in controlling the activity of the two most anteriorly expressed Wnts.

In addition to this short range function in the anterior body region, our experiments in Wnt secretion mutants showed that SFRP-1 also has a long-range inhibitory activity. Thus, mutation of *sfrp-1* rescued the posterior migration of the QL.d and the polarity of the V5 and T cell divisions in hypomorphic Wnt secretion mutants, consistent with a function of SFRP-1 in modulating Wnt activity in the mid to posterior body region. This long-range inhibition may fine tune the activity gradients of the posteriorly expressed Wnt genes.

An evolutionarily conserved function of Wnts and Wnt inhibitors in patterning the primary body axis

We found that four of the five *C. elegans* Wnt genes are expressed in a series of partially overlapping domains along the anteroposterior axis. This staggered expression is remarkably similar to the expression of Wnt genes in the cnidarian *Nematostella vectensis* (Kusserow et al., 2005) and in the planarian *Schmidtea mediterranea* (Petersen and Reddien, 2008). It has been proposed that the staggered expression of Wnt genes provides an ancestral mechanism for positional information along the primary body axis (Guder et al., 2006a) and our results suggest that *C. elegans* has retained such a system.

Another important similarity is the anterior specific expression of *sfrp-1* and the mostly posterior expression of the Wnt genes. This opposite expression of Wnts and Wnt inhibitors is already present in cnidarians, where Wnt inhibitors

are expressed at the aboral side and Wnts at the oral side of the primary body axis (Guder et al., 2006b; Hobmayer et al., 2000; Kusserow et al., 2005; Lee et al., 2006). Posterior Wnt signaling and anterior Wnt inhibition is also a central feature of vertebrate neurectodermal patterning, with the formation of the eyes and anterior brain structures depending on the anterior activity of both intracellular and secreted Wnt inhibitory factors (Kiecker and Niehrs, 2001; Kim et al., 2000; Niehrs, 2006). In protostomes, anterior specific expression of an SFRP has been observed in *Schmidtea mediterranea* (Petersen and Reddien, 2008), but Wnt inhibitors have not been studied in any of the other protostome model organisms. Our studies in *C. elegans* show that the opposite expression of Wnts and Wnt inhibitors is also an important feature of nematode development, supporting the notion that a system of posterior Wnt activity and anterior Wnt inhibition is a unifying principle of primary body axis specification in animals (Petersen and Reddien, 2009).

ACKNOWLEDGEMENTS

We thank Dr. Bert Hobmayer for critically reading the manuscript, Dr. Andrew Fire for expression vectors and the *Caenorhabditis* Genetic Center (University of Minnesota, Minneapolis) for strains. This work was funded by a NWO

VIDI fellowship (016.076.317) to H.C.K., a Boehringer Ingelheim Foundation fellowship to M.H. and a NIH Pioneer award (1DP1OD003936) to A.v.O.

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SUPPLEMENTARY FIGURES

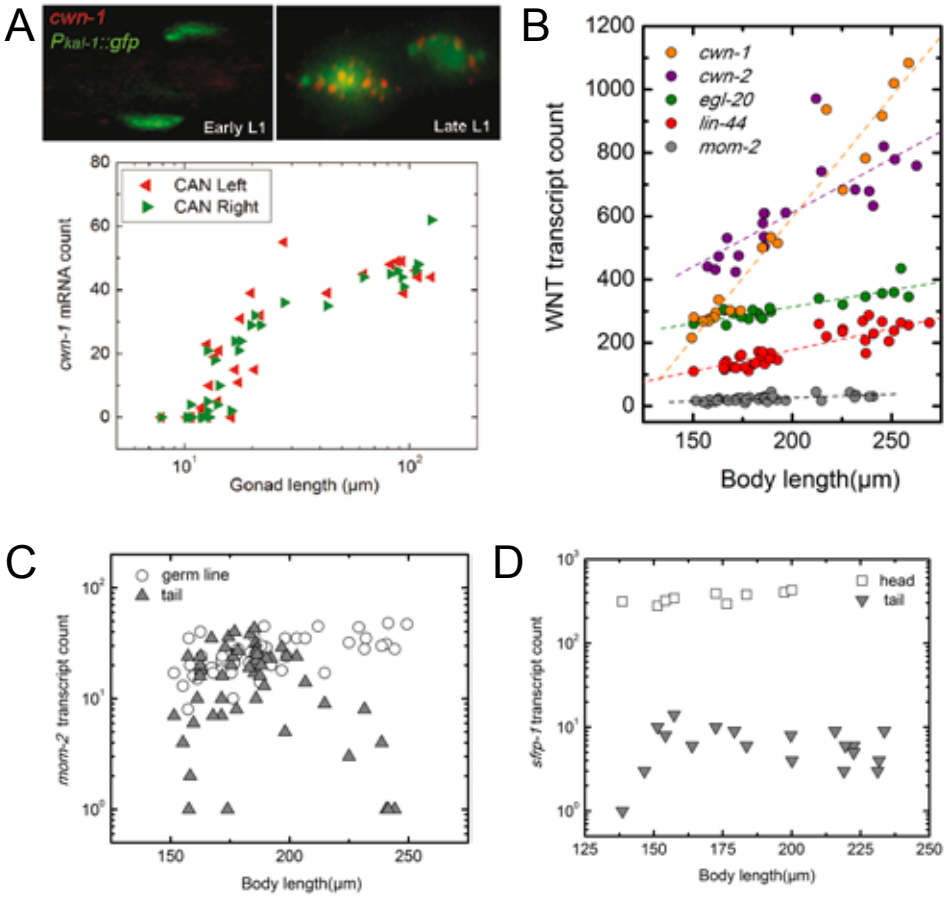

Figure S1. Expression of the *C. elegans* Wnt genes during the first stage of larval development. (**A**) The left and the right CAN neurons simultaneously induce *cwn-1* expression during L1 development. The CAN neurons are visualized using a *Pkal-1::gfp* reporter. (**B**) Quantification of total Wnt transcript numbers during L1 larval development. (**C**) Measurements of *mom-2* transcripts in the germline precursor cells and cells in the tail during L1 larval development. (**D**) Quantification of *sfrp-1* transcripts in the head and in tail neurons during L1 larval development.

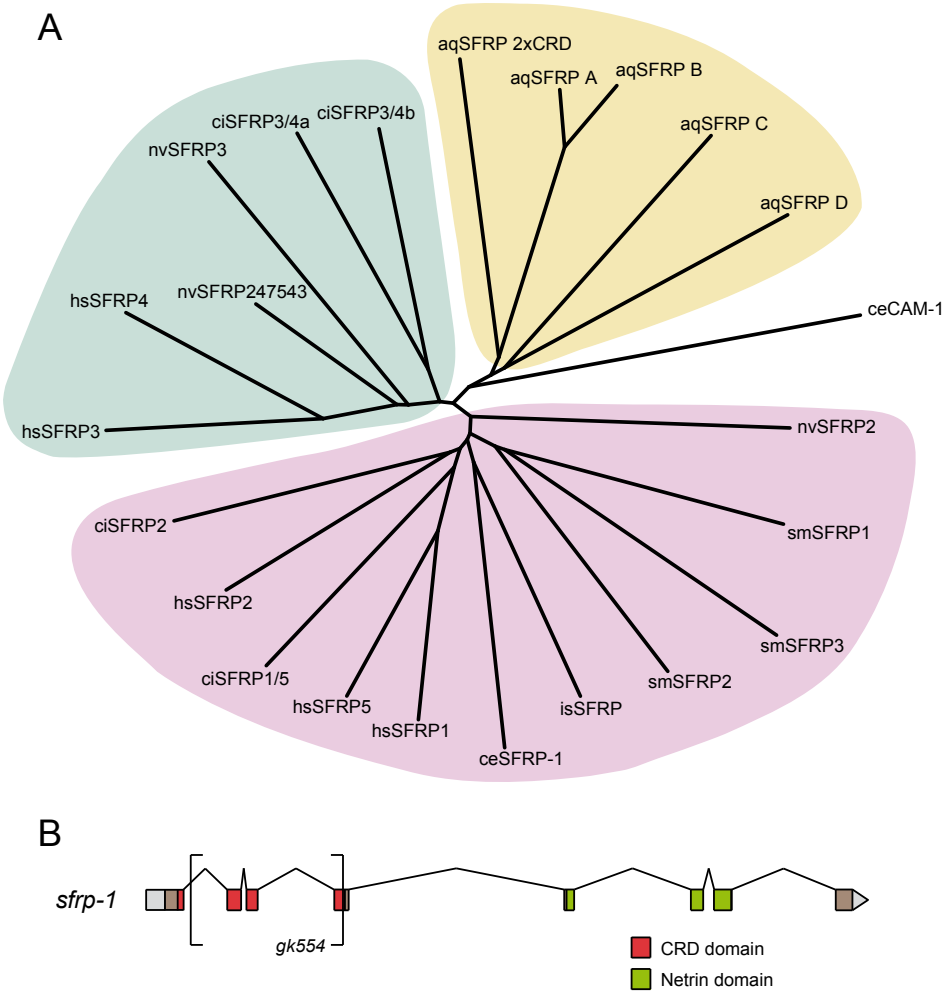

Figure S2. (**A**) Phylogenetic analysis of SFRPs family members of the chordates *Ciona intestinalis* (ci) and *Homo sapiens* (hs), the planarian *Schmidtea mediterranea* (sm), the nematode *Caenorhabditis elegans* (ce), the arthropod *Ixodes scapularis* (is), the cnidarian *Nematostella vectensis* (nv) and the sponge *Amphimedon queenslandica* (aq). SFRPs cluster in two clades (Adamska et al., 2010): the SFRP1/2/5 cluster (pink), which includes *C. elegans sfrp-1*, and the SFRP3/4 cluster (green). The *A. queenslandica* SFRPs cluster separately, suggesting that the species diverged before the SFRP duplication. *C. elegans* CAM-1/ROR contains a CRD domain that is related to the CRD domain of SFRPs. The phylogenetic tree was constructed with clustalw (using default parameter settings) (**B**) Schematic representation of the *C. elegans sfrp-1* (Y73B6BL.21) gene structure and protein domains. The *sfrp-1* deletion *gk554* is a 1261 base pair deletion which truncates the *sfrp-1* gene upstream of the CRD and NTR domains.

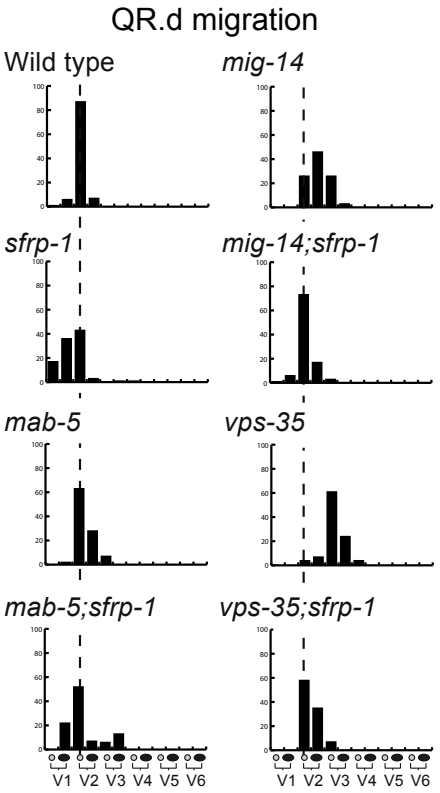

Figure S3. *sfrp-1* suppresses the QR.d undermigration induced by hypomorphic Wnt secretion mutants. The final positions of QR.paa and QR.pap cells are indicated relative to the seam cells V1 to V6 (n>50). The dashed line indicates the wild type position.

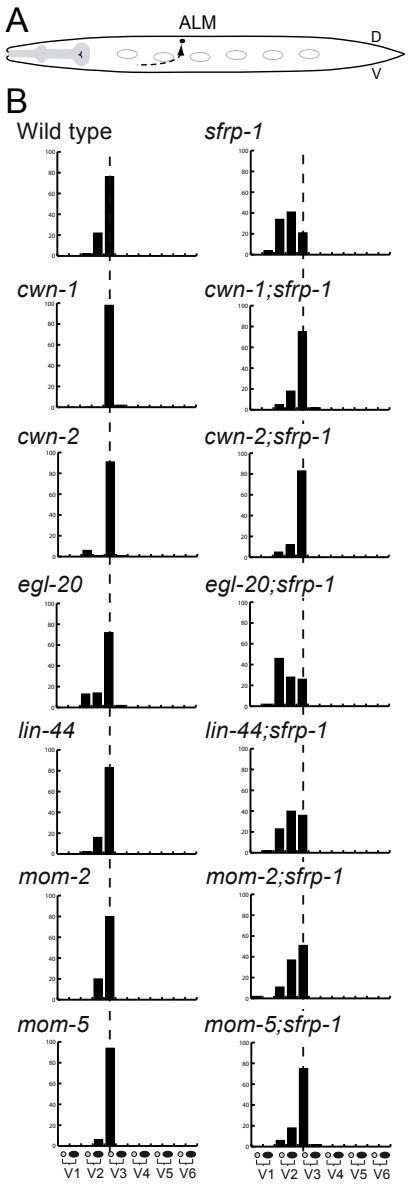

Figure S4. The *sfrp-1* induced undermigration of the ALM neurons is not suppressed by mutation of *lin-44*, *egl-20* or *mom-2*. (**A**) Schematic representation of ALM migration. (**B**) The final positions of the ALML and ALMR neurons are indicated relative to the seam cells V1 to V6 (n>50). The dashed line indicates the wild type position.

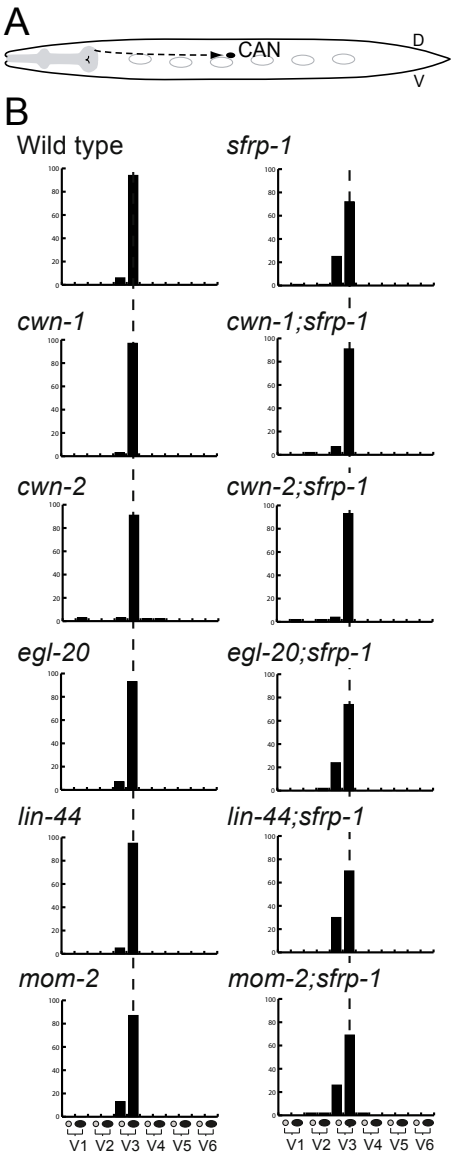

Figure S5. The *sfrp-1* induced undermigration of the CAN neurons is suppressed by mutation of *cwn-1* or *cwn-2*. (**A**) Schematic representation of the CAN neuron migration. (**B**) The final positions of the CANL and CANR neurons are indicated relative to the seam cells V1 to V6 (n>50). The dashed line indicates the wild type position.

6

DISSCUSSION

SUMMARIZING DISCUSSION

Wnt proteins are highly conserved signaling molecules regulating many important processes in development as well as in the adult. These proteins are secreted and form a morphogenetic gradient in the extracellular space to provide positional information to surrounding tissue. Much of the work described in this thesis has focused on the secretion of the Wnt morphogen. Wntless (Wls) plays a central role in this process, as it traffics Wnts from the Golgi to the plasma membrane for release. Wls is brought back to the Golgi by clathrin mediated endocytosis followed by a retromer dependent endosome to Golgi trafficking step (Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008). Loss of retromer results in lysosomal degradation of Wls, which becomes limiting, resulting in reduced Wnt secretion. The formation and shape of a morphogenetic gradient relies on several factors; the rate of secretion, the diffusion and the rate of degradation (Wartlick et al., 2009). Although reducing the rate of Wnt secretion would in theory not change the extent of the gradient, it will result in a shallower morphogenetic gradient, which can be insufficient for signal transduction by target cells.

In this thesis we have used the nematode *C. elegans* as a model organism to study Wnt signaling. The Q neuroblasts and its descendents have a characteristic anteroposterior migrating pattern, which depend on this signaling pathway (Maloof et al., 1999; Whangbo and Kenyon, 1999). In order to gain better insight in Wnt signaling we have used a genome-wide RNA interference (RNAi) library to search for genes that are required for Q cell migration. This resulted in the identification of several interesting genes required in Wnt producing cells for Wls trafficking, such as the novel retromer component *snx-3* and genes involved in the turnover of the endosomal enriched lipid phosphatidylinositol 3-monophosphate (PI3P) (Gillooly et al., 2000).

Sorting nexins and the retromer

The sorting nexin (SNX) protein family is classified by the presence of a particular lipid binding phox-homology (PX) domain (Teasdale et al., 2001). Since the SNX PX domains mainly bind the endosomal enriched PI3P lipid, SNXs are associated with the endosomal system, where they are involved in diverse functions, such as endosomal sorting, endocytosis and endosomal signaling (Cullen, 2008).

Several of these family members are involved in retromer dependent endosome to Golgi trafficking, aiding membrane tethering of the retromer core complex. These classical retromer sorting nexins contain a BAR domain next to the SNX PX domain, with which they can dimerize and induce membrane tubulation. Surprisingly, however, we have found that these classical retromer SNXs are dispensable for Wnt signaling, which to our knowledge is the first example of the retromer acting independently of these retromer SNX-BARs. In our screen we

identified a PX domain only sorting nexin, SNX3. We found that SNX3 is required for the retromer dependent endosome to Golgi retrieval of the Wnt sorting receptor Wls. This suggests that two retromer dependent endosome to Golgi trafficking routes exist, a SNX3/retromer route and a SNX-BAR/retromer route (Fig. 1). Since SNX3 has a much higher affinity for PI3P then the classical retromer SNXs (Yu and Lemmon, 2001) we propose that SNX3 associates with an earlier compartment in the endosomal maturation pathway then the PX-BAR SNXs. In the absence of SNX3, Wls is not Golgi retrieved and is degraded in lysosomes. As the SNX-BAR/retromer cannot compensate for this loss, this suggests that either Wls is not accessible for this retrieval pathway, possibly due to its internalization into intraluminal vesicles of the maturing endosome, or alternatively, Wls cannot be recognized by the SNX-BAR/retromer pathway.

In favor of the latter, yeast Grd19/SNX3 was found to bind both the retromer as well as to its cargo, the reductive iron transporter, Fet3p-Ftr1p, and therefore it was suggested to function as a cargo specific adaptor (Strochlic et al., 2007). However, no interaction was found between SNX3 and Wls. Furthermore yeast

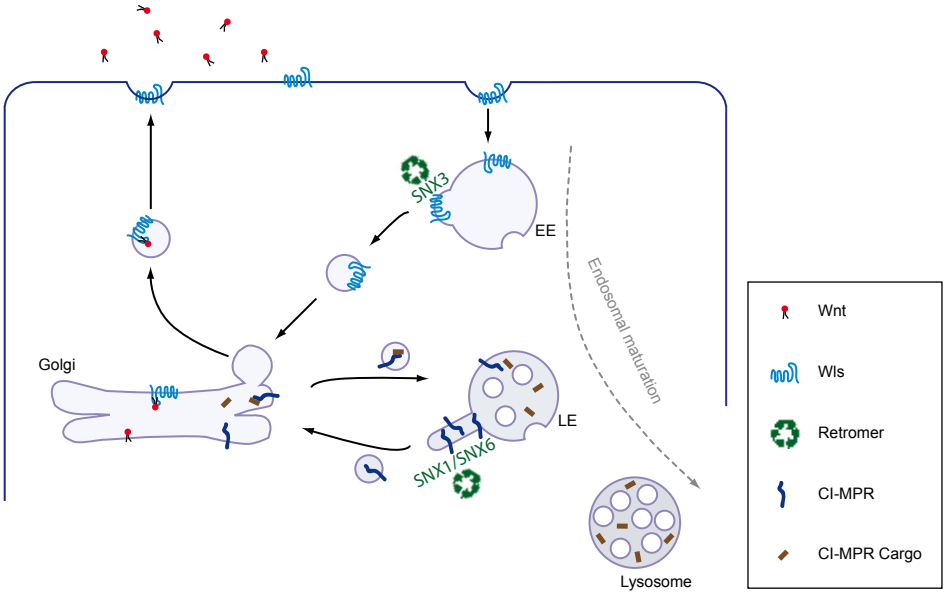

Figure 1. Model of two routes for retromer dependent endosome to Golgi traffic. The retromer cargo Wls traffics from the Golgi to the plasma membrane and via early endosomes (EE) back to the Golgi, whereas the classical retromer cargo CI-MPR traffics between the Golgi and the late endosome (LE). In this model, Wls is retrieved from an earlier endosomal compartment by the retromer assisted by SNX3. Here the Wls cargo buds off from the endosome, which might be clathrin dependent. CI-MPR is recognized by the retromer and the SNX-BARs, SNX1/2 and SNX5/6, at the late endosome where it tubulates away from the endosome and eventually pinches off for Golgi traffic.

Fet3p-Frt1p trafficking is fundamentally different to Wls trafficking since Golgi retrieval of Fet3p-Frt1p requires both the activity of Grd19/SNX3 as well as the classical retromer SNXs. Therefore it remains to be seen whether the SNXs are themselves involved in the recognition of the Wls cargo.

The cation independent mannose phosphate receptor (CI-MPR) is a well studied retromer cargo, which traffics between the Golgi and late endosomes (Arighi et al., 2004; Seaman, 2007). Upon SNX3 depletion the general distribution and levels of the CI-MPR are not affected, however a small fraction of the CI-MPR, which traffics via the plasma membrane, is sensitive to SNX3 depletion (Harbour et al., 2010; Wassmer et al., 2007). This suggests that the CI-MPR cargo can be Golgi retrieved by both the SNX3/retromer as well as the SNX-BAR/retromer.

This is in favor of a model where the spatial separation of classical and non-classical retromer complexes along the maturing endosomal pathway, coupled with the point of cargo entry, determines the preference for sorting via the SNX3 or the BAR-SNX retromer pathways.

PI3P and Wnt secretions

The endosomal enriched PI3P lipid plays important roles in endosomal functioning, since it regulates the recruitment PI3P binding proteins such as the SNXs. In our screen we identified several genes which are involved in the turnover of PI3P, including a member of the PI3P phosphatase family called myotubularins. Careful analyses of this myotubularin family revealed that specifically the complex formed by MTM-6 and MTM-9 play an important role in Wnt secretion. Since depletion of MTM-6/MTM-9 resulted in pronounced accumulation of SNX3, this suggests that the MTM-6/MTM-9 complex targets a specific pool of PI3P that is essential for retromer dependent trafficking. This raises the following question: if the myotubularins are required for the correct recruitment of the retromer, then

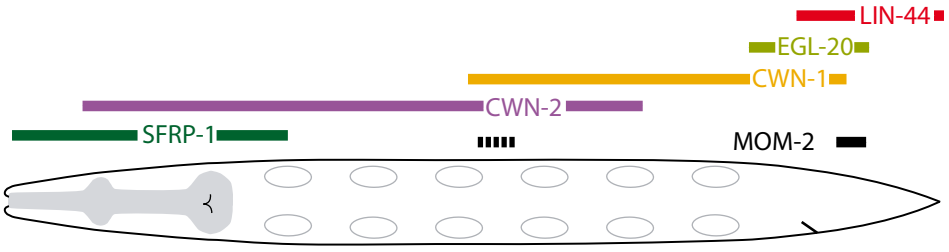

Figure 2. Model of Wnt and sfrp-1 expression domains. Schematic representation of the partially overlapping expression domains of the *C. elegans* Wnt genes along the antero-posterior axis observed using smFISH (see Figure 1 Chapter 5). The single *C. elegans* SFRP family member is expressed in the anterior, suggesting that opposing gradients of Wnts and Wnt inhibitors function to patters the AP axis.

what regulates the myotubularins in this process? Furthermore it is interesting to note that although MTM-6/MTM-9 depletion results in similar phenotypes as retromer depletion, there is also a difference. In the *Drosophila* wing imaginal disk, Wls is ubiquitously expressed. Upon retromer depletion, Wls levels are strongly reduced only in the Wnt producing cells, but it has no effect on non-Wnt producing cells, suggesting that Wls is not trafficked through the endosomal pathway in these cells. However, upon Dmtm6 depletion, Wls is lost both in the Wnt producing as well as in the non-producing cells. Recently Wls was shown to also have a role in the Wnt receiving cells at the neuromuscular junction (Korkut et al., 2009). Therefore, it is possible that the function and trafficking of Wls in the Wnt receiving cells is different from the Wnt producing cells, and whereas the retromer is only required in the producing cells, mtm-6 might be required in both. However, no other examples of Wls acting in the receiving cells have yet been described.

Wnt signaling and anteroposterior patterning

Wnt signaling plays important roles in *C. elegans* anteroposterior (AP) patterning, regulating processes such as cell migration and asymmetric cell division along the AP axis (Silhankova and Korswagen, 2007). In order to fully understand how Wnt signaling regulates these processes, it is essential to know where the Wnts are expressed. However, conflicting Wnt expression patterns have been reported (see Chapter 5). Gene expression analyses in *C. elegans* is usually performed using reporter constructs driven by the upstream sequence of a gene of interest. However promoter elements, which might be more distantly located, intronic or downstream of the gene, might be missed, resulting in an incorrect expression pattern.

We have used an adapted fluorescent in situ hybridization protocol, which enables individual transcript visualization (single mRNA molecule FISH or smFISH). Using this technique we were able to quantitatively analyze the expression patterns of the *C. elegans* Wnts in the larva, resolving the conflicting reports. Strikingly, the five Wnt genes are expressed in a series of partially overlapping expression domains along the AP axis. The single SFRP family member, *sfrp-1*, is expressed in the anterior muscles (Fig. 2). Since *sfrp-1* functions as a classical Wnt inhibitor in *C. elegans*, this suggests that opposing gradients of Wnts and Wnt inhibitors pattern the AP axis. Interestingly, anterior Wnt inhibition and posterior Wnt signaling seems to be a recurring theme in evolution (Petersen and Reddien, 2009), which we now show is also the case for a member of the Ecdysozoa superphylum.

Outlook and Perspectives

In recent years, our lab and others have extensively studied the Wnt production and secretion pathway. Although a detailed picture is forming about the

mechanism of this pathway, some key questions remain to be addressed, which have been further discussed in Chapter 1. For example, what are the functions of the Wnt lipid and glycosyl modifications in secretion and signaling? Where and how do Wnts dissociate from Wls? Additionally, it is important to consider that alternative Wnt secretion mechanisms exist. Korkut and colleagues made the interesting observation that Wls is secreted along with Wnt at the *Drosophila* neuromuscular junction, which is required for signaling (Korkut et al., 2009). It is not known if a similar signaling mechanism is present elsewhere.

Since Wnt signaling plays such crucial functions in development it is likely that its secretion is highly regulated both at the protein as well as the transcriptional level, although in vivo evidence for this is still sparse. Modulation of the Golgi retrieval of Wls seems an efficient way to control the levels of Wnt secretion. Another attractive mechanism to tightly control Wnt secretion is by regulating either the activity of porcupine or the trafficking of Wls to the plasma membrane. In our screen (Chapter 2) we did not identify obvious genes that, like Wls and Porcupine, are indispensable for Wnt secretion. A plausible explanation for this is that MOM-2/Wnt is essential for embryogenesis (Rocheleau et al., 1997; Thorpe et al., 1997). Therefore, depletion of any gene, which results in a complete block of Wnt secretion, results in embryonic lethality. In order to identify such genes, alternative approaches should be considered, such as tissue specific gene depletion or screens using a different readout, such as embryonic lethality.

Since deregulation of Wnt signaling is tightly linked to human disease, much effort has been undertaken to find drugs to regulate this signaling pathway. Since most mutations identified affect signaling in the Wnt receiving cells, much work has focused on drugs modulating signal transduction, such as inhibitors targeting the destruction complex, which positively influence Wnt signaling or inhibitors which negatively influence Wnt signaling by targeting genes like Disheveled or ß-catenin. Alternatively, several extracellular Wnt signaling inhibitors are under research, such as antibodies targeting the Wnts or its receptors (Curtin and Lorenzi, 2010).

We would like to finish with the idea that targeting Wnt production is worth to consider as well. Recently, inhibitors of the acyltransferase Porcupine were developed, which surprisingly were identified in a screen searching for Wnt/ß-catenin signaling inhibitors in cells harboring mutations in APC (Chen et al., 2009). Furthermore, depletion of Porcupine in lung cancers cells lead to a loss of Wnt signaling and was able to induce apoptosis specifically in the cancer cells (Chen et al., 2008). Care has to be taken using such compounds, as they target secretion of all Wnts, which could have far ranging consequences. However, targeting all Wnts makes it a broadly applicable strategy especially in cases where signaling is not well understood. Further research is needed to address whether targeting Wnt production can be used as a therapeutic approach for the treatment of human diseases.

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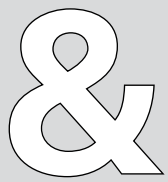

NEDERLANDSE SAMENVATTING
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NEDERLANDSE SAMENVATTING

Het menselijk lichaam is opgebouwd uit miljarden cellen, waarvan de meeste gespecialiseerd zijn in een bepaalde taak, zoals zenuw-, spier- en afweercellen. Iedere cel heeft een kopie van het erfelijk materiaal, het DNA, opgeslagen in de celkern. In dit DNA staat gecodeerd hoe en wanneer eiwitten gemaakt moeten worden. Eiwitten zijn misschien wel de belangrijkste component van de cel. Zo zijn eiwitten betrokken bij processen in de cel zoals celdeling, de energievoorziening alsook de stevigheid van de cel. Daarnaast worden sommige eiwitten uitgescheiden om een belangrijke rol buiten de cel te vervullen, zoals bepaalde hormonen, verteringsenzymen in de maag of het eiwit keratine waaruit haren en nagels zijn opgebouwd.

Door de samensmelting van een eicel en een zaadcel ontstaat de eerste cel van een organisme, wat via de nodige celdelingen binnen een paar dagen tot een klompje cellen uitgroeit. Echter, voordat dit klompje cellen zich in enkele weken tot een foetus ontwikkelt zijn flinke vormveranderingen nodig om de cellen op de goede plek te krijgen en de juiste rol te geven. Om dit geordend te laten verlopen zijn er uitgebreide communicatiemechanismen tussen de cellen nodig. Een van deze essentiële communicatiemechanismen maakt gebruik van het Wnt-eiwit, dat fungeert als een signaal. Daarom wordt dit mechanisme "Wnt-signalering" genoemd. Het Wnt-eiwit wordt in cellen aangemaakt en vervolgens uitgescheiden om omliggende cellen te instrueren, bijvoorbeeld dat ze zich moeten verplaatsen of delen. Behalve dat Wnt-signalering essentieel is tijdens de ontwikkeling van een organisme, is dit mechanisme ook in volwassenen erg belangrijk. De cellen in je darmen hebben een vrij korte levensduur (enkele dagen) en moeten zich dus regelmatig opnieuw delen zodat het aantal cellen gelijk blijft. Wnt-signalering zorgt in je darmen voor dit proces door de deling te stimuleren waardoor er weer nieuwe cellen bijkomen. Het is voor te stellen dat hier gelijk ook het gevaar in zit. Als er iets mis gaat met dit mechanisme en het overmatig actief wordt zullen deze cellen meer gestimuleerd worden om zich te delen, waardoor ze in aantal sterk zullen toenemen hetgeen het begin van kanker zou kunnen zijn. In bijna alle gevallen van darmkanker zijn er veranderingen gevonden in Wnt-signalering waardoor dit mechanisme overactief is geworden. Het is dus van essentieel belang om goed te begrijpen hoe dit signaleringsmechanisme precies werkt.

In onze onderzoeksgroep bestuderen wij het mechanisme van Wnt-signalering door gebruik te maken van een modelorganisme, de rondworm *C. elegans*. Deze wormpjes zijn erg klein, maar 1 mm lang, en hebben ongeveer 1000 cellen. Wnt-signalering is in deze wormpjes niet betrokken bij (darm)kanker, maar wel bij de deling en verplaatsing van andere cellen, zoals zenuwcellen. In ons werk hebben we veelvuldig gebruik gemaakt van de verplaatsing van een bepaalde zenuwcel, genaamd de QL-cel. Wnt-signalering is verantwoordelijk voor de verplaatsing van de QL-cel richting staart. Indien het Wnt-signaal niet aanwezig is of niet gevoeld wordt door de QL-cellen zullen ze zich richting het hoofd van de worm verplaatsten.

Door te analyseren of de QL-cel zich richting de staart of richting het hoofd heeft verplaatst weten we of het Wnt-signaleringsmechanisme goed gewerkt heeft.

Om een beter begrip te krijgen over hoe dit mechanisme precies in elkaar zit, hebben we één voor één de productie van elk eiwit uitgeschakeld (Hoofdstuk 2). Dit hebben we gedaan door gebruik te maken van een techniek genaamd RNA-interferentie, waaraan in 2006 de Nobelprijs is toegekend. Indien een eiwit belangrijk is voor Wnt-signalering, zal de uitschakeling van zijn productie leiden tot een verlies van het Wnt-signaleringsmechanisme. Hierdoor zullen de QL-cellen zich niet naar de staart, maar naar het hoofd verplaatsen. Wij hebben dit getest voor bijna alle 20.000 eiwitten van de worm, en hebben vele nieuwe eiwitten gevonden die een rol spelen in de QL-celverplaatsing. Aangezien defecten in QL-verplaatsing ook andere oorzaken kunnen hebben dan een verstoorde Wnt-signalering, is het belangrijk om dieper in te gaan op de manier waarop deze nieuw gevonden eiwitten werken.

Een aantal van de eiwitten die we verder hebben gekarakteriseerd, zijn betrokken bij de uitscheiding van Wnt-eiwitten. Wnt-eiwitten kunnen in tegenstelling tot andere eiwitten niet zomaar de standaard uitscheidingsroute nemen. Om uitgescheiden te worden moeten Wnt-eiwitten een transporteiwit binden, genaamd Wntless. Dit eiwit escorteert het Wnt-eiwit als het ware uit de cel. Echter, om meerdere keren Wnt-eiwitten te transporteren moet Wntless weer teruggebracht worden naar de plek in de cel waar het een nieuw Wnt-eiwit kan binden. In ons werk hebben we gevonden dat SNX-3 (Hoofdstuk 3) en MTM-6 (Hoofdstuk 4) een belangrijke rol spelen in het terugbrengen van het transporteiwit Wntless. Zonder SNX-3 of MTM-6 wordt Wntless niet teruggebracht maar afgebroken, waardoor te weinig Wnt-eiwitten uitgescheiden kunnen worden, wat leidt tot verlies van Wnt-signalering. Opmerkelijk is dat deze SNX-3 en MTM-6 transport-route anders lijkt te zijn dan de route die genomen wordt door andere eiwitten die naar dezelfde plek in de cel gebracht worden.

Als laatste hebben we ook nog gewerkt aan een tweede eiwit dat uitgescheiden wordt, genaamd SFRP-1. Aangezien het lijkt op een eiwit dat Wnts kan binden, wordt er verwacht dat SFRP-1 fungeert als een remmer van Wnt-signalering, door buiten de cel Wnts te binden. Om de functie van SFRP-1 in de worm te bestuderen hebben we gebruik gemaakt van een worm mutant waarin dit eiwit ontbreekt. We hebben gevonden dat deze wormen defecten hebben die verwacht worden als Wnt-signalering overmatig actief is (Hoofdstuk 5). Dit is in lijn met een Wnt-signalering remmende rol van dit eiwit. Aangezien SFRP-1 bijna uitsluitend in het hoofd gemaakt wordt, suggereert dit dat Wnt-signalering geremd moet worden in het hoofd voor correcte ontwikkeling.

Met ons werk hebben we een aantal nieuwe puzzelstukjes gevonden die ons meer inzicht geven hoe Wnt-eiwitten gemaakt en uitgescheiden worden en hoe de Wnt activiteit buiten de cel gereguleerd wordt. Een beter begrip van dit mechanisme biedt de mogelijkheid om hiermee geassocieerde ziektes beter te begrijpen en adequater te bestrijden.

DANKWOORD

Eindelijk is het dan zover, het werk van de afgelopen jaren heeft geresulteerd tot dit proefschrift. Echter, voor de totstandkoming van dit boekje ben ik een hoop mensen dank verschuldigd.

Allereerst Rik, bedankt voor de kans die je me hebt gegeven. Het vertrouwen en de vrijheid die je me gaf heb ik altijd zeer gewaardeerd en maakte het erg prettig om voor jou te werken. Ik heb (hopelijk) veel geleerd van je perfectionisme en manier van aanpakken. Als ik weer eens met één of ander wild idee langskwam had jij dat in 2 seconden omgebogen in een simpel en uitvoerbaar plan.

Ik zou graag alle leden van het Korswagen lab willen bedanken voor de fantastische tijd die ik gehad heb. De leuke discussies, niet politiek correcte humor en kopjes koffie zal ik zeker gaan missen. Jammer alleen dat er zo veel over fietsen gesproken werd, en niet over voetbal... Magdalena and Marie, it was great to work with you. Thanks for all the help you gave me. Marco iedere keer dat erweer eens gescreend moest worden, kon ik op je rekenen. Teije, Reinout, Remco, Kristy, Jan-Willem, Gwen en Peggy het was me een waar genoegen met jullie in het lab te staan.

Lars, Roy en Thang; super bedankt voor jullie inzet en enthousiasme tijdens jullie stage. Hopelijk heb ik jullie nog iets bij kunnen brengen. Ik heb in ieder geval veel van jullie geleerd en veel gehad aan jullie resultaten.

I would also like to thank our wonderful collaborators; Pete, Ian, Fillip, Dong hyun and Alexander. It would not have been the same without your help.

Furthermore I would like to thank the Boehringer Ingelheim Fonds not only for funding, but also for all the scientific input. Matilde, super leuk dat jij deze ook gekregen hebt, en dat we hier samen naartoe hebben kunnen gaan.

Daarnaast zou ik ook alle mensen van het Hubrecht willen bedanken, voor alle gezelligheid het goede onderzoek en discussies. Ook de ondersteunende diensten van het Hubrecht, bedankt! Mede door jullie is dit instituut een fantastische plek om te werken. De Utrechtse Wormen Club labs bedankt voor de interessante meetings en feedback, mijn kamergenoten, the Berezikovjes, voor de gezelligheid en Bas, Frank en Guy voor het organiseren van het voetbal. Uiteindelijk mijn AIO-commissie, Sander, René en Hans, bedankt voor de feedback en ondersteuning. En Hans bedankt dat je mijn promotor wilde zijn.

Papa, maman, zusjes, (schoon-)familie en vrienden, heel erg bedankt voor jullie steun. Ook al heb ik niet altijd duidelijk kunnen maken waar ik precies mee bezig was, jullie betrokkenheid en interesse heb ik altijd erg op prijs gesteld.

Als laatste, Judith, lief vriendinnetje, inmiddels mijn vrouw, bedankt voor je onvoorwaardelijke steun en liefde.

CURRICULUM VITAE

Martin Harterink werd geboren op 26 Juli 1981 te Utrecht. Zijn VWO voltooide hij in 1999 aan het St. Bonifatius College te Utrecht. Vervolgens is hij Scheikunde gaan studeren aan de Universiteit van Utrecht, en heeft hij de Master Biomolecular Sciences gevolgd. Hierbij heeft hij stage gelopen bij de vakgroep Membraan Enzymologie onder begeleiding van Klazien Huitema en Joost C. Holthuis, en in het lab van Christian R. Eckmann aan het Max Planck instituut in Dresden. Voor zijn afstudeer project heeft hij de Organon Jong Talent Afstudeerprijs voor biochemie en biotechnologie ontvangen. In 2006 in hij afgestudeerd en is hij begonnen aan het in dit proefschrift omschreven promotieonderzoek bij Dr. Hendrik. C. Korswagen aan het Hubrecht Instituut waarvoor hij een Boehringer Ingelheim fellowship heeft gekregen.

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