Mechanisms of Wntless trafficking and Wnt signaling

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Mechanisms of Wntless trafficking and Wnt signaling

Mechanismen van Wntless transport en Wnt signalering

(met een samenvatting in het Nederlands)

Proefschrift

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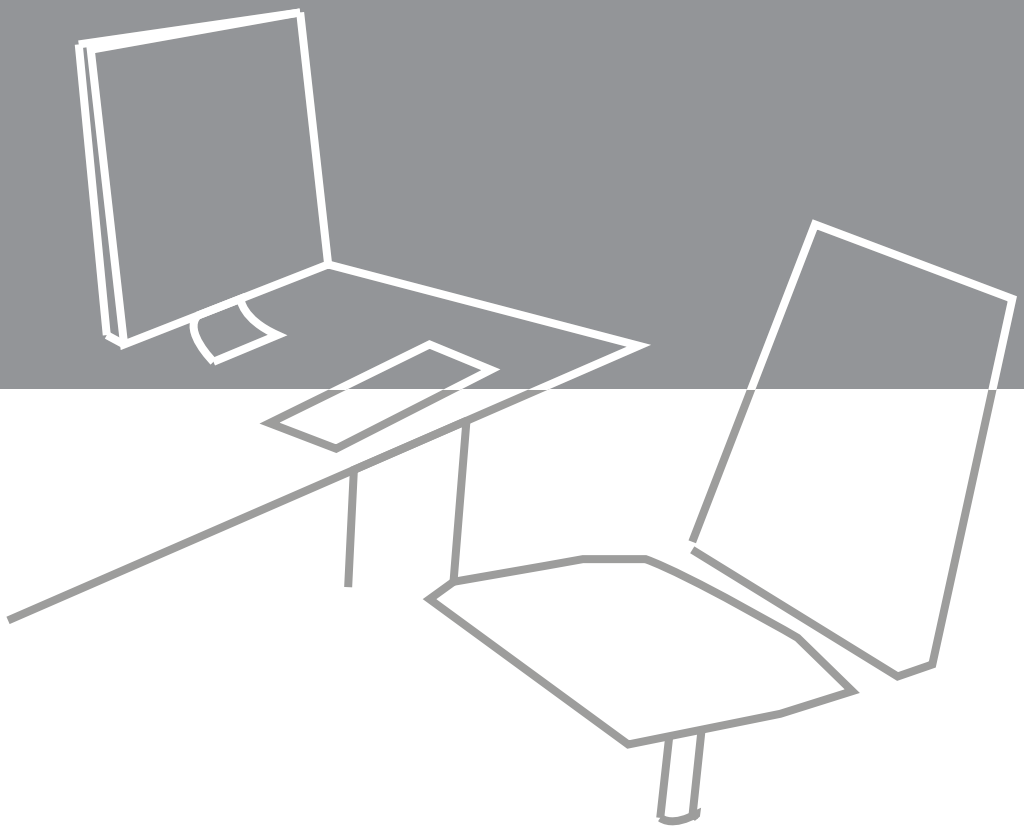
Scope of this thesis

During my graduate studies I have investigated the mechanisms of Wnt secretion and Wnt signaling. I used *C. elegans,* mouse and human tissue culture cells as model systems in my studies. In the first chapter of this thesis I will give an introduction to the molecular mechanisms of β-catenin dependent Wnt signaling with a special emphasis on the role of ubiquitin mediated regulation of Wnt signaling. I will then describe the role of β-catenin dependent Wnt signaling in two biological processes: QL.d migration during *C. elegans* development, which I use as a read-out for β-catenin dependent Wnt signaling in chapters 2, 3, 5 and 6, and stem cell maintenance in the mammalian intestine. I will describe the role of Wnt signaling in the mammalian intestine as an introduction to chapter 4. In addition, I will discuss the mechanisms of Wnt secretion and Wnt gradient formation in the introductory chapter. Retrograde trafficking from endosomes to the trans-Golgi network is important for Wnt secretion. For this reason, I will discuss the retrograde trafficking route of Wntless, which is the relevant protein for Wnt secretion. I will draw parallels between Wls trafficking and retrograde transport of the cation independent mannose-6-phosphate receptor (CI-MPR), a protein that undergoes a well-characterized retrograde trafficking route.

In chapters 2 to 6 I will describe the experimental work that I performed during my studies. In chapter 2, I describe an RNAi screen that I performed to identify novel regulators of Wnt signaling. The role of *eel-1*/Huwe1 as a negative regulator of Wnt signaling will be discussed in chapter 3. In chapter 4, I describe the consequences of deletion of the retrograde trafficking factor Vps35 from the murine intestinal epithelium for Wls trafficking, Wnt signaling and intestinal stem cell maintenance. We will describe the identification of an evolutionary conserved lipid flippase complex and its role in retrograde Wls trafficking in chapter 5. In chapter 6 we will discuss the role of protein kinase CK2 in Wnt secretion. A summarizing discussion will be provided in chapter 7.



CHAPTER 1

Introduction

In 1982, a year before the author was born, the observations that form the basis of this thesis were published. Roel Nusse, working in the lab of Harold Varmus, discovered that the murine retrovirus MMTV causes breast cancer in mice when it integrates in a specific locus in the mouse genome. The MMTV virus induces expression of a proto-oncogene at the integration site. This gene was named Int1[1]. In the same period, Eric Wieschaus and Christiane Nüsslein-Volhard investigated the genetics of embryonic development of *Drosophila melanogaster*. They studied mutant embryos that displayed patterning defects and investigated the responsible genes. One class of genes they identified, the 'segment polarity genes', included Armadillo, Arrow and Wingless[2]. It became apparent that Wingless and Int1 were orthologues. This discovery motivated the research community to call these genes, and their homologues, Wnt genes (for Wingless and Int1). Wnt was one of the first example of a gene with a function in development that, when mis-regulated, causes cancer in adult animals[3].

The Wnt gene family is evolutionary conserved in metazoans[4]. This suggests an ancient evolutionary origin of this gene family. In *Hydra* and *Nematostella*, Wnts define the oral-aboral axis. In most bilaterally symmetrical organisms, Wnt genes determine the anterio-posterior body axis. Anterior Wnt signal inhibition, and posterior Wnt expression, is a common mechanism to pattern the primary body axis[4,5].

There is a particularly striking role for Wnt signaling in the regulation of regeneration in planarian flatworms. *Schmidtea mediterranea* worms will regenerate head-tissue when the head is removed, and tail when the tail is amputated. The correct orientation of regenerating head and tail tissue depends on Wnt signaling. Knockdown of β-catenin, a central player in Wnt signal transduction, causes the ectopic regeneration of head tissue. In fact, when multiple cuts are made in a worm that is subjected to β-catenin RNAi, every regenerating wound will generate a head[6,7]. *S. mediterranea* has an exceptionally high regenerative capacity, many other species of flatworms fail to regenerate head tissue after injury. It was recently shown that interfering with Wnt signaling promotes regenerative capacity in flatworms that normally cannot regenerate. The *Phagocata kawakatsui, Procotyla fluviatilis* and *Dendrocoelum lacteum* species have limited regenerative capacity, but when the Wnt pathway is inactivated in these worms, head tissue can regenerate after transection[8–10], illustrating the importance of Wnt signaling in AP patterning and regeneration.

The molecular mechanisms of β-catenin dependent Wnt signal transduction

Wnt ligands can elicit a variety of responses in Wnt signal receiving cells. Broadly, these responses can be divided in a 'canonical', β-catenin dependent, response and a number of 'non-canonical', β-catenin independent responses. The non-canonical Wnt responses can result in changes to the cytoskeleton, changes in cell polarity and cell migration and changes in gene expression[11]. The β-catenin dependent Wnt response exclusively changes gene expression[12]. β-catenin dependent Wnt signal transduction is the main subject of this thesis. For this reason, a short summary of the molecular mechanisms of β-catenin dependent Wnt signaling will be provided here.

The canonical Wnt signal transduction cascade centers on the regulation of free, cytoplasmic β-catenin, i.e. β-catenin that is not engaged in cell adhesion through incorporation in adherens junctions[12]. In the absence of a Wnt signal, β-catenin is downregulated through the action of the β-catenin destruction complex. This cytoplasmic protein complex consists of scaffold proteins Axin and APC and kinases CK1α and GSK3β. The destruction complex phosphorylates β-catenin on serines located in the N-terminus of the protein. These

phosphorylated serine residues form a phosphodegron that is recognized and subsequently ubiquitylated by $SCF^{\beta TrCP}$. Ubiquitylated β-catenin is targeted for proteasomal degradation. As a result, cytoplasmic β-catenin levels are low in the absence of Wnt signaling (reviewed by MacDonald *et al.*[12] and Clevers[13]) (Fig. 1).

When a Wnt ligand binds to a receptor of the Frizzled (Fz) family and the co-receptor Lrp6, the destruction complex is inhibited. The upstream events in the Wnt signal transduction cascade are incompletely understood, but they involve phosphorylation of the cytoplasmic tail of Lrp6, phosphorylation of the cytoplasmic protein Dishevelled (Dvl) and the formation of signalosomes that consist of phosphorylated Dvl and Lrp6 at the plasma membrane[14–16]. Subsequently, the function of the destruction complex is inhibited. Several mechanisms of destruction complex inhibition have been proposed in the literature. Most reports suggest that the destruction complex is inhibited by dissociation of its components. An important role is reserved for Axin, because Axin is a limiting component of the destruction complex[15,17]. During Wnt pathway activation, Axin is recruited into signalosomes at the plasma membrane. When recruited to signalosomes, Axin is no longer available for the destruction complex and the destruction complex is inhibited. Recently, this model was refined by Kim *et al.* who

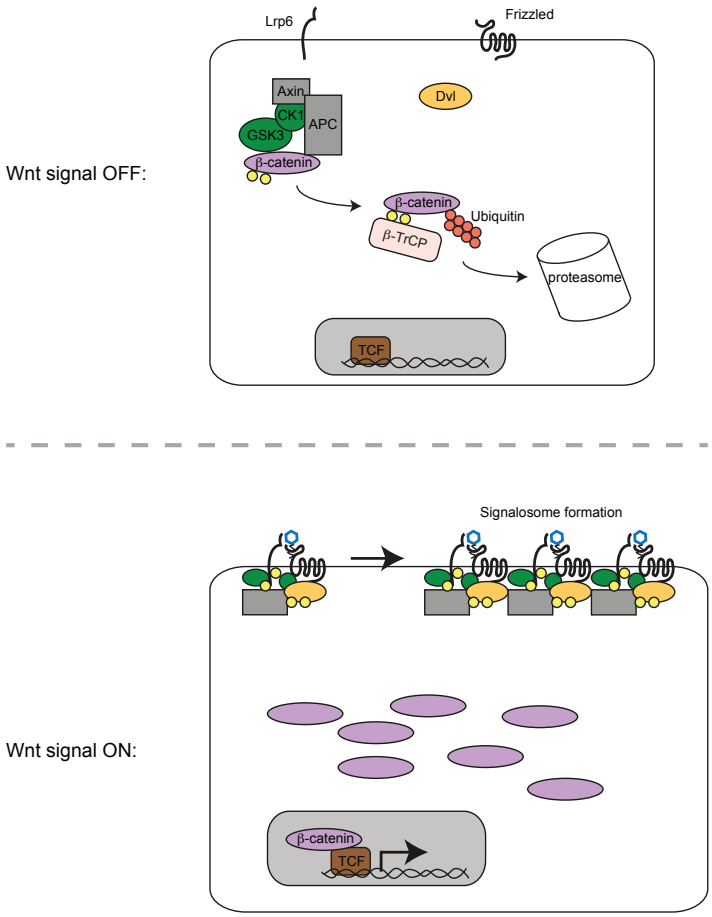


Figure 1: Schematic view of β-catenin dependent Wnt signal transduction See text for discussion.

propose the model that GSK3 phosphorylates Axin to form an active destruction complex. When Wnt signaling is activated, Axin is recruited to phosphorylated Lrp6, this inhibits the kinase activity of GSK3 towards Axin and allows the phosphatase PP1 to dephosphorylate Axin. Dephosphorylated Axin cannot induce β-catenin degradation, resulting in Wnt pathway activation[18]. Furthermore, Axin is degraded when the Wnt pathway is activated during a longer time period, providing an additional mechanism of destruction complex inhibition[19–21]. The molecular mechanisms of destruction complex inhibition remain under intense investigation. In recent publications, alternative models for the molecular mechanisms of destruction complex inhibition have been proposed. Taelman *et al.* suggest that the destruction complex is inhibited through sequestration of GSK3 inside multivesicular bodies (MVBs)[22]. Li *et al.* suggest that βTrCP associates with the destruction complex and that Wnt signaling prevents the release of phosphorylated β-catenin from the destruction complex. In this way, the destruction complex is clogged up with phosphorlyated β-catenin and newly formed β-catenin accumulates[23]. Although there is no consensus about the exact molecular mechanisms of destruction complex inhibition, the inhibition of β-catenin phosphorylation is sufficient to accurately model the kinetic responses of β-catenin accumulation in response to Wnt signals[24].

When cytoplasmic β-catenin levels rise as a result of Wnt pathway activation, β-catenin can translocate to the nucleus[12]. There, it can replace the transcriptional repressor Groucho/TLE from TCF transcription factors that are bound to TCF consensus motifs in the DNA. β-catenin acts as a transcriptional co-activator in combination with TCF to induce Wnt target gene expression (reviewed by Cadigan[25]) (Fig. 1).

Regulation of the β-catenin dependent Wnt pathway by ubiquitylation

Ubiquitylation is a post-translational modification that involves ligation of the protein ubiquitin to lysine residues on target proteins[26]. Ubiquitin is an 8.5 kDa protein with 7 lysines in its primary amino acid sequence. The lysines of ubiquitin itself can be ubiquitylated, yielding ubiquitin polymers. These polymers are characterized by the lysine to which the following lysine of the polymer is attached. K48 and K63 linked polyubiquitin chains are best characterized, but also the other lysine residues of ubiquitin can be ubiquitylated and mixed ubiquitin chains exist as well[27,28].

Ubiquitylation is well known for its role in the regulation of proteolysis. Proteins that carry a specific type of ubiquitin polymer (K48 linked polyubiquitin), are earmarked for proteasomal degradation[26]. K63 linked polyubiquitylation is involved in sorting proteins to multivesicular bodies, the regulation of the DNA damage response, NFκB signaling and many other processes. Mono-ubiquitylation is involved in the regulation of endocytosis of membrane proteins, the regulation of subcellular localization and signaling functions of proteins[27,29–31].

Ubiquitin is ligated to a target protein in a three-step process. First, an E1 enzyme activates ubiquitin. Subsequently, ubiquitin is transferred to an E2 enzyme. In the last step, ubiquitin is transferred to its target by an E3 ubiquitin ligase[26]. This final step confers substrate specificity to the ubiquitylation reaction. Ubiquitylation is a reversible process, de-ubiquitylating enzymes (DUBs) can remove ubiquitin modifications from proteins and counteract the function of ubiquitin ligases[32].

Knowing that ubiquitylation is involved in the regulation of a plethora of biological processes, it is not surprising that the Wnt pathway is also regulated by ubiquitylation[33]. We

will now discuss the various steps of the Wnt signal transduction cascade that are regulated by this post-translational modification.

In certain contexts, Wnt, together with its sorting receptor Wntless (Wls), can be secreted on exosomes[34–36]. Exosomes are secreted intraluminal vesicles of multivesicular bodies that are released from cells when MVBs fuse with the plasma membrane[37]. Sorting of membrane proteins to intraluminal vesicles of MVBs requires the action of the endosomal sorting complexes required for transport (ESCRT)[38]. ESCRT sorts ubiquitylated membrane proteins to intraluminal vesicles of MVBs[39]. This implies that ubiquitylation may be involved in exosomal release of Wnt. However, so far, no direct evidence for a role for ubiquitylation in Wnt secretion has been reported.

There are reports that indicate a role for ubiquitylation in the post-translational regulation of Wnt receptors. The Lrp6 coreceptor is palmitoylated in the secretory pathway. This palmitoylation is required for ER exit and cell surface localization of Lrp6. A quality control mechanism for correct palmitoylation is in place that involves ubiquitylation of Lrp6 at lysine 1403. A Lrp6 K1403R mutant, which cannot be ubiquitylated, has reduced signaling activity[40]. It is not known which ubiquitin ligase mediates Lrp6 ubiquitylation.

Intricate ubiquitin mediated regulation of the Wnt receptor Frizzled (Fz) has been discovered. Fz levels at the plasma membrane are regulated by the RING E3 ligases Znrf3 and Rnf43. Fz ubiquitylation by Rnf43/Znrf3 causes internalization of Fz and desensitizes cells for Wnt signals[41,42]. Chen *et al.* propose a further layer of regulation of Rnf43. Chen *et al.* solved the structure of R-spondin, a secreted Wnt agonist that promotes Wnt signaling by binding to the Lgr4 and Lgr5 receptors. They conclude that R-spondin also binds Rnf43. In this way, Lgr4/5, together with R-spondin, sequesters Rnf43 away from Fz. This results in reduced Fz ubiquitylation and increased plasma membrane localization of Fz. Chen *et al.* propose this is the mechanism by which R-spondin and Lgr4/5 promote Wnt signaling[43]. This model is not uncontroversial since Peng *et al.* and Wang *et al.* agree that R-spondin binds to the Leucine Rich Repeats of Lgr4/5, but they do not concur that R-spondin also binds Rnf43[44,45]. Further studies will have to determine which model for R-spondin and Lgr5 mediated Wnt signal amplification is correct.

The DUB UBPY reverses Fz ubiquitylation. UBPY regulates cellular responsiveness to Wnt signals by de-ubiquitylating Fz. In this way, UBPY prevents lysosomal sorting of Fz and it promotes recycling of Fz to the plasma membrane[46].

Another player in Wnt signaling that is regulated by ubiquitylation is Dishevelled (Dvl). Dvl is a cytoplasmic protein that acts downstream of the Wnt receptors in Wnt signal transduction. Dvl is the bifurcation point between β-catenin dependent and β-catenin independent Wnt signal transduction pathways[47]. This may be a reason why Dvl is under tight control by post-translational modifications. Dvl is phosphorylated upon Wnt signaling, which is important for Wnt signal transduction[47–49]. Furthermore, Dvl is under tight control of ubiquitin ligases and DUBs. Dvl ubiquitin ligases can be divided in two categories; those that regulate Dvl stability and those that regulate Dvl signaling through different mechanisms. Klhl12, Nedl1, ITCH, Neddl4 and the APC/C are E3 ubiquitin ligases that can regulate Dvl stability by targeting Dvl for protesomal degradation[50–54]. Dvl can also be destined for lysosomal degradation. When autophagy is induced through starvation or rapamycin treatment, VHL promotes Dvl ubiquitylation, Dvl aggregates, is sorted into autophagosomes and subsequently targeted for lysosomal degradation[55].

A different layer of ubiquitin-mediated regulation of Dvl signaling is through K63 linked ubiquitylation of the DIX domain of Dvl. The tumor suppressor Cyld was identified as a DUB for K63 linked polyubiquitin chains ligated to the DIX domain of Dvl. Cyld acts as a

negative regulator of Wnt signaling[56]. In chapter 3 we describe the role of the *eel-1*/Huwe1 ubiquitin ligase in Wnt signaling. We show that *eel-1*/Huwe1 is a negative regulator of Wnt signaling and we propose that Huwe1 promotes K63 linked ubiquitylation of the DIX domain of Dvl. Although Cyld and Huwe1 both act as negative regulators of Wnt signaling, these two proteins may together regulate K63 linked ubiquitylation of the DIX domain of Dvl. The dynamic regulation of DIX domain ubiquitylation appears to be important for proper Dvl signaling function.

Given the key role of Axin in the function of the destruction complex, it is not surprising that Axin levels are under tight control in the cell as well. Wnt signaling results in Axin degradation[19–21]. Kim *et al.* identified the ubiquitin ligase Smurf2 as a negative regulator of Axin protein levels[57]. Axin2 expression is upregulated as a result of Wnt pathway activation, resulting in negative regulation of Wnt pathway activity[58]. Recently, a molecular mechanism that post-transcriptionally regulates Axin levels was elucidated. Huang *et al.* discovered that Tankyrase inhibition results in Wnt pathway inhibition. They discovered that Tankyrase poly-ADP-ribosylates (PARPylates) Axin. Parpylated Axin is targeted for proteasomal degradation[59]. Zhang *et al.* subsequently showed that Rnf146 is the ubiquitin ligase that ubiquitylates PARPylated Axin[60].

The DUB USP34 positively regulates the stability of Axin. Knockdown of USP34 results in reduced Axin levels, but this can be rescued by Tankyrase inhibition, indicating that USP34 antagonises Tankyrase and Rnf146 mediated Axin degradation. Paradoxically, USP34 seems to act as a positive regulator of Wnt signaling. It is suggested that this is caused by a positive regulatory role of Axin in the nucleus[61].

APC, another component of the destruction complex, is also under ubiquitin-mediated regulation. There is a positive role for K63 linked ubiquitylation of APC in Wnt signaling. K63 linked ubiquitylation of APC promotes the Axin-APC interaction and destruction complex function. The HECT domain E3 ligase HECTD1 is the responsible ubiquitin ligase[62,63]. The DUB Trabid antagonizes K63 linked ubiquitylation of APC to inhibit destruction complex function[64].

The regulation of β-catenin protein levels is central to Wnt signal transduction. Phosphorylation of β-catenin by CK1 and GSK3 on a DSGXX(X)S motif forms a phosphodegron on β-catenin which is recognized by βTrCP, the substrate binding subunit of a SCF ubiquitin ligase complex. β-catenin is then ubiquitylated and earmarked for proteasomal degradation[65–67]. In specific contexts, β-catenin levels can be regulated by other ubiquitin ligases. Siah ubiquitylates and down-regulates β-catenin in response to genotoxic stress. β-catenin regulation by Siah is independent of phosphorylation, providing a mechanism of β-catenin regulation that bypasses the destruction complex[68,69]. Recently, c-Cbl was identified as a β-catenin ubiquitin ligase. During Wnt pathway activation, c-Cbl can translocate to the nucleus and ubiquitylate nuclear β-catenin. In this way, c-Cbl acts as a negative regulator of Wnt signaling[70]. Dao *et al.* report a positive role for β-catenin ubiquitylation in Wnt signaling. They show that the Fanconi anemia gene Fancl can modify β-catenin with K-11 linked ubiquitin chains. In this way, Fancl protects β-catenin from degradation and promotes TCF mediated transcription[71].

The most downstream node of the Wnt pathway that is regulated by ubiquitylation is the TCF/LEF transcription factor that mediates Wnt target gene transcription. NARF, an E3 ligase that interacts with Nemo-like kinase, ubiquitylates TCF/LEF transcription factors and targets them for proteasomal degradation. In this way, NARF negatively regulates the Wnt pathway[72]. The switch between TCF binding to Groucho/TLE to act as a transcriptional repressor in the Wnt off state, to TCF binding to β-catenin in the Wnt on state, is also regulated

by ubiquitylation. The E3 ligase XIAP is recruited to Groucho upon Wnt pathway activation. XIAP mono-ubiquitylates Groucho to decrease its affinity for TCF, resulting in a release of the transcriptional repression of Wnt target genes[73].

In summary, the β-catenin dependent signal transduction pathway is under tight control by ubiquitin ligases and DUBs. There is evidence for ubiquitin mediated regulation of Wnt signaling at almost every step of the Wnt pathway.

β-catenin dependent Wnt signaling in C. elegans: QL.d migration

Since the 1960's, *Caenorhabditis elegans* is a popular model organism among geneticists. Its ease of manipulation, transparency, short generation time and stereotyped cell lineage make *C. elegans* a convenient model organism to study developmental biology[74]. Furthermore, generation of transgenic animals is straight-forward, reverse genetics can easily be performed and the genome sequence of *C. elegans* is known. For these reasons, we used *C. elegans* as a model system in the research described in this thesis.

Wnt signaling regulates various processes during *C. elegans* development. Examples are: endoderm induction in the 4 cell stage embryo, regulation of neuronal polarity and the induction of vulva precursor cells[75]. In the research described in this thesis, QL.d migration is often used as a read-out of β-catenin dependent Wnt signaling. For this reason, I introduce this process in more detail (Fig. 2A).

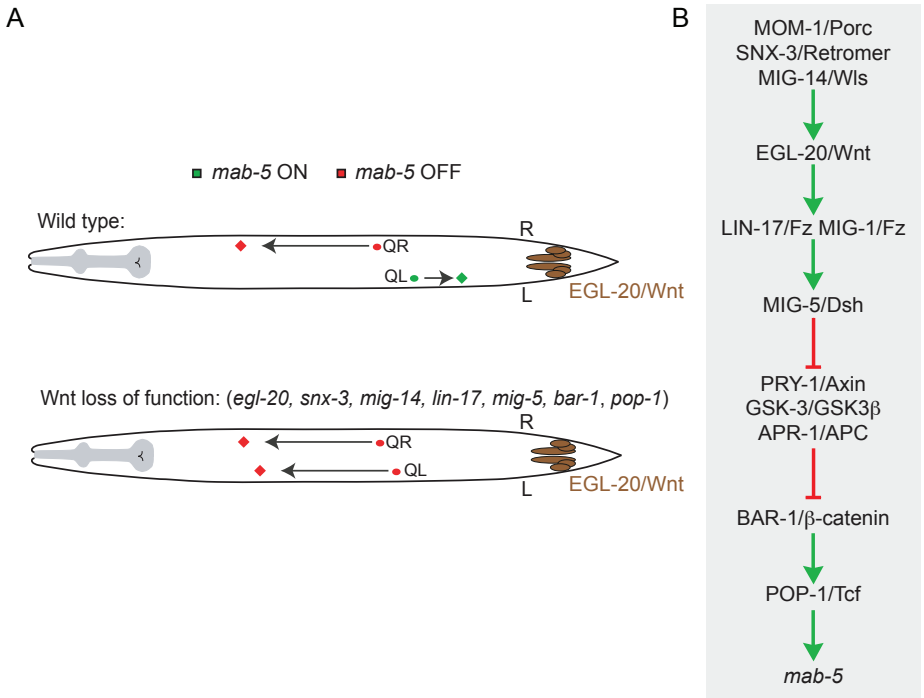


Figure 2: β-catenin dependent Wnt signaling in *C. elegans:* QL.d migration (A) Schematic view of Q neuroblast migration in *C. elegans*. (B) Schematic view of the β-catenin dependent Wnt signal transduction pathway that controls *mab-5* expression in QL.

At the time of hatching, the QL neuroblast of *C. elegans* is located at the left lateral side of the larva between seam cells V4 and V5. Under the influence of an EGL-20/Wnt signal, *mab-5* is expressed in QL. MAB-5 expression is necessary and sufficient to induce posterior migration of the QL daughter cells[76–79]. Cells in the tail of the worm produce the EGL-20/Wnt signal[80,81]. Dedicated Wnt secretion machinery is in place in the EGL-20 producing cells to establish a long range EGL-20 gradient, from the tail to the mid-body, which allows Wnt pathway activation in QL[80,82–86]. The Wnt signal transduction machinery that activates *mab-5* expression in response to *egl-20* signaling involves the Frizzled receptors *lin-17* and *mig-1*, *mig-5/*Dvl and a destruction complex that consists of *pry-1/*Axin, *apr-1/*APC, *gsk-3/*GSK3. This destruction complex regulates *bar-1/*β-catenin, which activates *mab-5* expression through the interaction with the TCF/LEF homologue *pop-1*[87] (Fig. 2B). Intricate feedback regulation between *mab-5* and the Frizzled receptors is in place in QL to make sure that *mab-5* is expressed in sufficient levels upon *egl-20* signaling (Ni *et al.*, in press). In this way, *mab-5* expression in QL and posterior migration of the QL daughter cells is robustly achieved.

A role for β-catenin dependent Wnt signaling in mammals: intestinal stem cell maintenance

Stem cells are characterized by their ability to self-renew and give rise to daughter cells that can differentiate. Totipotent stem cells, such as embryonic stem cells, can give rise to tissue of all three germ layers. Somatic stem cells have a restricted differentiation potential. For example, the stem cells of the skin or intestine can only form skin or intestinal cells[88]. Wnt signaling has important functions during stem cell maintenance in the intestine, I will discuss this in the following section of this chapter.

The intestinal epithelium absorbs nutrients from food and provides a protective barrier between the intestine and the lumen of the gastrointestinal tract[89]. The intestinal epithelium is organized along the crypt-villus axis. Villi are protrusions into the intestinal lumen. Between the villi, invaginations into the intestinal lining are located, these are the crypts of Lieberkühn. Villi are covered with enterocytes, the cells that absorb nutrients. In addition to enterocytes, there are mucus producing goblet cells, hormone producing entero-endocrine cells and Tuft cells distributed among the enterocytes. Paneth cells and crypt base columnar cells (CBCs) are located at the base of the crypts. Paneth cells are differentiated cells that have an antibacterial function[89]. The CBCs are the stem cells of the intestine[90]. The rest of the crypt consists of highly proliferating cells in the transient amplifying compartment. These cells give rise to the differentiated cells in the villus, but these cells do not posses self renewing capacity[89].

The proliferating cells in the crypt are essential to maintain intestinal homeostasis. The mammalian intestine is a fast renewing tissue; every 5-7 days, the complete lining of the intestine is replaced[89]. The proliferative capacity of the intestine depends on the proliferating cells in the transient amplifying compartment. The transient amplifying compartment is fueled by intestinal stem cells. The stem cells are actively cycling, with a cell cycle of approximately 24 hours. Intestinal stem cells are characterized by the expression of stem cell markers such as Lgr5, Olfm4 and Ascl2[91].

The stem cells of the intestine require signals from the surrounding niche to maintain their stem cell characteristics. The EGF and Notch signaling pathways have important roles. The Wnt signaling pathway is essential to maintain proliferating stem cells in the intestine[89]. Many genetic mouse models have underlined the requirement for Wnt signaling for intestinal stem cell maintenance. For example, conditional inactivation of TCF4, the main TCF transcription

family member in the intestine, results in a loss of proliferating crypts[92]. Loss of β-catenin in the intestinal epithelium, or overexpression of the Wnt antagonist Dickkopf1 causes a similar phenotype[93,94]. Conversely, overexpression of the Wnt agonist R-spondin1, or inactivation of the Wnt antagonists Rnf43 and Zrnf3, causes overproliferation in the intestine[42,95].

Wnt3 is the critical Wnt in intestinal stem cell maintenance. Wnt3 is expressed at the bottom of the crypt - where the stem cells are located - and Wnt3 is essential to maintain cultured intestinal organoids[96,97]. Exogenous Wnt3a can rescue loss of Wnt3 from organoids. Wnt3a can also rescue the effect of a Porcupine inhibitor, which inhibits the secretion of all Wnts and causes organoid death. The main sources of Wnt are the Paneth cells and cells in the mesenchyme of the intestine[97,98].

The importance of Wnt signaling in intestinal stem cell homeostasis is further illustrated by the fact that Wnt pathway overactivation, through mutations in APC or stabilizing point mutations in β-catenin, causes constitutive Wnt pathway activation and adenoma formation. This is the first step towards cancer formation. It is relevant that the Wnt pathway is activated in the intestinal stem cells, since loss of APC specifically in the Lgr5 expressing cells causes adenoma development, while loss of APC in the transiently amplifying cells does not[99]. The role of the Wnt pathway in adenoma formation is conserved in humans. People that carry congenital mutations in the APC gene develop many intestinal polyps and are prone to develop intestinal cancer, this syndrome is called familial adenomatous polyposis. Also in the majority of sporadic colon cancer cases, mutations in Wnt pathway components can be identified[100].

The molecular mechanisms of Wnt production and secretion

As mentioned above, Wnt signaling is of key importance for many biological processes. The molecular mechanisms of Wnt signal transduction have already been discussed, we will now focus on the post-translational modifications of Wnt and the mechanisms of Wnt secretion and production.

Acylation

Wnt proteins are cysteine rich glycoproteins with a molecular mass of approximately 40 kDa. The *C. elegans* genome contains 5 Wnt genes, mice and humans have 19 Wnt genes[101,102]. Wnt proteins, with the exception of *Drosophila* WntD, are lipid modified on a conserved serine residue (S209 in mouse Wnt3a)[102–105]. Until recently, it was believed that Wnt was also lipid modified on a conserved cysteine residue (C77 in mouse Wnt3a)[106]. However, with the elucidation of the structure of XWnt8 in complex with the cysteine-rich domain of mFz8, this assumption has been refuted. In XWnt8, C55 (the equivalent of C77 in Wnt3a) is engaged in a di-sulfide bridge and thus cannot be lipid modified. This disulfide bond is predicted to be conserved among Wnt proteins, suggesting that Wnt proteins are only lipid modified on the conserved serine residue[102,103].

It has been observed that the C77A mutant of Wnt3a loses its hydrophobic character, which led to the conclusion that this residue is acylated[106]. There is an alternative explanation for the fact that the C77A mutant Wnt becomes less hydrophobic: the free cysteine in the C77A mutant proteins is available to form inter-molecular disulfide bridges. As a result, WntC77A proteins can oligomerize and aggregate to form large complexes. These aggregates are water soluble, but have reduced signaling activity, hence explaining the decreased hydrophobicity and signaling function[107]. The Wnt antagonist Tiki exploits a similar mechanism to inhibit

Wnt signaling. Tiki is a membrane protein that cleaves the N-terminus of Wnt, resulting in the formation of Wnt aggregates that lose signaling activity. In this way, Tiki inhibits Wnt receptor activation[108].

Wnt is lipid modified by Porcupine, a member of the membrane-bound-O-acyltransferase (MBOAT) family. Depletion of Porcupine causes a complete loss of Wnt signaling[104,109,110]. The lipid modification on S209 likely serves two purposes. To begin with, the lipid inserts into a pocket in the cysteine rich domain (CRD) of the Frizzled receptor. The lipid-binding domain is conserved among Frizzled proteins, it is therefore highly likely that the lipid-Frizzled interaction is of functional importance[103]. Indeed, a S239A mutation in the *Drosophila* Wnt protein Wingless (Wg) results in a much-reduced signaling capability compared to wild-type Wg[111]. Furthermore, the serine acylation is important for Wnt secretion. In cells depleted of Porcupine, Wnt fails to interact with Wls - a protein that is essential for Wnt secretion - and Wnt is not secreted[112].

Glycosylation

Wnt proteins undergo N-linked glycosylation. The functional relevance of this post-translational modification is not completely clear. Glycosylation of *Drosophila* Wg seems to be dispensable for Wg secretion and signaling *in vivo*[111]. In contrast, researchers from the Kikuchi lab found that glycosylation is required for Wnt3a and Wnt5a secretion[113,114]. The role of glycosylation in Wnt secretion may be more subtle, since Yamamoto *et al.* recently reported that differential glycosylation of Wnt3a and Wnt11 determines basolateral versus apical secretion of these Wnt proteins in a polarized cell system[115]. In their studies, Yamamoto *et al.* relied on Wnt overexpression in tissue culture cells. These conditions may not adequately reflect the *in vivo* pathway. Therefore, it will be interesting to further characterize the role of specific N-linked glycans on Wnt proteins in an physiological *in vivo* system.

The route of Wnt through the secretory pathway

We will discuss the mechanism of Wnt secretion in the following section (Fig. 4). Wnt proteins contain a signal sequence and are co-translationally translocated into the endoplasmic reticulum (ER) of Wnt producing cells. In the ER, Wnt is lipid modified by Porcupine. Wnt follows the secretory pathway from the ER to the Golgi[116]. Export of Wnt from the ER requires the action of p24 proteins, which sort cargo proteins into COPII vesicles for exit from the ER[117,118]. In the Golgi complex, Wnt associates with Wls. This interaction is crucial for export of Wnt to the cell surface; in Wls depleted cells, Wnt is trapped intracellularly[119–121]. The interaction of Wnt with Wls requires the acylation of Wnt by Porcupine[112]. Only WntD, which is not acylated, does not bind Wls and is secreted in a Wls independent way[105].

Wls is a rate-limiting factor for Wnt secretion. In order to maintain sufficient Wnt secretion, Wls needs to travel back to the Golgi complex. This transport route involves internalization of Wls from the plasma membrane and retrograde transport of Wls from endosomes to the TGN[83,84,122–124] (Fig. 4). We will discuss this trafficking route in more detail later in this chapter.

Spreading of Wnt in the tissue

Wnt proteins are highly hydrophobic due to their lipid modification. Paradoxically, Wnt can spread through the aqueous extracellular environment to establish long-range concentration gradients. How can this paradox be explained? Wnt may spread through the tissue while attached to membranes, or extracellular matrix components. Alternatively, various mechanisms have been described that may shield the hydrophobic lipid moieties of

Wnt from the aqueous environment[125]. One example is the Swim protein, a lipocalin family member that facilitates the spreading of Wg in the wing disc of *Drosophila*[126]. Furthermore, in *Drosophila*, lipoprotein particles were shown to contain Wnt and facilitate Wnt spreading[127]. Also in human cells, Wnt can be secreted on lipoprotein particles[128]. Another mechanism of Wnt spreading has recently gained attention. Several labs reported that Wnt, together with Wls, can be secreted on exosomes[34–36]. Exosomes are small vesicles that are released from cells when multi-vesicular bodies (MVBs) fuse with the plasma membrane and release their intraluminal vesicles[37]. These studies agree on the fact that Wnt can be secreted on exosomes from tissue culture cells, but contradict each other when it comes to the *in vivo* relevance of this secretion mechanism. One lab uses the neuromuscular junction of the *Drosophila* larva to study Wg signaling *in vivo*. In this context, Wnt and Wls are secreted from Wnt producing cells on exosomes[36]. Using the *Drosophila* wing disc as a model, Gross *et al.* and Beckett *et al.* reach conflicting conclusions. Gross *et al.* express the exosome marker CD63::GFP in Wg producing cells, they find punctae where Wg and Wls colocalise with CD63 outside the Wg expression domain. In addition, Gross *et al.* identify the SNARE protein Ykt6 as a protein that is enriched in exosomes. Knockdown of Ykt6 results in reduced Wnt loading on exosomes obtained form tissue culture cells. Moreover, knockdown of Ykt6 in Wg expressing cells results in loss of Wg secretion, loss of expression of the Wg target gene Senseless and wing margin defects. Based on these results, Gross *et al.* conclude that Wg secretion on exosomes is important for Wg signaling in the wing disc. In contrast, Beckett *et al.* conclude that exosomes are not required for the formation of the Wg gradient in the wing disc. They find that Rab11 is important for

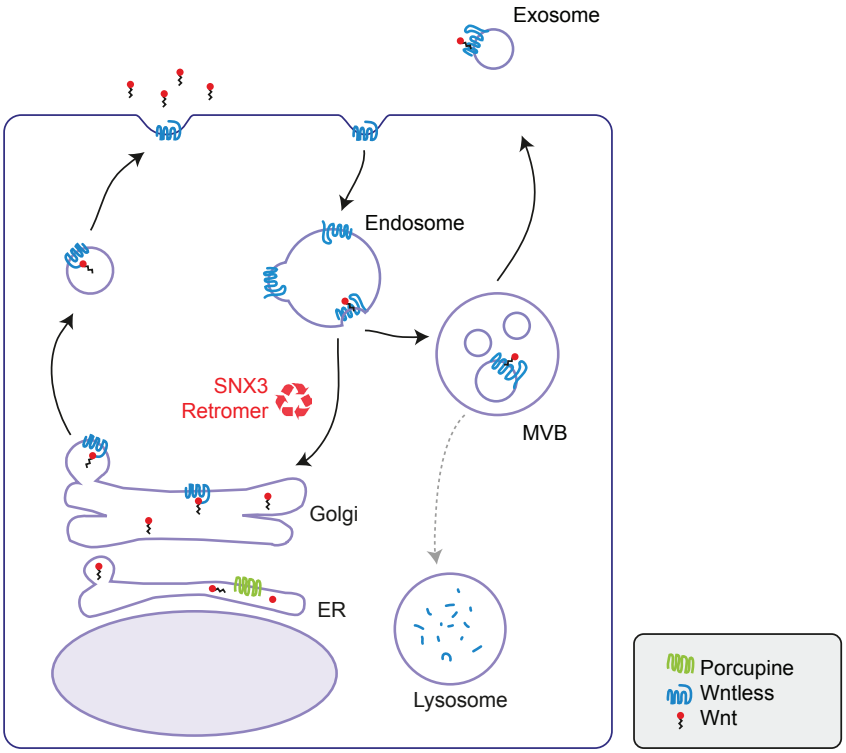


Figure 4: Schematic view of the Wnt secretion pathway See text for discussion.

loading of Wnt on exosomes obtained from S2 cells, but knockdown of Rab11 does not affect Wg signaling in the wing disc. Neither do Beckett *et al.* observe an extracellular Wls gradient in the wing disc epithelium.

Gross *et al.* rely on overexpressed CD63::GFP to mark exosomes. As a consequence, the observed punctae may not represent endogenous exosomes, but may be an artifact of CD63 overexpression. Also, it seems that only a very limited amount of Wg and Wls is localized on exosomes in the wing disc. At the same time, knockdown of Ykt6 causes a dramatic reduction in Wg signaling. No explanation is provided for this discrepancy. On the other hand, the fact that Beckett *et al.* do not observe a Wls gradient in the Wing disc may not be a very strong argument. Wls is consitutively expressed in the wing disc and a subtle gradient may be difficult to detect. Also, the cellular components that facilitate exosome secretion may differ in various tissues and contexts. This may explain why Rab11 is essential for Wnt secretion on exosomes from S2 cells, but not for Wg secretion in the wing disc. Concluding, there is ample evidence that Wg and Wnt can be secreted on exosomes from tissue culture cells. The *in vivo* relevance for this secretion mechanism in the wing disc is still under debate.

Shaping the Wnt gradient

Once transported to the cell surface, Wnt forms a concentration gradient, either attached to the plasma membrane of cells, or solubilized in one of the previously mentioned ways. We will now discuss the mechanisms that establish and shape the Wnt gradient. We will mainly discuss the Wg gradient in the *Drosophila* wing disc because this is a suitable model to study morphogen gradients[129].

The wings of *Drosophila* develop from wing imaginal discs, sheets of epithelium that will give rise to the wings after metamorphosis[88]. During larval development, the imaginal discs are patterned in order to form the structures of the adult organ. Patterning of the wing disc involves several morphogens. Decapentaplegic (Dpp) is expressed in a stripe that is oriented along the dorso-ventral axis of the embryo. The Dpp signal patterns the wing disc in the anterior-posterior direction. Hedgehog is expressed in the posterior region of the wing disc and is required for specific patterning along the A-P axis. Patterning along the dorsoventral axis is mediated by Wg which is expressed in a narrow stripe of cells that is directed along the A-P axis of the wing disc. The Wg expressing cells demarcate the D/V boundary and form the edge of the future wing[129].

Wg induces the expression of several target genes in a concentration dependent manner (Wg is a morphogen). At the highest concentration of Wg, expression of Senseless is induced. Senseless is expressed in a narrow stripe along the middle of the wing disc, close to the region of Wg expression. The low threshold gene Distalless is expressed in a wider region around the Wg expression domain[130].

In the wing disc, two discrete Wg gradients are observed. There is a short-range Wg gradient at the apical side of the epithelium. A long range gradient is observed at the basal side[127,130]. Distinct mechanisms form the short and long range gradients. Both gradients require Wls to transport Wg to the plasma membrane. Apically secreted Wg can spread a short distance, possibly while associated with cell membranes. Specific molecules are required to form the long range concentration gradient at the basal side of the epithelium. Reggie1/Flottillin is specifically required for the spreading of the long range form of Wg[131]. Reggie1 overexpression causes a decrease in expression of short range Wg target genes, while the expression of long range targets is increased. This suggests more Wg is secreted in the long range form at the expense of the short range form. Indeed, erosion of the apical, short range Wg gradient was observed, while the basolateral gradient of Wg was increased upon Reggie1

overexpression. In contrast, when Reggie1 function was lost, long range Wg spreading was reduced[131].

Another class of molecules that is important for the spread of Wg in *Drosophila* are the glypicans Dally and Dally-like (Dlp)[132–134]. Glypicans are GPI-linked heparansulphateproteoglycans (HSPG). HSPG are components of the extracellular matrix and are characterized by sulfated carbohydrate modifications. The *Drosophila* HSPG Dally and Dlp can bind Wg and especially Dlp has an important role in Wg spreading. Dlp overexpression causes an increase in long range Wg signaling (as determined by Distalles expression) while short range signaling is reduced. Dlp loss of function causes a reduction of the Wg gradient[135]. Results by Gallet *et al.* implicate transcytosis of Dlp as an important step for the formation of a long range Wg gradient. They show that Wg is secreted apically. Furthermore, they find that the GPI anchor of Dlp is required for Dlp trancytosis and that this is required for the formation of the basal Wg gradient[136]. Wg trancytosis by Dlp is a possible mechanism for the formation of the basal, long range Wg gradient in the wing disc.

Several factors can influence the shape and range of a morphogen gradient. The stability of the morphogen is an important factor. A very stable morphogen will spread further compared to a morphogen with a short half-life time, provided all other factors are the same. One way in which the stability of a morphogen can be influenced is by intracellular degradation. Indeed, in the *Drosophila* embryo, the spread of Wg is limited through internalization and subsequent HRS dependent lysosomal degradation[137]. Internalization pathways are also important to shape the Wg gradient in the *Drosophila* wing disc. The Wg receptors Fz, Dfz2 and Arrow can internalize Wg in the wing disc, limiting the Wg gradient[132,138–140].

Wnt antagonists

Another mechanism to shape the Wnt signaling gradient is mediated by Wnt antagonists. Secreted Frizzled related protein (Sfrp), Wnt inhibitory factor (Wif) and Cerberus are secreted Wnt antagonists that function by sequestering Wnt, and preventing Wnt induced receptor activation[141]. The aforementioned Tiki protein is a membrane bound protease that cleaves Wnt to render it inactive[108]. Dickkopf (Dkk), a different type of secreted Wnt antagonists, inhibits Wnt signaling by binding to the Wnt receptor Lrp6. Dkk induces Lrp6 internalization via clathrin mediated endocytosis[142]. In this way, Lrp6 is not available at the plasma membrane to bind Wnt and initiate Wnt signal transduction.

Taken together, extracellular matrix components, internalization pathways, and the action of Wnt antagonists shape the Wnt gradient in tissues.

The molecular mechanisms of plasma membrane to TGN transport of Wntless

In the preceding section, we mentioned that Wls undergoes retrograde traffic from the plasma membrane to the trans-Golgi network (TGN). Wls endocytosis is clathrin and AP-2 mediated[83,84,122–124]. An endocytosis motif in the third intracellular loop of Wls is essential for Wls internalization and for Wg secretion and Wg signaling in *Drosophila*[143]. In chapter 6 of this thesis, we describe that CK2, a serine-threonine protein kinase, is involved in Wnt secretion. We propose that CK2 plays a role in Wls internalization.

After internalization, Wls is transported to early endosomes and is then retrieved from the endosomal system and transported to the TGN. Retrograde trafficking from endosomes to the TGN requires the following steps: (I) cargo sorting in the endosome, (II) deformation of the endosomal membrane to form a transport carrier, (III) carrier fission from the endosome,

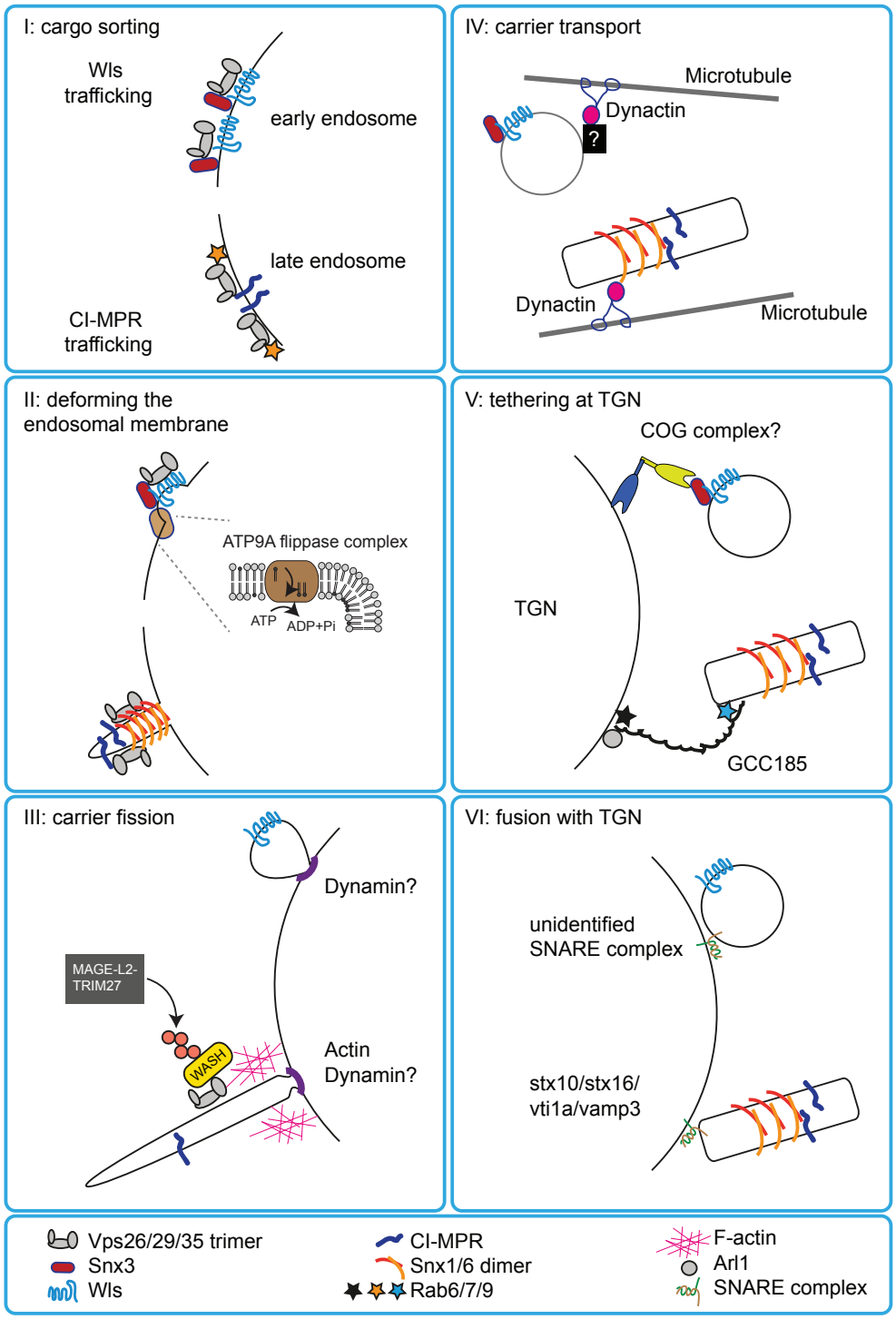


Figure 5: Schematic view of the retrograde trafficking pathways of Wls and the CI-MPR See text for discussion.

(IV) carrier transport to the TGN, (V) tethering at the TGN, (VI) fusion of the carrier with the TGN membrane[144,145]. In this section, we will discuss these retrograde trafficking steps for our favorite retrograde cargo Wls, and we draw parallels with retrograde trafficking of the cation independent mannose-6-phosphate receptor (CI-MPR), another protein that undergoes a well-studied itinerary from endosomes to the TGN (Fig. 5).

The retromer complex is essential for retrograde cargo sorting and cargo transport from endosomes to the TGN. Retromer cargos include the CI-MPR, Wls, Sortilin and the polarity determinant Crumbs[146]. The cargo-selective sub-complex (CSC) of retromer is composed of the subunits Vps35, Vps26 and Vps29[147]. This protein complex mediates cargo sorting and the recruitment of membrane deforming factors. Rab7 recruits the CSC to the endosomal membrane and knockdown of Rab7 results in missorting of the CI-MPR[148,149]. Rab7 seems not to be involved in Wls sorting as knockdown of *rab-7* has no effect on Wnt signaling in *C. elegans*. In contrast, *rab-7* knockdown rescues retrograde Wls transport in a retromer reduction-of-function mutant (Lorenowicz *et al.* in press). This raises the question, how is the CSC recruited to the endosomal membrane during Wls sorting? The Snx-PX domain containing sorting nexin Snx3 is required for this process[86]. The PX domain of Snx3 has high affinity for the early endosomal enriched PI3P phosphoinsositide and Snx3 forms a complex with the CSC. Importantly, Snx3 is required for Wls transport from endosomes to the TGN[86,150]. The importance of PI3P for Wls trafficking is further illustrated by the findings of Silhankova *et al.* They show that a PI3P phosphatase, which consists of MTM-6 and MTM-9, is required in the Wnt producing cells of *C. elegans* and *Drosophila* for retrograde trafficking of Wls and Wnt secretion. These results indicate that PI3P levels need to be tightly controlled for correct recruitment of SNX-3 to endosomal membranes[85].

The CSC dependent retrograde trafficking routes of Wls and the CI-MPR do not only differ in their requirement for Snx3 or Rab7, these retromer routes are also spatially separated. Wls is transported from early endosomal membranes while the CI-MPR needs to traffic through acidic late endosomes. The low pH is essential to release the mannose-6-phosphate tagged cargos from the CI-MPR for transport to lysosomes[150].

The CSC coordinates retrograde cargo sorting and the recruitment of membrane deforming factors. In the case of the CI-MPR, and many other retromer cargos, the membrane deforming factors are members of the Snx-BAR sorting nexin family (Snx1 or Snx2 together with Snx5 or Snx6)[151]. The BAR domain of these proteins can dimerize and induce and stabilize membrane curvature[152]. The combination of CSC and Snx-BAR sorting nexins results in the formation of higher order structures that induce membrane tubule formation. The CSC sorts cargo into these tubules that emanate from the endosomal membrane. These tubules form the tubular carriers that are used to transport the CI-MPR (and other retromer cargo proteins) to the TGN. Wls transport does not require the Snx-BAR sorting nexins, but rather the Snx-PX sorting nexin Snx3[86]. Snx3 does not have a membrane tubule forming BAR domain, and the carriers that transport Wls are not tubular shaped, but vesicular. Since Snx3 does not contain membrane deforming protein domains, a different mechanism is in place to deform the endosomal membrane to form Wls carriers. We describe this mechanism in chapter 5 of this thesis. Membrane curvature generation by the Snx3-retromer is induced by a phospholipid flippase, a protein that uses ATP to transport phospholipids from the luminal to the cytoplasmic leaflet of the membrane. As a result, the cytoplasmic leaflet of the membrane increases relative to the luminal leaflet. In this way, flippases can induce membrane curvature[153]. We show that the flippase ATP9A forms a complex with associated factors Mon2 and Dopey2. This complex localizes to 'hotspots' on endosomal membranes together with Snx3, CSC components and Wls. Importantly, this complex is required for Wls

trafficking and Wnt secretion *in vivo*.

After deformation of the endosomal membrane and sorting of the retrograde cargo into tubules (as is the case for the CI-MPR) or buds (as for Wls), these nascent carries need to separate from the endosome. The mechanisms of Wls carrier abscission are thus far unknown. In the case of CI-MPR sorting, an important role is reserved for dynamic actin[154,155]. The WASH complex is an endosomal localized protein complex that activates Arp2/3 mediated actin polymerization. Inhibition of the WASH complex results in tubulation of endosomes, indicating a defect in tubule abscission, and defects in CI-MPR sorting. Recently, a ubiquitin ligase, MAGE-L2-TRIM27, was identified as a regulator of the WASH complex and CI-MPR sorting[156]. MAGE-L2-TRIM27 interacts with CSC components and modifies WASH with K63 linked ubiquitin chains. As a result, Arp2/3 is recruited to WASH and actin polymerization is promoted, which is required for retrograde transport of the CI-MPR. At this time, it is not clear if there is a role for F-actin, or the WASH complex, in Wls transport.

The final step in retrograde sorting from the endosome is fission of the retrograde carrier from the endosome. The molecular mechanisms of this process are still poorly understood. Abscission may be achieved through actin dynamics. Alternatively, dynamin or dynamin-like proteins may be involved in carrier abscission. The GTPase dynamin is well-known for its role in membrane fission in the process of endocytosis. Dynamin polymerizes at the neck of clathrin coated pits at the plasma membrane, just prior to vesicle abscission. Dynamin is also involved in intracellular membrane fission events[157,158]. Possibly, dynamin or dynamin-like proteins mediate Wls carrier fission from the endosome. It will be challenging to address this question with the current genetic techniques, because dynamin is also required for Wls endosytosis.

Retrograde transport of the CI-MPR requires the interaction of Snx6 with the $p150^{glued}$ subunit of the dynactin motor complex[159,160]. Dynactin is required for the transport of cargo along microtubules and in this way mediates long range transport of CI-MPR carriers from endosomes to the TGN. Interestingly, Snx6 needs to substitute binding to $p150^{glued}$ for binding to PI4P at the TGN for proper delivery of the CI-MPR at the TGN[161]. EGL-20/Wnt signaling in *C. elegans* also requires dynactin/*dnc-1* as *dnc-1* mutants display Wnt signaling defects, suggesting that dynactin is also required for the transport of Wls carriers. However, Snx3 does not interact with $p150^{glued}$, suggesting that other proteins mediate the interaction between the Wls carriers and dynactin[159].

After long-range transport from endosomes to the TGN along microtubules, retrograde carriers need to come into close proximity to TGN membranes. Tethering factors 'catch' incoming carriers and bring them close to the TGN membrane[162]. An important tethering factor for retrograde CI-MPR transport is the Rab9 effector GCC185[163]. GCC185 binds Rab9 which is located on retrograde carriers. GCC185 is attached to the TGN by the action of two Rab6 and two Arl1 molecules[164,165]. The CI-MPR requires both Rab9 and GCC185 for retrograde transport to the TGN.

The conserved oligomeric Golgi (COG) complex may be the tethering factor for Wls carriers. In chapter 5, we describe an interaction between the COG complex and Snx3. The COG complex is involved in intra Golgi retrograde transport and retrograde transport from endosomes to the TGN[166]. Potentially, the COG complex tethers Wls carriers at the Golgi during retrograde transport.

The last step of retrograde trafficking pathways is fusion of the carrier membrane with the TGN membrane. Membrane fusion requires close proximity of the two donor membranes so that the two lipid bilayers can fuse. Water needs to be displaced between the membranes, a reaction that is energetically unfavorable. SNARE proteins on vesicles (v-SNARES) can interact

with SNARES on the target membranes (t-SNARES) to provide the necessary energy to induce membrane fusion[167]. The SNARE proteins that are important for Wls trafficking have not been identified yet. Fusion of CI-MPR carriers requires a SNARE complex that includes Syntaxin10, Syntaxin16, Vti1a and Vamp3[168].

In summary, many molecular details of the retrograde trafficking route of the CI-MPR have been identified. The retrograde trafficking route of Wls shares some of these components, such as the CSC, but differs in many ways. The Snx-BAR and Snx3 sorting nexins form mutually exclusive complexes with the CSC components[86]. Furthermore, Wls traffics from early endosomal membranes, it requires Snx3 and the endosomal membrane is deformed through the generation of lipid asymmetry. The CI-MPR traffics from late endosomal membranes and it requires the Snx-BAR sorting nexins to generate membrane curvature[150].

The molecular components of many steps in the retrograde trafficking route of Wls remain to be identified. Important questions are: how is the induction of membrane curvature by the ATP9A flippase complex regulated? What is the role for dynamic actin in retrograde trafficking of Wls? How are the Wls carriers abscised from the endosome? How are the Wls carriers linked to dynactin? What is the exact role of the COG complex in Wls carrier tethering at the TGN? How does the Wls carrier fuse with the TGN membrane? These questions will be the subject of future investigations.

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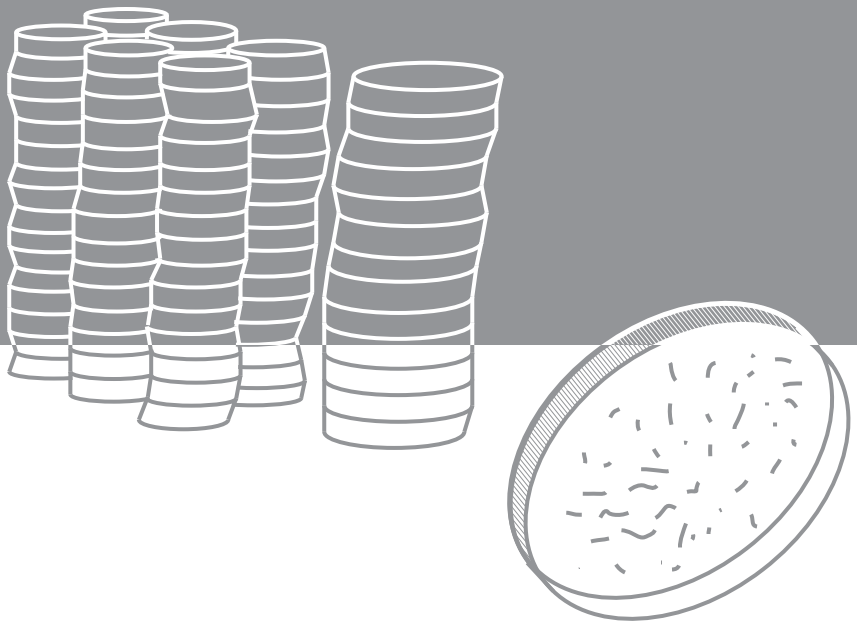

CHAPTER 2

A reverse genetics screen of ubiquitin ligases and DUBs to identify novel regulators of Wnt signaling

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Abstract

Wnt signaling is an evolutionary conserved signaling pathway that controls many biological processes during development and adult tissue homeostasis. Deregulation of Wnt signaling can cause disease, most notably cancer.

Wnt pathway components are tightly controlled by post-translational modifications such as ubiquitylation. We aimed to further investigate ubiquitin mediated regulation of Wnt signaling. To address this point, we screened by RNAi the HECT domain E3 ligases, RING domain proteins, E2 enzymes and de-ubiquitylating enzymes of *C. elegans*. As an assay, we used the EGL-20/Wnt dependent posterior migration of the QL descendants. To be able to identify both positive and negative regulators of Wnt signaling, we performed the screen in a mutant background that reduces Wnt secretion and produces a partially penetrant defect in Wnt signaling.

We identified the genes *eel-1, sel-10, vps-41, apc-11, dcn-1* and *d2089.2* in our screen. We determined that the HECT domain E3 ligase *eel-1* acts as a negative regulator of Wnt signaling, upstream of β-catenin. We hypothesize about potential roles for the other identified genes in Wnt signaling, but further research is required to substantiate those speculations.

Introduction

The Wnt signal transduction cascade controls many developmental and homeostatic processes. Deregulation of Wnt signaling is often associated with disease[1,2].

In the β-catenin dependent Wnt signal transduction pathway, the central player β-catenin is constitutively degraded by a destruction complex that contains the kinases GSK3β and CK1α, and the scaffold proteins Axin and APC (reviewed by MacDonald *et al.*[3]). In the absence of Wnt signaling, the destruction complex phosphorylates β-catenin. The phosphorylated residues of β-catenin form a phosphodegron that is recognized by the F-box protein βTrCP. βTrCP is the substrate specific component of the Skp1-cullin-Fbox (SCF) ubiquitin ligase that ubiquitylates phosphorylated β-catenin and targets it for proteasomal degradation[4,5]. As a consequence, cytoplasmic β-catenin levels are very low in the absence of Wnt signals.

When Wnt binds to its receptor Frizzled and co-receptor Lrp6, a signaling cascade is initiated that involves the phosphorylation of the cytoplasmic protein Dishevelled (Dvl), phosphorylation of the cytoplasmic tail of Lrp6 and the formation of signalosomes[6–8]. This results in the inhibition of the destruction complex. Consequently, β-catenin is no longer phosphorylated or ubiquitylated. β-catenin levels in the cytosol rise and β-catenin can translocate to the nucleus to act as a transcriptional co-activator to induce Wnt target gene transcription[2,3,9].

The Wnt pathway is tightly regulated by post-translational modifications such as phosphorylation, poly-ADP-ribosylation (PARPylation) and ubiquitylation[10–12]. Ubiquitylation involves the ligation of ubiquitin to target proteins by the action of E1, E2 and E3 enzymes (reviewed by Hershko and Ciechanover[13]). E3 enzymes are ubiquitin ligases that confer substrate specificity to the ubiquitylation reaction. The best-characterized types of E3 ligases are the HECT domain containing ubiquitin ligases and SCF ubiquitin ligases[14,15].

Ubiquitin modification of a substrate can elicit pleotropic effects; the substrate will be degraded by the proteasome if K48 linked ubiquitin chains are ligated to the substrate[13]. The addition of a single ubiquitin protein can change protein-protein interactions that may

influence the subcellular localization or signaling function of a substrate[16]. Furthermore, K63 linked poly-ubiquitin chains can target proteins for ESCRT mediated sorting to the intraluminal vesicles of multivesicular bodies, or change specific characteristics of the substrate, such as activity, binding partners or signaling function[17]. Ubiquitylation can be reversed by the action of de-ubiquitylating enzymes (DUBs)[18].

Ubiquitylation or modification by ubiquitin-like proteins is involved in the regulation of various steps of the Wnt signal transduction cascade[12]: plasma membrane localization of the Frizzled receptor is regulated by the concerted activity of ubiquitin ligases and DUB enzymes[19–21]. Furthermore, Dvl protein levels are under tight control by the ubiquitin-proteasome system[22] and Dvl signaling function is promoted by K63 linked ubiquitylation of the DIX domain of Dvl[23]. In addition, Axin and APC, components of the β-catenin destruction complex, are regulated by ubiquitylation. Also nuclear factors, such as TCF/LEF transcription factors, are under control of ubiquitin ligases[12].

We hypothesized that there are more roles for ubiquitin ligases, or DUBs in the Wnt pathway. To identify these players, we performed an RNAi screen in *Caenorhabditis elegans*. We identified several genes that are potential novel regulators of Wnt signaling.

Results and Discussion

Screen for novel regulators of Wnt signaling

We assayed genes that function in ubiquitin regulation for a role in Wnt signaling. We used the final position of the QL neuroblast descendants of *C. elegans* as a read-out of β-catenin dependent Wnt signaling. During the first stage of larval development, the Q neuroblast on the left side of the animal (QL) is induced by an EGL-20/Wnt signal, through a BAR-1/β-catenin dependent Wnt signal transduction cascade, to express the homeobox transcription factor *mab-5*. Activation of *mab-5* expression drives migration of the descendants of QL (QL.d) towards the posterior[24]. The QR neuroblast, which is located at an equivalent position on the right side of the animal, does not activate *bar-1*/β-catenin dependent Wnt signaling and its descendants migrate in the opposite, anterior direction. Interfering with EGL-20/Wnt signaling inhibits *mab-5* expression in QL and as a consequence, the QL descendants migrate towards the anterior instead of the posterior. Overactivation of EGL-20/Wnt signaling results in the opposite phenotype, with the QR descendants migrating towards the posterior instead of the anterior (Fig. 1A)[25,26]. To sensitize the screen and to identify both positive and negative regulators of EGL-20/Wnt signaling, we performed the screen in a *vps-29* mutant background. In *vps-29* mutants, EGL-20/Wnt secretion is reduced, resulting in a partially penetrant defect in the anterior migration of the QL.d. Interfering with negative regulators of the Wnt signaling pathway will suppress this phenotype, while knock down of positive components of the pathway will enhance the phenotype[27].

We screened a library of RNAi clones that included the HECT domain ubiquitin ligases, SCF ubiquitin ligases with clear human homologues, RING domain proteins, E2 enzymes, and DUBs of *C. elegans* (Table S1). RNAi clones that suppressed the QL.d migration phenotype of *vps-29* mutants from 30% in control RNAi treated animals to below 11%, or enhanced the phenotype to above 80%, were retested two more times. Hits that reproducibly enhanced or suppressed the *vps-29* QL.d phenotype were *eel-1*, *vps-41*, *apc-11*, *dcn-1*, *sel-10* and *d2089.3* (Fig. 1B). We used *vps-35*, an essential component of the Wnt secretion machinery, as a positive control in our screen.

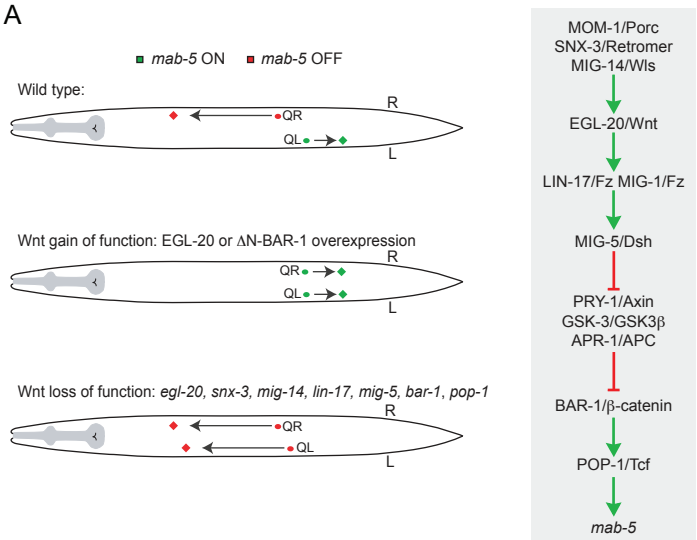
To further characterize the role of the identified genes in the Wnt pathway, we used

transgenic animals that heat-shock inducibly express EGL-20/Wnt or constitutively active ΔN-BAR-1/β-catenin. In this way, we could activate the Wnt pathway at the level of the Wnt ligand, or at the level of β-catenin, in animals that were subjected to RNAi. We assayed posterior displacement of the QR.d as a read-out of Wnt pathway overactivation and examined if the animals became more, or less, sensitive to Wnt pathway acrivation when we targeted *eel-1, vps-41, apc-11, dcn-1, sel-10* or *d2089.3* by RNAi (Fig 2). In this way, we aimed to genetically place the hits relative to *egl-20* and *bar-1*.

We will briefly discuss the results:

eel-1

We observed that *eel-1* knockdown rendered the worms more sensitive to EGL-20, but not to ΔN-BAR-1 expression (Fig 2). This result, in combination with the fact that *eel-1* RNAi suppressed the QL.d migration defect of vps-29 mutants (Fig. 1B), suggests that *eel-1* acts as a negative regulator of Wnt signaling at a level upstream of bar-1 in the Wnt pathway. We will discuss the role of *eel-1* as a negative regulator of Wnt signaling in more detail in chapter 3 of this thesis.



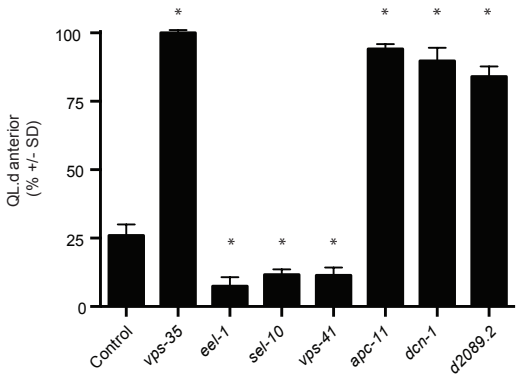


Figure 1: Screen to identify novel regulators of Wnt signaling from genes involved in ubiquitylation (A) Schematic view of Q daughter cell migration in *C. elegans* as a read-out of β-catenin dependent Wnt signaling. (B) Hits from the screen for genes with a role in ubiquitylation that enhance or suppress the QL.d phenotype of *vps-29(tm1320)* mutants (data are presented mean +/- SD and include results from 3 experiments, n>50 per experiment, * indicates p<0.05 (Student's t-test)).

sel-10

sel-10 is an F-box protein that is involved in the regulation of Notch signaling, sex determination and *lin-45*/Braf signaling in *C. elegans*[28–30]. FBW7 is the mammalian homologue of *sel-10*. FBW7 regulates a plethora of substrates including myc, cyclin E and Jun[31]. *sel-10* RNAi suppressed the *vps-29* QL.d migration defect (Fig. 1B), but *sel-10* RNAi did not change the sensitivity to EGL-20 or ΔN-BAR-1 overexpression (Fig. 2). A viable *sel-10(ok1632)* mutant was available. We crossed this mutant to the *vps-29(tm1320)* mutant and observed a similar suppression of the QL.d migration phenotype, confirming our RNAi results (Fig. 3A).

To further investigate the function of *sel-10*, we determined the final positions of the QL.d and QR.d in the *sel-10(ok1632)* mutant. We found no significant changes compared to wild-type animals, neither did we observe other Wnt related phenotypes in these mutants (Fig. 3B and data not shown). Subsequently, we used single molecule mRNA fish to measure *mab-5* expression in the Q neuroblasts of the *sel-10(ok1632)* mutants as a sensitive measure of Wnt pathway activity. We did not observe changes in the number of *mab-5* transcripts in the QL and QR neuroblasts of *sel-10* mutants compared to wild-type animals, indicating that the Wnt pathway is not over-activated in this mutant (Fig. 3C).

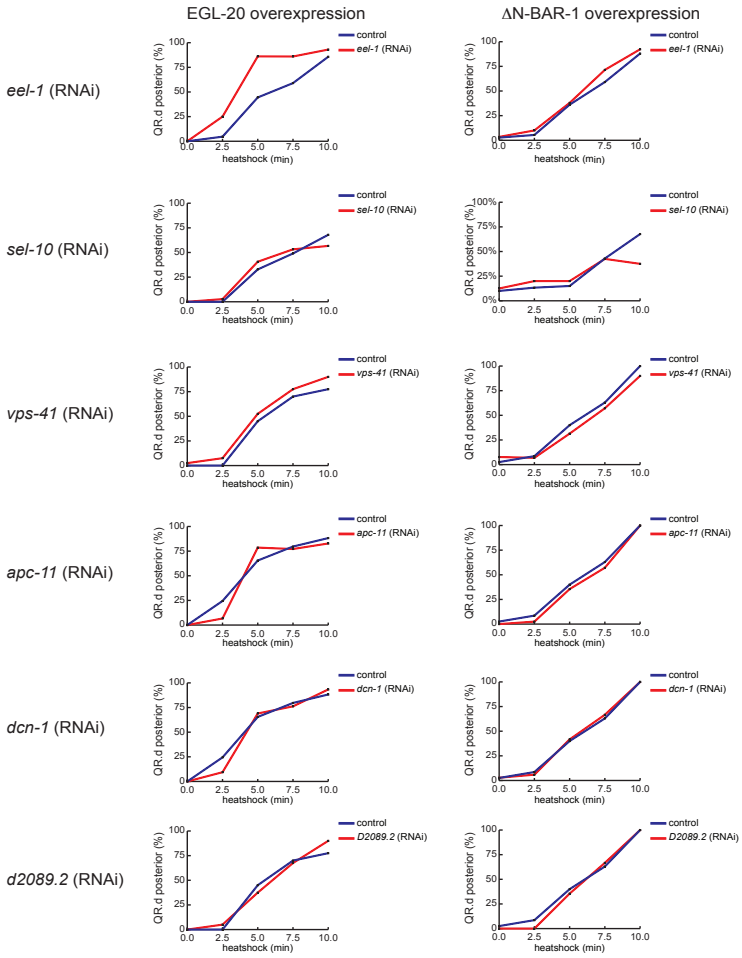


Figure 2: Epistatic analysis of the hits in Wnt pathway Transgenic animals that heat-shock inducibly express EGL-20/Wnt or ΔN-BAR-1/β-catenin were subjected to RNAi targeting *eel-1, sel-10, vps-41, apc-11, dcn-1* or *d2089.2*.

We conclude that *sel-10* suppresses the QL.d migration defect of *vps-29* mutants, but at this time we cannot explain the mechanism of this suppression. An interesting possibility is that Notch signaling is involved in the regulation of QL.d migration in *vps-29* mutants. We aim to further explore this possibility.

vps-41

Knock down of *vps-41* suppressed the QL.d migration defect of *vps-29* mutants (Fig. 1B), but it did not change sensitivity to EGL-20 or ΔN-BAR-1 overexpression (Fig. 2). This result suggests that *vps-41* RNAi promotes Wnt signaling in *vps-29* mutants. We propose an explanation for this observation based on our research that indicates that inhibition of endosome to lysosome maturation promotes Wnt secretion in *vps-29* mutants (Lorenowicz *et al.* in press).

Wnt secretion requires trafficking of Wls, a membrane protein that is essential for Wnt secretion, from the Golgi to the plasma membrane. To maintain sufficient levels of Wnt secretion, Wls needs to return to the Golgi, a transport route that requires endocytosis

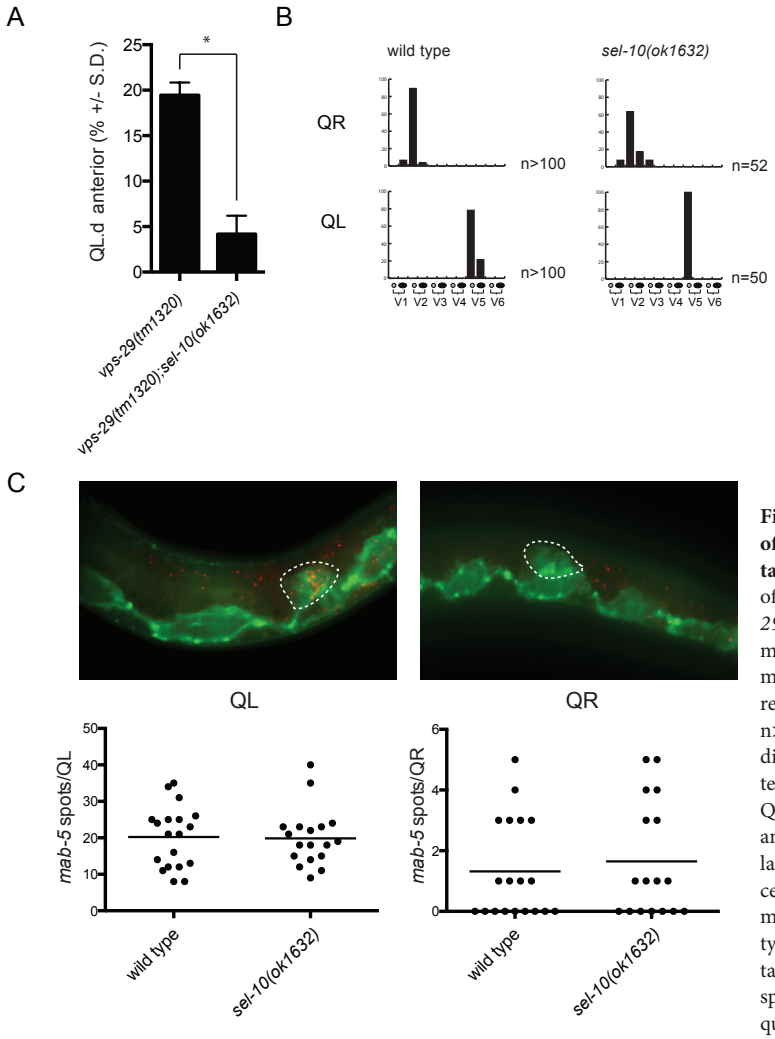


Figure 3. Characterization of the *sel-10(ok1632)* mutant (A) QL.d phenotype of *vps-29(tm1320)* and *vps-29(tm1320);sel-10(ok1632)* mutants (data are presented mean +/- SD and include results from 3 experiments, n>50 per experiment, * indicates $p<0.05$ (Student's t-test)). (B) Final positions of QL.d and QL.d in wild type and *sel-10(ok1632) C. elegans* larvae, relative to the seam cells. (C) Single molecule mRNA fish for *mab-5* in wild type and *sel-10(ok1632)* mutants, the number of *mab-5* spots per Q neuroblast was quantified.

and transport from early endosomes to the trans-Golgi network (TGN). SNX-3 and the core retromer complex, which is made up of VPS-29, VPS-35 and VPS-26, mediate the endosome to TGN transport step[27,32–37]. The *vps-29(tm1320)* mutation causes a partial reduction of retromer function (Lorenowicz *et al.* in press). As a result, transport of Wls from early endosomes to the TGN is compromised in *vps-29* mutants and a portion of Wls protein is missorted to lysosomes, where it is degraded. In this way, less Wls is available and Wnt secretion is reduced in *vps-29* mutants.

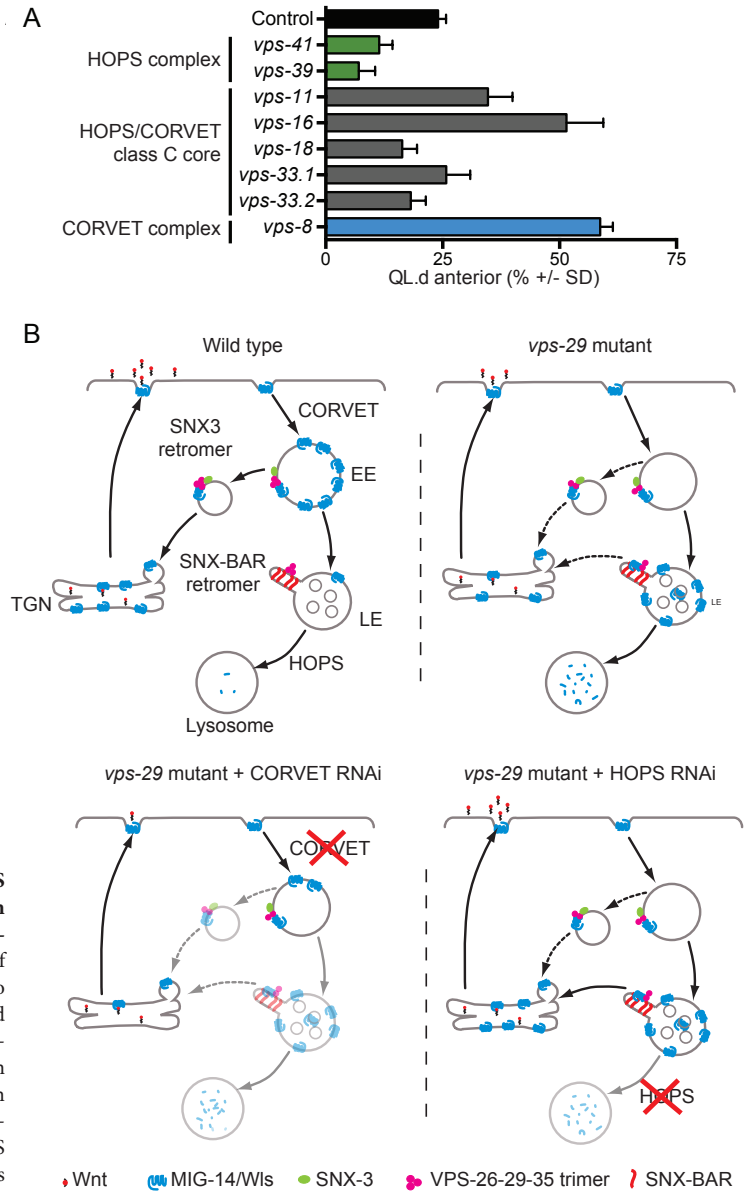


Figure 4: The role of the HOPS and CORVET complexes in Wnt secretion in *vps-29* mutants (A) QL.d phenotype of *vps-29* mutants subjected to RNAi targeting HOPS and CORVET complex components (data are presented mean +/- SD and include results from 3 experiments, n>25 per experiment). (B) Model for HOPS and CORVET function in Wls trafficking in *vps-29* mutants, see text for explanation.

Inhibiting endosome to lysosome maturation, by interfering with *rab-7* function, restores Wnt secretion in *vps-29* mutants. This is the result of increased residence of the Wnt secretion factor Wls/MIG-14 in a late endosomal compartment. In this compartment, MIG-14 is available for SNX1-retromer mediated transport to the TGN. Furthermore, blocking an earlier step of endosome maturation, by interfering with SAND-1 function, precludes access of MIG-14 to the SNX-1 retromer route and thus enhances the *vps-29* phenotype (Lorenowicz *et al.* in press).

The VPS-41 protein contains a RING domain and it is part of the class C vacuolar sorting/homotypic fusion and vacuolar protein sorting (HOPS) complex. The HOPS complex in yeast and mammals is an effector of *rab-7* and it is involved in lysosome maturation[38]. We propose that *vps-41* RNAi rescues the *vps-29* QL.d phenotype through inhibition of endosome to lysosome maturation, which results in increased retrograde transport of Wls via the SNX1-retromer route. Following form our hypothesis, knockdown of other HOPS complex components should also rescue the *vps-29* QL.d phenotype. We tested this hypothesis and observed suppression of the *vps-29* phenotype when we targeted *vps-39* by RNAi. Knockdown of other HOPS components had no effect, or increased, the *vps-29* phenotype (Fig. 3A). We can explain this observation because *vps-11, vps-16, vps-18* and *vps-33* form the class C subunit of the HOPS complex, which is also part of the class C core vacuole/endosome tethering (CORVET) complex. The CORVET complex is an effector of *rab-5* and involved in the regulation of early endosomes[38]. In addition to the class C core proteins, the CORVET complex contains the *vps-3* and *vps-8* subunits, Knockdown of *vps-8* enhances the *vps-29* phenotype (Fig. 3A). We conclude that interfering with the early steps of endosome maturation, through knockdown of the CORVET complex, enhances the *vps-29* phenotype, similar to knockdown of *sand-1*. Blocking endosomal maturation at a later stage through inhibition of the HOPS complex, or *rab-7*, rescues the *vps-29* phenotype (Fig. 4B) because this promotes Wls retrieval via the SNX1-retromer route.

apc-11

apc-11 RNAi enhanced the *vps-29* QL.d phenotype (Fig. 1B), but it did not change sensitivity to EGL-20 or ΔN-BAR-1 overexpression (Fig. 2). This result suggests that *apc-11* acts as a positive regulator of Wnt signaling in the *vps-29* Wnt secretion hypomorph. *apc-11* is part of the Anaphase Promoting Complex/Cyclosome (ACP/C), which is a ubiquitin ligase complex with important functions during cell-cycle progression. Wnt signaling is known to regulate the cell cycle by promoting the G1 phase of the cell cycle. Moreover, Wnt signaling is highly active at the end of G2 and β-catenin levels oscillate with the cell cycle and peak in mitosis (reviewed by Niehrs and Acebron[39]). Interestingly, our results might indicate that the APC/C may be involved in regulating Wnt signaling. A link has been described between the APC/C and Wnt signaling in human cells where APC^{Cdc20} regulates Conductin/Axin2 levels. Knockdown of Cdc20 results in elevated Axin2 levels and inhibition of Wnt signaling[40]. It will be interesting to determine if *apc-11* regulates QL.d migration and Wnt signaling in *C. elegans* via a similar mechanism.

dcn-1

dcn-1 RNAi did not significantly change sensitivity to EGL-20 or ΔN-BAR-1 overexpression (Fig. 2). *dcn-1* RNAi enhanced the *vps-29* QL.d migration defect, which suggests *dcn-1* may act as a positive regulator of Wnt signaling (Fig. 1B). *dcn-1* (defective in cullin neddylation) is a protein that can ligate the small ubiquitin-like protein NEDD8 to cullin proteins, and in this way promotes activity of SCF ubiquitin ligases[41,42]. DCN-1 regulates many SCF complexes,

some of which may have a positive regulatory function in Wnt signaling. However, we have not identified which SCF complex is regulated by *dcn-1* in this context.

d2089.2

The last hit in our screen was *d2089.2*, a gene that contains a RING domain and a transmembrane domain. The mammalian homologue of *d2089.2*, March2, is localized to endosomes and may be involved in the regulation of intracellular trafficking[43]. March2 controls ubiquitylation and internalization of carvedilol-bound β2-andrenergic receptor[44]. Recently, the RING and transmembrane domain containing proteins, Rnf43 and Znrf3, were identified as regulators of Frizzled internalization. In this way, Rnf43 and Znrf3 act as a negative regulators of Wnt signaling[19,20]. An appealing hypothesis is that *d2089.2* may be involved in the regulation of a membrane protein that is involved in Wnt signaling, potentially Wls. Future research will be aimed at elucidating the role of *d2089.2* in Wnt signaling.

Conclusion

We performed a stringent and sensitive RNAi screen in *C. elegans* to identify novel regulators of Wnt signaling. We focused our screen on genes that have a role in ubiquitylation. We identified 5 potential novel regulators of Wnt signaling: *eel-1*, *vps-41*, *apc-11*, *dcn-1* and *d2089.2*. We will discuss the role of *eel-1* in Wnt pathway regulation further in chapter 3 of this thesis. We formulated a model for the role of *vps-41*, the HOPS complex and CORVET complex in Wnt secretion in *vps-29* mutants. More research is required to elucidate the roles of *sel-10*, *d2089.2*, *dcn-1* and *apc-11* in Wnt signaling and QL neuroblast migration in *C. elegans*.

There are various ubiquitin ligases and DUBs with known roles in Wnt signaling that we did not identify in our screen, for example β-TrCP/*lin-23*, Cyld, Trabid, Klhl12, Itch, Rnf43 and Rnf146. For β-TrCP/*lin-23* there was no RNAi clone available. Rnf146/*y47d3b.11* and Cyld/*f40f12*.5 modulated the *vps-29* QL.d migration defect, but failed to reproducibly meet our stringent cutoff limits. The worm homologues of Itch/*y65b4br.4* and Trabid/*y50c1a.1* had no effect on the QL.d migration phenotype of *vps-29* mutants.

The knockdown efficiency that can be achieved by feeding RNAi in *C. elegans* varies among RNAi clones resulting in significant rates of false-negatives in RNAi screens. Furthermore, there may be tissue, species and context dependent roles for specific ubiquitin ligases or DUBs in the regulation of β-catenin dependent Wnt signal transduction that may explain why we did not identify these regulators of Wnt signaling in our screen.

Methods

C. elegans strains and transgenes

C. elegans were cultured in standard conditions at 20°C as described[45]. Strains and transgenes used in this study: *vps-29(tm1320)*, *sel-20(ok1632)*, *muIs32[Pmec-7::gfp]*, *huIs7[Phs::ΔNbar-1]*, *muIs53[Phs::egl-20]*, *heIs63[Pwrt-2::H2b::gfp; Pwrt2::PH::gfp]*.

RNAi screen

A list of ubiquitin conjugating genes, DUB's, RING domain containing genes, HECT domain containing genes was obtained from Kipreos[46]. We isolated RNAi clones targeting these genes form the Vidal or Ahringer RNAi libraries[47,48]. RNAi clones were grown overnight in LB

supplemented with 50 µg/ml Ampicillin. Bacterial cultures were grown for 3 days on NGM plates supplemented with Ampicillin, Tetracylin, Fungizone and IPTG. 2 *vps-29(tm1320);muIs32* L4 larvae were placed on the bacterial lawn and allowed to give rise to progeny. The position of the QL.d cell relative to the vulva was scored when the progeny reached young-adult stage[49]. If RNAi clones modulated the *vps-29* QL.d phenotype to >80% or <11%, the RNAi experiment was repeated twice. RNAi clones that consistently enhanced or suppressed the *vps-29* phenotype we considered a hit and confirmed by sequencing.

EGL-20 and ΔN-BAR-1 overexpression

huIs7 and *muIs53* worms were grown on RNAi food. Gravid adults were bleached and the progeny was allowed to hatch overnight in M9. The following morning, 100 staged L1 larvae were subjected to heat shock at 33°C in 100 ml M9. The heatshocked worms were allowed to develop on OP50 plates and the QR.d position relative to the vulva was scored when the worms reached young adult stage.

Single molecule mRNA FISH

Single molecule mRNA FISH was performed as described[50,51]. Imaging was performed using a Leica DM6000 microscope equipped with a Leica DFC360FX camera, 100x objective and Tx2 filter cube. Images were acquired at 1024x1024 resolution and 2x2 binned before analysis using ImageJ. *mab-5* mRNA spots were manually counted in the Q neuroblasts using *heIs63* as a marker to outline the cells.

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Table S1: List of screened genes.

Category	Gene	Category	Gene	Category	Gene	Category	Gene	Category	Gene	Category	Gene
DUB	C04E6.5	E2	F29B9.6	RING	C06A5.8	RING	F26G5.9	RING	R06F6.2	RING	ZK1240.3
DUB	C08B11.7	E2	F40G9.3	RING	C06A5.9	RING	F32A6.3	RING	R10A10.2	RING	ZK1240.6
DUB	C34F6.9	E2	F49E12.4	RING	C11H1.3	RING	F35G12.9	RING	T01C3.3	RING	ZK287.5
DUB	E01B7.1	E2	F58A4.10	RING	C12C8.3	RING	F36F2.3	RING	T01G5.7	RING	ZK637.14
DUB	F07A11.4	E2	M7.1	RING	C15F1.5	RING	F40G9.12	RING	T02C1.1	RING	ZK809.7
DUB	F14D2.7	E2	R01H2.6	RING	C16C10.5	RING	F40G9.14	RING	T05A12.4		
DUB	F21D5.2	E2	R09B3.4	RING	C16C10.7	RING	F42C5.4	RING	T08D2.4	SKR	C06A8.4
DUB	F28F8.6	E2	Y110A2AR.2	RING	C17E4.3	RING	F42G2.5	RING	T13A10.2	SKR	C42D4.6
DUB	F29C4.5	E2	Y54E5B.4	RING	C17H11.6	RING	F43C11.7	RING	T20F5.6	SKR	C52D10.6
DUB	F30A10.10	E2	Y54G2A.31	RING	C18B12.4	RING	F43G6.8	RING	T20F5.7	SKR	C52D10.7
DUB	F35B3.1	E2	Y69H2.6	RING	C18H9.7	RING	F45G2.6	RING	T22B2.1	SKR	C52D10.8
DUB	F38B7.5	E2	Y71G12B.15	RING	C26B9.6	RING	F46F2.1	RING	T23F6.3	SKR	C52D10.9
DUB	F46E10.8	E2	Y87G2A.9	RING	C30F2.2	RING	F47G9.4	RING	T24D1.2	SKR	F13A7.9
DUB	F59E12.6	E2	Y94H6A.6	RING	C32D5.10	RING	F53F8.3	RING	T26C12.3	SKR	F44G3.6
DUB	H19N07.2			RING	C32D5.11	RING	F53G2.7	RING	W02A11.3	SKR	F46A9.4
DUB	H34C03.2	HECT	C34D4.14	RING	C32E8.1	RING	F54B11.5	RING	W04H10.3	SKR	F46A9.5
DUB	K02C4.3	HECT	D2085.4	RING	C34E10.4	RING	F55A11.7	RING	Y105C5B.11	SKR	F47H4.10
DUB	K08B4.5	HECT	F36A2.13	RING	C36A4.8	RING	F55A3.1	RING	Y105E8A.14	SKR	F54D10.1
DUB	K09A9.4	HECT	F45H7.6	RING	C45G7.4	RING	F56D2.2	RING	Y38H8A.2	SKR	K08H2.1
DUB	R10E11.3	HECT	Y39A1C.2	RING	C49H3.5	RING	F58B6.3	RING	Y45F10B.8	SKR	R12H7.3
DUB	T05H10.1	HECT	Y48G8AL.1	RING	C52E12.1	RING	F58E6.1	RING	Y45F10B.9	SKR	R12H7.5
DUB	T22F3.2	HECT	Y65B4BR.4	RING	C53A5.6	RING	H05L14.2	RING	Y47D3B.11	SKR	Y105C5B.13
DUB	T24B8.7	HECT	Y67D8C.5	RING	C53D5.2	RING	H10E21.5	RING	Y47G6A.14	SKR	Y37H2C.2
DUB	T27A3.2			RING	C55A6.1	RING	K01G5.1	RING	Y52E8A.2	SKR	Y47D7A.1
DUB	Y106G6H.12	Ubox	F59E10.2	RING	C56A3.4	RING	K02B12.8	RING	Y53G8AM.4	SKR	Y47D7A.8
DUB	Y40G12A.1	Ubox	T05H10.5	RING	D2089.2	RING	K04C2.4	RING	Y55F3AM.6		
DUB	Y50C1A.1	Ubox	T09B4.10	RING	F08B12.2	RING	K09F6.7	RING	Y57A10B.1	FBOX	C02F5.7
DUB	Y71A12B.9			RING	F08G12.5	RING	K11D12.9	RING	Y71F9AL.10	FBOX	C14B1.3
DUB	ZK328.1	RING	B0281.3	RING	F10D7.5	RING	K12B6.8	RING	Y73C8C.7	FBOX	DY3.6
		RING	B0281.8	RING	F10G7.10	RING	M02A10.3	RING	Y73C8C.8	FBOX	F55B12.3
E2	B0403.2	RING	B0393.6	RING	F11A10.3	RING	M110.3	RING	Y75B8A.10	FBOX	K04A8.6
E2	C06E2.3	RING	B0416.4	RING	F16A11.1	RING	M142.6	RING	Y7A9C.1	FBOX	T01E8.4
E2	C28G1.1	RING	C01B7.6	RING	F19G12.1	RING	M88.3	RING	ZC13.1		
E2	D1022.1	RING	C01G6.4	RING	F23B2.10	RING	R02E12.4	RING	ZK1240.1		
E2	F25H2.8	RING	C02B8.6	RING	F26F4.7	RING	R05D3.4	RING	ZK1240.2		

CHAPTER 3

Huwe1-mediated ubiquitylation of Dvl defines a novel negative feedback loop in the Wnt signaling pathway

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Abstract

Wnt signaling plays a central role in development, adult tissue homeostasis and cancer. Several steps in the canonical Wnt/β-catenin signaling cascade are regulated by ubiquitylation, a protein modification that influences the stability, subcellular localization or protein-protein interactions of target proteins. To identify novel regulators of the Wnt/β-catenin pathway, we performed RNAi screens in *C. elegans* and human tissue culture cells and identified the HECT domain containing ubiquitin ligase *eel-1*/Huwe1 as a negative regulator of Wnt signaling. Huwe1 functions cell autonomously in signal-receiving cells and genetically acts upstream of β-catenin. Mechanistically, Huwe1 binds to and ubiquitylates the cytoplasmic Wnt pathway component Dishevelled (Dvl) in a Wnt3a and CK1ε dependent manner. Huwe1 mediated ubiquitylation does not target Dvl for degradation. Instead, we found that Huwe1 decreases Dvl signaling by inhibiting Dvl multimerization. We conclude that Huwe1 is part of an evolutionarily conserved negative feedback loop in the Wnt/β-catenin pathway.

Introduction

In the canonical Wnt signaling pathway, the stability of the Wnt pathway effector β-catenin is regulated by a destruction complex composed of the Adenomatous Polyposis Coli protein (APC), Axin and the kinases casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β), which phosphorylate β-catenin and target it for βTrcp-dependent ubiquitylation and proteasomal degradation. Binding of Wnt to the receptors Frizzled (Fz) and LRP6 leads to inhibition of destruction complex function and translocation of β-catenin to the nucleus, where it interacts with TCF/Lef transcription factors to induce target gene expression[1].

One of the early events in the Wnt signal transduction cascade is the activation of the cytoplasmic protein Dishevelled (Dvl) by CK1ε dependent phosphorylation[2,3]. The activated Dvl protein interacts with Fz at the cell membrane and is required for the formation of signalosomes, multiprotein complexes in which the proximal events in the Wnt signal transduction cascade take place[4]. Dvl also binds to Axin through an amino-terminal DIX domain, an interaction that is required to inhibit destruction complex function. The DIX domain has the ability to multimerize, leading to the formation of dynamic Dvl assemblies in which the clustering of low affinity binding sites is thought to increase the overall avidity of Dvl for its interaction partners[5–8].

In addition to phosphorylation, Dvl is also regulated by ubiquitylation[9–11]. Ubiquitylation is a versatile post-translational modification in which specific E3 ubiquitin ligases mediate the addition of ubiquitin molecules, either as single ubiquitin proteins, or as ubiquitin polymers, to substrate proteins. Moreover, ubiquitin chains can be linked through different lysines within the ubiquitin sequence to further increase the functional versatility of this modification. Depending on the type of ubiquitylation, the modified protein is targeted for proteasomal degradation, endocytosed and routed into the lysosomal degradation pathway or is affected in its ability to interact with other proteins[12]. In the case of Dvl, it has been shown that K48-linked ubiquitylation by KLHL12 controls Dvl stability[9], but there is evidence for additional ubiquitin modifications that may control other aspects of Dvl function[10,13].

In the present study, we have identified the E3 ubiquitin ligase Huwe1 as a novel evolutionary conserved negative regulator of the Wnt/β-catenin signaling pathway. We demonstrate that Huwe1 binds, ubiquitylates and controls the signaling properties of Dvl as part of a negative feedback loop in the Wnt signaling pathway.

Results

Huwe1/EEL-1 is a negative regulator of Wnt/β-catenin signaling in *C. elegans* and mammalian cells

In order to identify additional roles for ubiquitylation in Wnt/β-catenin signaling, we performed an RNAi screen in *C. elegans* in which we targeted 22 predicted E2 ubiquitin ligases, 173 RING domain E3 ubiquitin ligases, 9 HECT domain E3 ubiquitin ligases and 34 deubiquitylating enzymes (DUBs) (data not shown). We used the final position of the left Q neuroblast descendants (QL.d) as a sensitive measure of Wnt/β-catenin pathway activity (Fig. S1a). During early larval development, the Wnt protein EGL-20 activates a BAR-1/β-catenin dependent Wnt pathway in QL that induces expression of the homeobox gene *mab-5* and migration of the QL.d towards the posterior[14]. In the absence of EGL-20/Wnt signaling, *mab-5* is not expressed and as a consequence, the QL.d migrate in the opposite, anterior direction. To screen for both positive and negative regulators of Wnt signaling, we used a *vps-29(tm1320)* retromer mutant background, in which EGL-20/Wnt secretion is reduced, resulting in a partially penetrant defect in *mab-5* expression and QL.d migration. Interfering with positive regulators of EGL-20/Wnt signaling enhances this phenotype, while knock down of negative regulators restores posterior QL.d migration in this mutant background[15].

We found that knock down of the HECT domain containing ubiquitin ligase *eel-1* suppressed the QL.d migration phenotype of *vps-29(tm1320)* (Fig. 1a). Furthermore, using a quantitative single molecule mRNA FISH approach[16], we found that loss of *eel-1* induces a significant increase in expression of the EGL-20/Wnt target gene *mab-5* in the Q neuroblasts (Fig. 1b), indicating that *eel-1* functions as a negative regulator of EGL-20/Wnt signaling.

To investigate the function of *eel-1* in other Wnt dependent processes, we examined vulva formation, in which Wnt/β-catenin signaling plays a permissive role in preventing fusion of the vulva precursor cells (VPCs) with the surrounding hypodermal syncytium[17]. In hypomorphic mutants of the Wnt secretion factor *mig-14*/Wntless, a decrease in Wnt signaling leads to a partially penetrant defect in vulva induction[18]. *eel-1*(RNAi) significantly rescued this defect (Fig. S1b), supporting the notion that *eel-1 f*unctions as a general regulator of Wnt/β-catenin signaling in *C. elegans*.

To address whether *eel-1* is required in Wnt responding cells, we used a tissue-specific RNAi approach to knock-down *eel-1* in the Q neuroblast lineage. Similar to systemic RNAi, knock-down of *eel-1* in the Q neuroblasts significantly rescued QL.d migration in the *vps-29* mutant background (Fig. 1a). In contrast, knock down of *eel-1* in EGL-20/Wnt producing cells had no effect (data not shown). These results are consistent with a cell autonomous function of *eel-1* in Wnt responding cells.

To determine the position of *eel-1* in the Wnt/β-catenin pathway that regulates *mab-5* expression in the Q neuroblasts, we performed epistasis analysis with loss of function mutations in different Wnt pathway components. As shown in Table S1, *eel-1*(RNAi) suppressed the QL.d migration phenotype of *vps-29*/retromer and *mig-14*/Wntless, but had no effect in *bar-1*/β-catenin or *pop-1*/TCF mutants. Taken together with the cell autonomous function of *eel-1* in the Q neuroblast lineage, these results place *eel-1* between the Wnt ligand *egl-20* and *bar-1*/β-catenin. This conclusion is further supported by experiments in which we overexpressed EGL-20/Wnt or constitutively active BAR-1/β-catenin (ΔN-BAR-1)[18] to induce ectopic expression of *mab-5* in QR and posterior migration of the QR.d. Thus, *eel-1*(RNAi) strongly enhanced the EGL-20/Wnt induced posterior migration of the QR.d (Fig. 1c), whereas no effect was observed when ΔN-BAR-1 was overexpressed (Fig. 1d), consistent with a function of *eel-1* downstream of EGL-20/Wnt but upstream of BAR-1/β-catenin. Interestingly, *eel-1*(RNAi) did

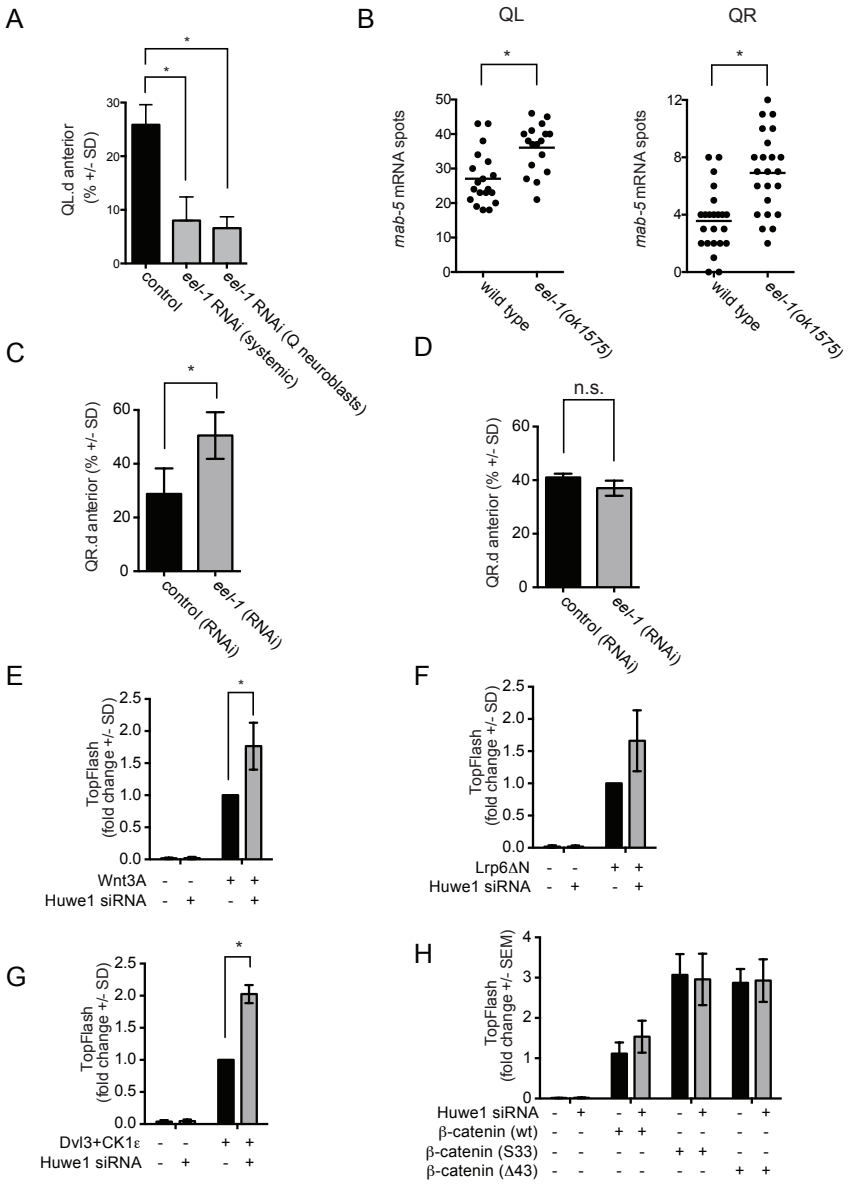

Figure 1: *eel-1*/Huwe1 is a negative regulator of Wnt signaling that acts upstream of β-catenin in the Wnt pathway in *C. elegans* and mammalian cells. (A) Systemic *eel-1* knock-down or tissue-specific knock-down by *egl-17* promoter directed expression of *eel-1* dsRNA rescues posterior migration of the QL.d in *vps-29(tm1320)* mutants. Data from 3 independent experiments are presented as mean +/- SD. In each case, n>30. *p=0.0063 for systemic RNAi, *p=0.0037 for tissue specific RNAi (Student's t-test). (B) Quantitative single molecule mRNA FISH shows increased expression of the Wnt target gene *mab-5* in the QL and QR neuroblasts of *eel-1(ok1575)* null mutants. **p=4.9*10-5 for QL, **p=9.6*10-4 for QR (Student's t-test). (C) *eel-1*(RNAi) enhances the response to heat-shock promoter directed expression of EGL-20/Wnt. Data from 4 experiments are presented as mean +/- SD. In each case, n>30. *p=0.015 (Student's t-test). (D) *eel-1*(RNAi) does not influence the response to heat-shock promoter directed expression of ΔN-BAR-1. Data from 4 experiments are presented as mean +/- SD. In each case, n>30. Huwe1 knock-down increases Topflash reporter activity in HEK293T cells treated with recombinant Wnt3a (E), HEK293T cells expressing LRP6ΔN (F) or HEK293T cells express-

not restore QL.d migration in *egl-20*/Wnt null mutants (Table S1), indicating that loss of *eel-1* is not sufficient to activate the EGL-20 pathway in the absence of Wnt signaling.

In parallel to the screen in *C. elegans*, we performed a genome-wide siRNA screen in HEK293T cells stably transfected with a Dvl-estrogen receptor fusion protein and a TOP-luciferase TCF reporter (7DF3 cells)[19]. We found that knock-down of the mammalian ortholog of *eel-1*, Huwe1 (Fig. S1c), enhanced the estradiol-dependent activation of the TCF reporter (Fig. S1d), an effect that was confirmed using non-overlapping siRNAs in 7DF3 and U2OS cells (Fig. S1e, f). Next, we activated the Wnt pathway in HEK293T cells by stimulation with recombinant Wnt3a, expression of constitutively active N-terminally-truncated LRP6 (LRP6ΔN)[20], expression of Dvl3 and CK1ε[21] or expression of constitutively active β-catenin[22]. Knock-down of Huwe1 increased Wnt reporter activity when the pathway was activated at the level of Wnt3a, LRP6ΔN or Dvl3 and CK1ε, but not when the pathway was activated by expression of constitutively active β-catenin (Fig. 1e-h). Moreover, Wnt reporter activity induced by Dvl1 or LRP6ΔN was significantly inhibited by overexpression of a Huwe1 fragment containing the HECT domain (HA-ΔN-Huwe1)[23], while reporter activity induced by β-catenin was not influenced by HA-ΔN-Huwe1 (Fig. S1g-i). Taken together with the *C. elegans* data discussed above, these results show that Huwe1 functions as an evolutionarily conserved negative regulator of Wnt signaling that acts upstream of β-catenin in the Wnt pathway.

Huwe1 binds and ubiquitylates Dishevelled

We obtained further mechanistic insight into the role of Huwe1 in Wnt signaling when we immunoprecipitated endogenous Dvl2, Dvl3, Axin1 and CK1ε from mouse embryonic fibroblasts (MEFs) and analyzed the associated proteins by mass-spectrometry. We found that Huwe1 forms a complex with endogenous Dvl2 and Dvl3. Importantly, this interaction could be confirmed by co-immunoprecipitation (co-IP) experiments with antibodies directed against endogenous Huwe1 and Dvl3 (Fig. 2a, b). Next, we used Dvl1 and Dvl3 truncations to map the domain of Dvl that is required for binding to HA-ΔN-Huwe1 in co-IP experiments and found that a proline-rich region between the PDZ and DEP domains of Dvl is essential for interaction (Fig. 2c, d, S2a, b). Dvl proteins form dynamic protein assemblies that are visible as distinct punctae within the cytoplasm[6]. Consistent with the interaction between Huwe1 and Dvl, immunofluorescence experiments using tagged versions of Huwe1 and Dvl2 or Dvl3 showed that Huwe1 colocalizes with Dvl in these punctate structures (Fig. 2e, S2c).

Huwe1 is a HECT domain E3 ubiquitin ligase that ubiquitylates a variety of substrates and is involved in processes ranging from regulation of apoptosis and neuronal differentiation to p53 regulation and DNA repair[23–28]. To investigate whether Huwe1 also ubiquitylates Dvl, we coexpressed Flag-Dvl1 and His-ubiquitin with wild type ΔN-Huwe1 or a catalytically inactive ΔN-Huwe1(CA) mutant[27]. Pull-down of His-ubiquitin modified proteins followed by detection of Flag-Dvl1 showed that ΔN-Huwe1 promotes Dvl1 ubiquitylation, while ΔN-Huwe1(CA) does not (Fig. 2f). Furthermore, deletion of the Huwe1 interaction region of Dvl1 blocked Huwe1 dependent ubiquitylation, indicating that binding of Huwe1 to Dvl is required for ubiquitylation (Fig. S2d).

To investigate whether the Huwe1 mediated ubiquitylation of Dvl is regulated by Wnt signaling, we treated cells with Wnt3a conditioned medium (Wnt3a CM) prior to the ubiquitylation assay. As shown in Fig. 3a, incubation with Wnt3a CM resulted in a

ing Dvl3 and CK1ε (G). Data from 3 independent experiments are presented as mean +/- SD. *p=0.0095 for control vs. Wnt3a treated (Student's t-test) *p=0.0062 for control vs. Dvl3 and CK1ε (Student's t-test). Huwe1 knock-down does not change TopFlash induction in cells that express stable β-catenin mutants (H). Data from 7 experiments are presented as mean +/- SEM.

clear increase in Huwe1 mediated ubiquitylation of Flag-Dvl1, indicating that the Huwe1 dependent ubiquitylation of Dvl is stimulated by Wnt signaling. To address whether this effect is mediated through CK1ε, the kinase that phosphorylates Dvl in response to Wnt3a signaling[3], we performed co-IP experiments with Flag-Dvl3 and HA-ΔN-Huwe1 in cells overexpressing CK1ε. As shown in Fig. 3b, CK1ε promoted the Huwe1-Dvl3 interaction, while

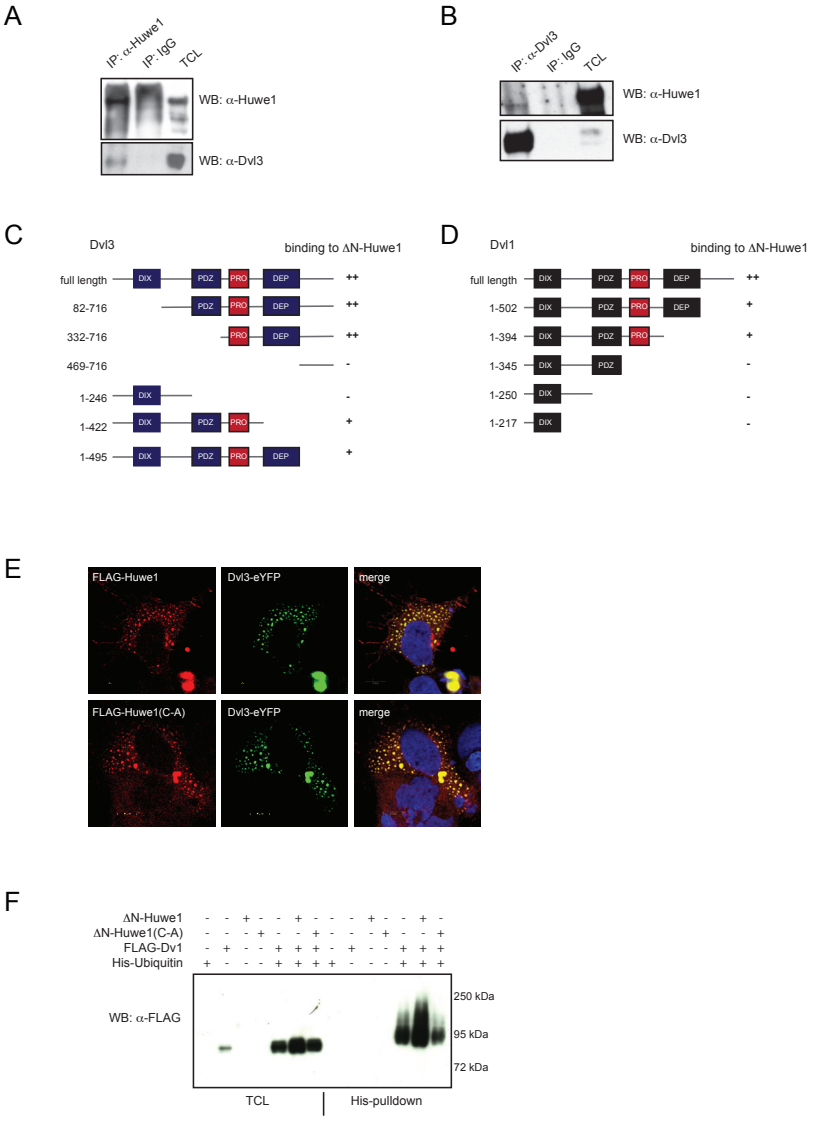

Figure 2: Huwe1 interacts with Dvl and promotes Dvl ubiquitylation. Immunoprecipitation of endogenous Huwe1 (A) or Dvl3 (B) from mouse embryonic fibroblast cell lysate was performed followed by Western blot detection using Dvl3 or Huwe1 antibodies. (C, D) Summary of co-immunoprecipitation of HA-ΔN-Huwe1 with truncated versions of Dvl3 and Dvl1 presented in Fig. S2A and B. + and – symbols indicate the binding efficiency of different mutants with HA-ΔN-Huwe1. (E) Co-localization of Flag-Huwe1 and Dvl3-eYFP in cytoplasmic Dvl punctae. (F) HA-ΔN-Huwe1 promotes Dvl1 ubiquitylation. HEK293T cells were transfected with Flag-Dvl1, His-ubiquitin and ΔN-Huwe1 or ΔN-Huwe1(CA). After pull-down of His-ubiquitin modified proteins, Flag-Dvl1 was detected by Western blotting.

expression of dominant negative CK1ε or treatment with the chemical CK1ε inhibitors D4476 (CK1 inhibitor I) or PF-670462 (CK1 inhibitor II) reduced the interaction. Importantly, CK1 inhibition prevented the Huwe1 dependent ubiquitylation of Flag-Dvl1 (Fig. 3c), indicating that CK1ε activity is a prerequisite for the binding and ubiquitylation of Dvl by Huwe1.

Huwe1 does not target Dvl for degradation but influences Dvl multimerization

Next, we set out to elucidate the mechanism by which the Huwe1 dependent ubiquitylation of Dvl inhibits Wnt signaling. First, we investigated whether Huwe1 targets Dvl for degradation. We observed a clear reduction in Flag-Dvl1 levels and stability after co-expression of KLHL12 – an adaptor protein which recruits the Cullin-3 ubiquitin ligase towards Dvl and targets Dvl for proteasomal degradation[9] – but found no reduction in Flag-Dvl1 levels or stability when HA-ΔN-Huwe1 was expressed (Fig. 4a). Neither did we observe increased levels of endogenous Dvl when we knocked down Huwe1 (Fig. 4b).

We then set out to determine the type of polyubiquitin chains that Huwe1 adds to Dvl. We used a two step purification approach, which consisted of a His-ubiquitin pull-down followed by a Flag-IP to isolate ubiquitylated Flag-Dvl1. Next, we probed the ubiquitylated Dvl sample with antibodies that specifically recognize K48 or K63 linked polyubiquitin chains. We observed an increase in K63-modified Dvl1 when Huwe1 was expressed, but not when the catalytically inactive Huwe1(C-A) mutant was expressed (Fig. 4c). In addition, Huwe1 induced a mild

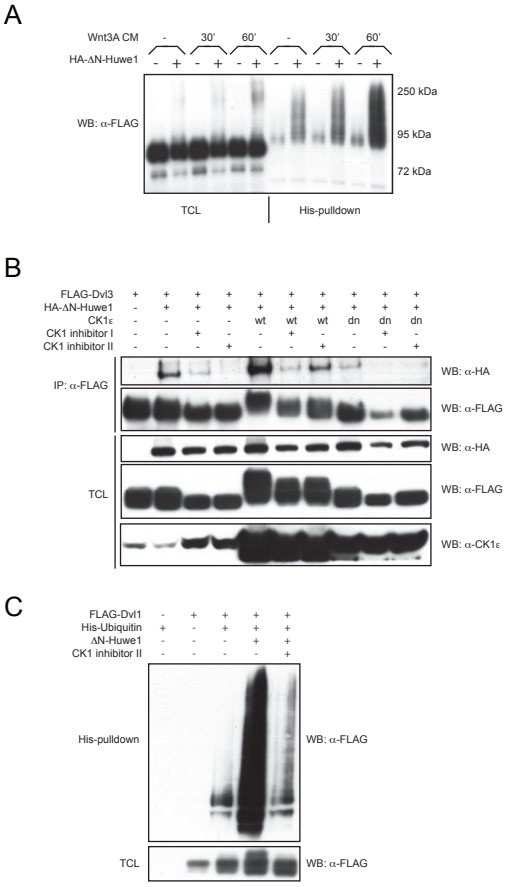

Figure 3: Wnt stimulation and CK1ε promote Huwe1-Dvl interaction and Dvl ubiquitylation. (a) Wnt3a stimulation enhances Huwe1 mediated ubiquitylation of Dvl1. HEK293T cells transfected with Flag-Dvl1, His-ubiquitin and HA-ΔN-Huwe1 were treated with control medium or Wnt3a conditioned medium (CM) for 30 or 60 minutes prior to His-pull-down and Flag-Dvl1 detection. (b) CK1ε promotes the interaction between Huwe1 and Dvl3. Co-IP of HA-ΔN-Huwe1 with Flag-Dvl3 in HEK293T cells expressing wild type or dominant negative CK1ε or treated with the chemical CK1 inhibitors D4476 (inhibitor I) or PF-670462 (inhibitor II). (c) Inhibition of CK1ε decreases the HA-ΔN-Huwe1 mediated ubiquitylation of Dvl1. HEK293T cells expressing Flag-Dvl1, His-ubiquitin and HA-ΔN-Huwe1 were treated with control vehicle or PF-670462 prior to His pull-down and Flag-Dvl1 detection.

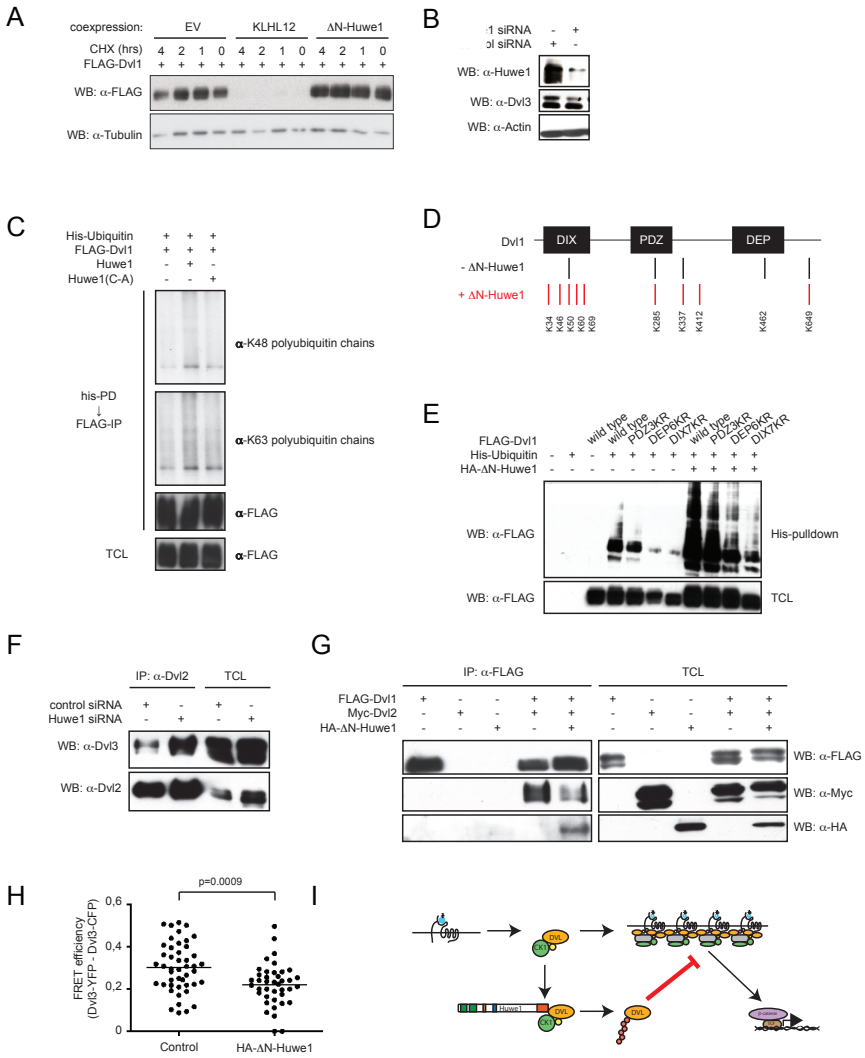

Figure 4: Huwe1 does not target Dvl for degradation, but inhibits Dvl polymerization. (A) HEK293T cells transfected with Flag-Dvl1 and control vector (EV), KLHL12 or HA-ΔN-Huwe1 expression constructs were treated with cycloheximide (CHX) for the indicated time prior to lysis and Flag-Dvl1 detection. (B) Dvl3 protein levels were determined by western blot in lysates obtained from control or Huwe1 siRNA treated OVCAR-5 cells. (C) Huwe1 promotes the formation of K63-linked polyubiquitin chains on Dvl. HEK293T cells were transfected with Flag-Dvl1, His-ubiquitin and Huwe1 or Huwe1(CA). After pull-down, the His-ubiquitin modified proteins were eluted from the Ni-NTA beads using imidazole. Ubiquitylated Flag-Dvl1 was immunoprecipitated and analyzed by Western blot using antibodies that specifically recognize K48 or K63 linked polyubiquitin chains. (D) Huwe1 promotes ubiquitylation of lysines in the DIX domain. Flag-Dvl1 was immunoprecipitated from cells HEK293T cells expressing HA-ΔN-Huwe1 and subjected to SDS PAGE followed by trypsin digestion. The peptides were analyzed by LC/MS (ubiquitylated lysines prevent trypsin digestion and the resulting peptides show a characteristic additional G-G mass). Ubiquitylated lysines are indicated. (E) HEK293T cells were transfected with Flag-Dvl1 mutants in which lysines in the PDZ, DEP and DIX domains are mutated into arginines. After pull-down of His-ubiquitin modified proteins, Flag-Dvl1 was detected by Western blotting. (F) Knock-down of Huwe1 increases co-IP between endogenous Dvl2 and Dvl3. OVCAR-5 cells were transfected with control or Huwe1 siRNA and endogenous Dvl2 was immunoprecipitated. (G) Overexpression of HA-ΔN-Huwe1 decreases co-IP between Flag-Dvl1 and Myc-Dvl2 in HEK293T cells. (H) FRET efficiency between Dvl3-YFP and Dvl3-CFP in HEK293T cells transfected with control or HA-ΔN-Huwe1 expression constructs. (I) Model of Huwe1 function in Wnt/β-catenin signaling. See text for explanation.

increase in K48 linked polyubiquitylation of Dvl1. Based on these results, and the fact that we did not observe changes in Dvl protein levels or stability when Huwe1 is overexpressed or knocked-down, we conclude that Huwe1 negatively regulates Wnt signaling by promoting K63-linked polyubiquitylation of Dvl.

We used a semi-quantitative mass-spectrometry approach to determine the lysine residues in Dvl that are ubiquitylated by Huwe1. In addition to 5 lysine residues that appear to be constitutively ubiquitylated, we found an additional 5 lysine residues that are ubiquitylated when ΔN-Huwe1 is expressed (K34, K46, K60, K69, K124). Most of these lysines are located within the DIX domain of Dvl (Fig 4d), indicating that this region is preferentially ubiquitylated by Huwe1. Consistently, we found that ΔN-Huwe1 efficiently ubiquitylates Dvl mutants in which the lysines in the PDZ domain (PDZ3KR) or DEP domain (DEP6KR) are mutated, while a Dvl mutant in which the 7 lysines in the DIX domain are mutated is much less efficiently ubiquitylated (Fig 4e). Taken together, we conclude that Huwe1 mainly ubiquitylates lysines within the DIX domain of Dvl.

The signaling activity of Dvl depends on the DIX domain dependent formation of multimeric aggregates[7,8]. To investigate whether Huwe1 influences the ability of Dvl to multimerize, we tested the interaction between Dvl2 and Dvl3 by co-IP. Knockdown of Huwe1 increased co-IP between Dvl2 and Dvl3 (Fig 4f). Furthermore, Huwe1 expression reduced co-IP between Flag-Dvl1 and Myc-Dvl2 (Fig. 4g). When we assayed the interaction between Dvl3-YFP and Dvl3-CFP using a FRET-based approach (Fig. 4h), we observed that expression of HA-ΔN-Huwe1 resulted in decreased FRET efficiency, indicating that Huwe1 interferes with Dvl3 multimerization. We therefore propose that Huwe1 inhibits Wnt signaling by decreasing the multimerization that is required for Dvl activity.

Discussion

In the present work, we identify the HECT-domain containing E3 ubiquitin ligase Huwe1 as an evolutionarily conserved negative regulator of the Wnt/β-catenin pathway. Our results from epistasis experiments in *C. elegans* and biochemical studies in mammalian cells suggest that Huwe1 acts at the level of Dvl.

The cytoplasmic protein Dvl functions at a key position in the Wnt/β-catenin pathway, interacting with Fz and Axin1 and assembling into multimeric aggregates to form signalosomes at the cell membrane[4]. It has previously been shown that Wnt3a signaling activates CK1ε[2,3] and that phosphorylation of Dvl by this kinase induces binding of Dvl to Axin1[21]. Our results show that this phosphorylation also induces binding of Huwe1, setting in motion a negative regulatory mechanism that inactivates Dvl (Fig. 4i). We show that Huwe1 ubiquitylates Dvl in response to Wnt3a and Ck1ε-dependent phosphorylation. Importantly, Huwe1-mediated ubiquitylation does not lead to Dvl degradation, as has been observed for the ubiquitin ligases KLHL12/Culin3 and ITCH[9,11]. Instead, we found that Huwe1 inhibits the polymerization of Dvl. As the formation of multimeric complexes is essential for Dvl activity[7,8], our results are consistent with a model in which Huwe1 negatively regulates Wnt/β-catenin signaling by inhibiting Dvl multimerization.

We found that Huwe1 promotes the formation of K63-linked polyubiquitin chains on lysine residues within the DIX domain of Dvl. It has been shown that K63-linked polyubiquitin chains are removed from the DIX domain by the de-ubiquitylating enzyme Cyld[10]. However, mutation of Cyld and the resulting hyper-ubiquitylation of Dvl was shown to activate Wnt/β-catenin signaling in mammalian cells. These results support the notion that K63-linked

polyubiquitylation is an important regulatory mechanism of Dvl activity, but do not explain why Huwe1 and Cyld both act as negative regulators of Wnt/β-catenin signaling. Cyld and Huwe1 may control the ubiquitylation of different lysines within the DIX domain to either promote signaling activity or to inhibit signaling by preventing Dvl multimerization. Alternatively, a tight balance in the dynamic addition and removal of K63-linked polyubiquitin chains may be required for the regulation of Dvl activity. Of note is that mutation or knock-down of the Cyld ortholog *cyld-1* did not lead to defects in Wnt/β-catenin signaling in *C. elegans* (data not shown). This indicates that Dvl activity is regulated independently of Cyld in nematodes and suggests that Huwe1 represents a more ancestral mechanism of Dvl regulation.

Huwe1 has recently been identified as an important driver of Wnt pathway dependent intestinal tumorigenesis in a mouse transposon insertional mutagenesis screen[29]. Our results showing that Huwe1 is a negative regulator of Wnt/β-catenin signaling provides an important mechanistic explanation for the role of Huwe1 in intestinal cancer.

Materials and Methods

C. elegans strains and culture

C. elegans strains were cultured at 20°C using standard conditions as described[30]. Mutants and transgenes used were: *vps-29(tm1320), egl-20(hu105), mig-14(ga62), mig-14(mu71), mig-5(rh147), bar-1(ga80), pop-1(hu9), eel-1(ok1575), muIs32[Pmec-7::gfp], muIs35[Pmec-7::gfp], huIs7[Phs::ΔNbar-1], muIs53[Phs::egl-20], heIs63[Pwrt-2::H2b::gfp; Pwrt2::PH::gfp], huEx273[Pegl-17::eel-1(RNAi)]*.

C. elegans RNAi and heat-shock experiments

Feeding RNAi was performed with bacterial clones as described[31,32]. The final positions of the Q descendants PVM and AVM were scored as anterior or posterior to the vulva in young adult animals[33]. Tissue specific RNAi was performed as described[34]. *egl-17* promoter sequence and a 1.1 kb fragment spanning exon 13 of *eel-1* were amplified from *C. elegans* genomic DNA. The *eel-1* fragment was fused to the *egl-17* promoter in a sense and antisense orientation using a PCR approach. The PCR products were injected in *vps-29(tm1320); muIs32* at 2.5 ng/µl with 7 ng/µl *Pmyo2:mCherry* injection marker and 200 ng/µl pBluescript DNA, resulting in transgenic line *huEx273*.

Embryos isolated from *huIs7* or *muIs53* animals grown on *eel-1* or control dsRNA expressing bacteria were allowed to hatch overnight in M9 buffer. Approximately 100 L1 larvae were heat-shocked in 100 µl M9 at 33°C for different lengths of time and the position of the Q cell descendants AVM and PVM was determined when the animals reached the young adult stage.

Single molecule mRNA FISH

Single molecule mRNA FISH was performed as described[16]. Imaging was performed using a Leica DM6000 microscope equipped with a Leica DFC360FX camera, 100x objective and Tx2 filter cube. Images were acquired at 1024x1024 resolution and 2x2 binned before analysis using ImageJ. *mab-5* mRNA spots were manually counted in the Q neuroblasts using *heIs63* as a marker to outline the cells.

Cell culture, plasmids and transfections

MEF and HEK293 cells were propagated in DMEM with 10% FBS, 5% L-glutamine and 5%

penicillin/streptomycin, while OVCAR-5 cells were grown in RPMI media with 10% FBS, 5% L-glutamine and 5% penicillin/streptomycin. Cells were seeded in 24 well plates on coverslips for FRET and immunocytochemistry and 10 cm dishes for immunoprecipitation. Cells were transfected using 2 µl of polyethylenimine per µg of DNA. Forty-eight hours post transfection, cells were harvested and processed. The following previously described expression constructs were used: Dvl2-Myc[35], Flag-hDvl3 and deletions[9], hCK1ε[36], Flag-mDvl1 deletions and lysine mutants[10], HA-Dvl2[8], HA-ΔN-Huwe1[23], FLAG-Huwe1 and FLAG-Huwe1(CA)[28], β-catenin, S33 β-catenin, Δ43 β-catenin[22], Lrp6ΔN[37], Super8X TopFlash[38] and VSV-KLHL12[9]. Dvl3-EYFP and Dvl3-ECFP were generated by Gateway cloning. Cells were stimulated with mouse rWnt3a (R&D Systems, Minneapolis, MN) for 3 hours if not stated otherwise. Control stimulations were done with 0.1% BSA in PBS. CK1 inhibition was performed using D4476 (Calbiochem) and/or PF-670462 (Roche) dissolved in DMSO or with 1 µl of Lipofectamine 2000 (Invitrogen) per well to increase cell penetration.

RNA interference

HEK-293 cells were transfected with Huwe1 esiRNA using Lipofectamine 2000. Huwe1 esiRNA (300 ng/well) was mixed with OptiMEM (Gibco) and 0.5µl of Lipofectamine 2000 and incubated at room temperature for 30 minutes, after which the mixture was added to trypsinized cells in 24 well plates. The medium was changed after 12 hours.

Dual Luciferase assay

HEK 293T cells were seeded in 24 well plates. After 24 hours, cells were transfected with 0.2 µg of Super8X TopFlash construct and 0.2 µg Renilla luciferase construct per well and the indicated expression plasmids. Twenty-four hours later, cells were lysed and Luciferase activity was measured using the Promega dual luciferase assay kit according to the manufacturer's instructions on a MLX luminometer (Dynex Technologies) and normalized using the Renilla luciferase measurements. Results are shown as means with standard deviations of at least three independent experiments.

High-throughput esiRNA screen and validation

384 well plates were preloaded with 10 ng esiRNA per well. Four sets of APC and β-catenin controls were placed on each of the 384 well plates. 7DF3 cells were thawed and resuspended at $1x10^7$ cells/ml in DMEM supplemented with 10% FBS without antibiotics and reverse-transfected using Interferin (Polyplus) following manufacturers guidelines using a Matrix Well-mate dispenser. Twenty-four hours after transfection, cells were induced by adding b-estradiol at a concentration of 4 mM. Forty-eight hours after transfection cells were washed in PBS, lysed in Glo-lysis buffer and incubated with luciferase substrate (Promega Bright-Glo). Plates were imaged on a GE LEADseeker™ imager. Non-overlapping secondary esiRNAs were generated against Huwe1 and reverse transfected as above in 7FD3 cells before estradiol treatment 24 hours post transfection followed by luminescence assays a further 24 hours later. U2OS eGFP-β-catenin cells (BioImage) were reverse transfected using Lipofectamine 2000 following manufacturers guidelines, luminescence assays were performed after 48 hrs.

Antibodies, immunoprecipitation and Western blotting

Twenty-four to 48 hours after transfection, cells were lysed in NP40 lysis buffer containing 50 mM Tris pH-7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP40 and protease inhibitors (Roche) for 20 minutes at 4°C and centrifuged at 13000 rpm for 30 minutes at 4°C. Supernatant was incubated with the following antibodies: mouse monoclonal anti-Dvl3 (Santa Cruz), mouse

monoclonal anti-Dvl2 (Santa Cruz), mouse monoclonal anti-FLAG (Sigma), rabbit polyclonal anti-HA (Abcam) or rabbit polyclonal anti-Huwe1 (Bethyl Laboratories) for 30 minutes on ice followed by incubation with G Sepharose beads (GE Healthcare) at 4ºC overnight. Samples were washed five times in NP40 lysis buffer and analyzed by Western blot. In addition to the above mentioned antibodies, the following antibodies were used for western blot: mouse monoclonal anti-Myc; goat polyclonal anti-actin; goat polyclonal anti-Ck1ε (all from Santa Cruz Biotechnology), K48 linked polyubiquitin chains and K63 linked polyubiquitin chains (Cell Signaling Technology); mouse monoclonal anti alpha-tubulin (Sigma); HRP-conjugated anti-mouse secondary antibody (GE Healthcare); and HRP-conjugated anti-rabbit secondary antibody (Sigma).

Immunocytochemistry

HEK293 cells were seeded onto gelatin coated coverslips in 24 well plates and transfection was carried out after 24 hours. Transfected cells were fixed after 24 hours in 4% paraformaldehyde for 15 minutes and blocked with PBTA (3% BSA, 0,25% Triton, 0,01% NaN3) for 1 hour at room temperature, followed by incubation in primary antibodies overnight at 4ºC. The following day, cells were washed in PBS and incubated with secondary antibodies: Alexa fluor 488, Alexa fluor 568 or Alexa fluor 594 (Invitrogen) for 1 hour at room temperature, then washed twice with PBS, stained for DAPI and mounted.

Ubiquitylation assay

In vivo ubiquitylation assays were performed as described[10]. HEK293T cells were transfected with expression constructs for Flag-Dvl1, His-ubiquitin and HA-ΔN-Huwe1. Forty-eight hours later, cells were washed in PBS supplemented with 10 mM NEM, lysed in denaturing guanidium buffer (10 mM Tris pH8, 0,1 M Na_2HPO_4, 0,1 M NaH_2PO_4, 20 mM imidazole, 6M guanidium-HCl and 10 mM β-mercaptoethanol) and sonicated. The lysate was centrifuged (10 minutes at 14000 rpm) and His-tagged proteins were isolated from the supernatant using Ni-NTA beads (Qiagen) for 4 hours at room temperature. The beads were washed in guanidium buffer and 3 times in ureum buffer before elution of His-tagged proteins in 1x Laemli Sample buffer supplemented with 10 mM Tris pH7, 0,1 M Na_2HPO_4, 0,1 M NaH_2PO_4, 8M urea and 200 mM imidazole. For the ubiquitin chain type experiment, the Ni-NTA beads were washed twice in IP buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100) after the ureum buffer washes. Ubiquitylated proteins were eluted in IP buffer supplemented with 250 mM imidazole. Ubiquitylated Dvl was isolated by immunoprecipitation using anti-FLAG antibody.

Mass spectrometric analysis

The following plasmids were overexpressed in HEK293 cell lines: His-Ub, FLAG-Dvl1 and ΔN-HA-Huwe1. Co-immunoprecipitation was performed using an antibody against FLAG (F1804) in NP40 lysis buffer (0.5% NP40; 150mM NaCl; 50 mM Tris pH 7.4), after which samples were run on SDS PAGE to separate the ubiquitylated complexes. Gels were then stained with 0.25% coomassie blue stain (R250). Corresponding 1-D bands were excised and destained followed by reduction and alkylation. Gel pieces were then subjected to digestion (trypsin, 2 hours at 40°C). Alternatively, immunoprecipitated Dvl was digested directly in solution.

LC-MS/MS analyses of peptide mixture were done using a RSLCnano system connected to an Orbitrap Elite hybrid spectrometer (Thermo Fisher Scientific). Prior to LC separation, tryptic digests were concentrated and desalted using a trapping column (100 µm × 30 mm) filled with 3.5-µm X-Bridge BEH 130 C18 sorbent (Waters). After washing with 0.1% FA, the peptides were eluted (flow 300 nl/min) from the trapping column onto a Acclaim

Pepmap100 C18 analytical column (2 µm particles, 75 µm × 250 mm; Thermo Fisher Scientific) by the following gradient program (mobile phase A: 0.1% FA in water; mobile phase B: ACN:methanol:2,2,2-trifluoroethanol (6:3:1; *v/v/v*) containing 0.1% FA): the gradient elution started at 2% of mobile phase B and increased from 2% to 45% during the first 90 min (11% in the 30th, 25% in the 60th and 45% in 90th min), then increased linearly to 95% of mobile phase B in the next 5 min and remained at this state for the next 15 min.

MS data were acquired in a data-dependent strategy with dynamic precursor exclusion selecting up to the top 20 of precursors based on precursor abundance in the survey scan (350-1700 m/z, resolution 120 000). Low resolution CID MS/MS spectra were acquired in rapid CID scan mode.

FRET analysis

Cells were seeded on gelatin coated cover slips in 24 well plates and transfected after 24 hours with 0.1 µg Dvl3-ECFP, 0.1 µg Dvl3-EYFP and 0.3 µg HA-ΔN-Huwe1 using polyethylenimine. Plasmids containing fused EYFP and ECFP together are used as additional controls. The next day, cells were fixed using 4% paraformaldehyde for 15 minutes and blocked with PBTA (3% BSA, 0,25% Triton, 0,01% NaN_3) for 1 hour at room temperature followed by incubation in primary antibodies (anti-HA) overnight at 4ºC. The following day cells were washed in 0.1M PIPES pH 6.9 and incubated with secondary antibody (anti rabbit Alexa fluor 594, Invitrogen) at room temperature for 1 hour. Cells were then washed twice with PIPES solution, once with 2 mM $MgCl_2$ and once with EGTA, followed by mounting. FRET between Dvl3-ECFP (donor) and Dvl3-EYFP (acceptor) was measured using a Leica confocal microscope. CFP, YFP and Alexa fluor 594 were excited using 455, 514 and 590 nm lasers, respectively. Region of Interest (ROI) was set on Dvl punctae. Dvl3-YFP punctae were irreversibly bleached for 15-30 seconds. Ratios of donor intensities before and after photobleaching were calculated to obtain FRET efficiencies. To avoid heterogeneity in donor and acceptor expression levels, experiments were performed thrice measuring FRET from 15-20 punctae/cell from 4-5 cells.

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Table S1. Effect of *eel-1*(RNAi) on the EGL-20/Wnt dependent migration of the QL.d

	QL.d anterior (%)	
	control (RNAi	*eel-1* (RNAi)
Wild type	0	0
mig-14(mu71)/Wls	100	86
vps-29(tm1320)/Retromer	33	8
egl-20(hu105)/Wnt	100	100
mig-5(rh147)/Dvl	100	100
bar-1(ga80)/β-catenin	100	100
pop1(hu9)/TCF	29	26
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 or using Nomarski optics. In each case, n>50.		

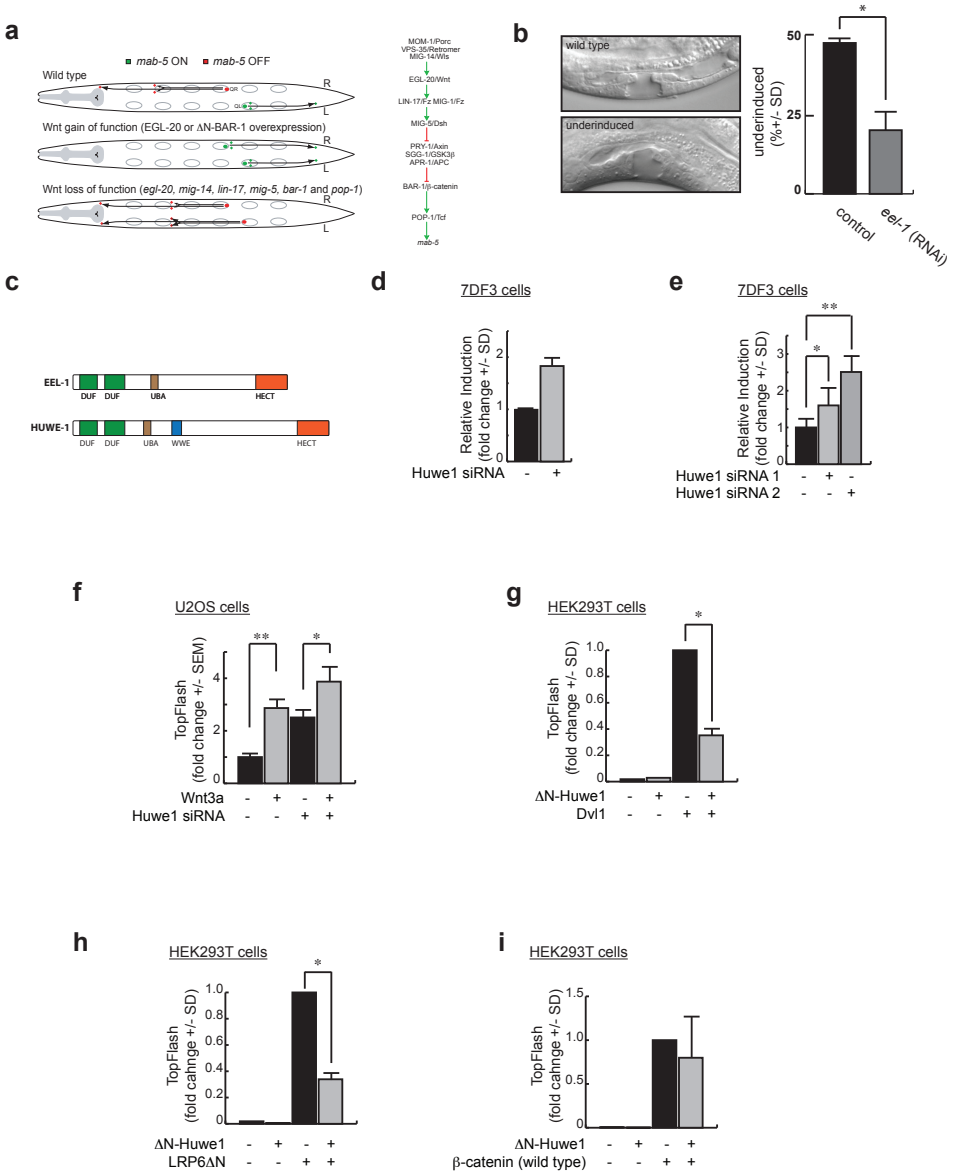


Figure S1. *eel-1*/Huwe1 is a negative regulator of Wnt signaling that acts upstream of β-catenin in the Wnt pathway. (a) Schematic representation of the migration of the Q neuroblast descendants in wild type L1 larvae and in animals with impaired EGL-20/Wnt signaling. Dorsal view. Cells expressing *mab-5* are depicted in green. (b) *eel-1*(RNAi) rescues the under-induced vulva phenotype of *mig-14(ga62)*/Wls. Data from 4 independent experiments are presented as mean +/- SD. In each case, n>10. *p=0.015. (c) Schematic representation of EEL-1 and the human ortholog Huwe1. (d) Huwe1 knockdown increases TCF reporter activity in 7DF3 cells after estradiol treatment. (e) Huwe1 knockdown using an independent siRNA increases TCF reporter activity in 7DF3 cells after estradiol treatment. (f) Knock-down of Huwe1 increases Topflash activity in U2OS cells *p=0.044 **p=0.00034 (Students t-test). (g, h, i) HA-ΔN-Huwe1 overexpression inhibits Topflash activity in HEK293T cells expressing Dvl1 (p=0.0022) or LRP6ΔN (p=0.015), but not in cells expressing β-catenin. Data from 3 independent experiments are presented as mean +/- SD.

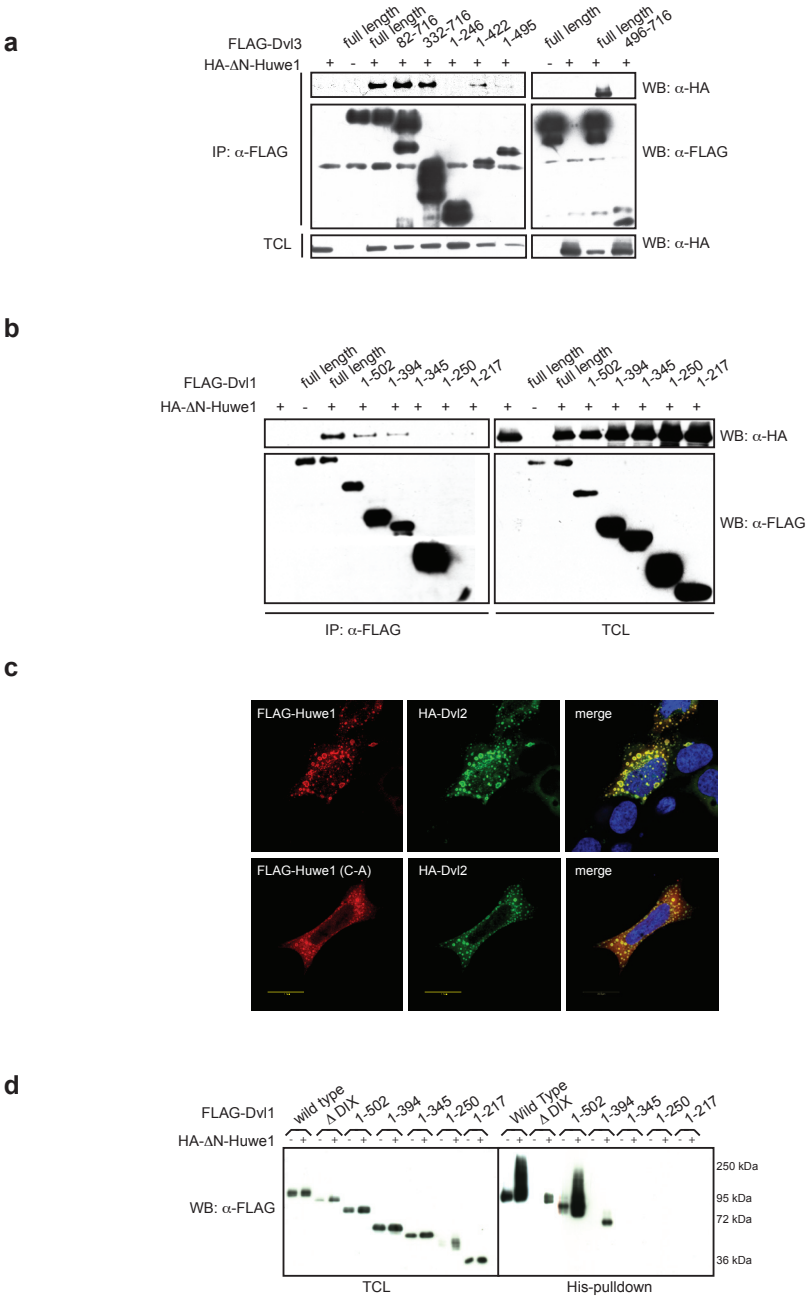
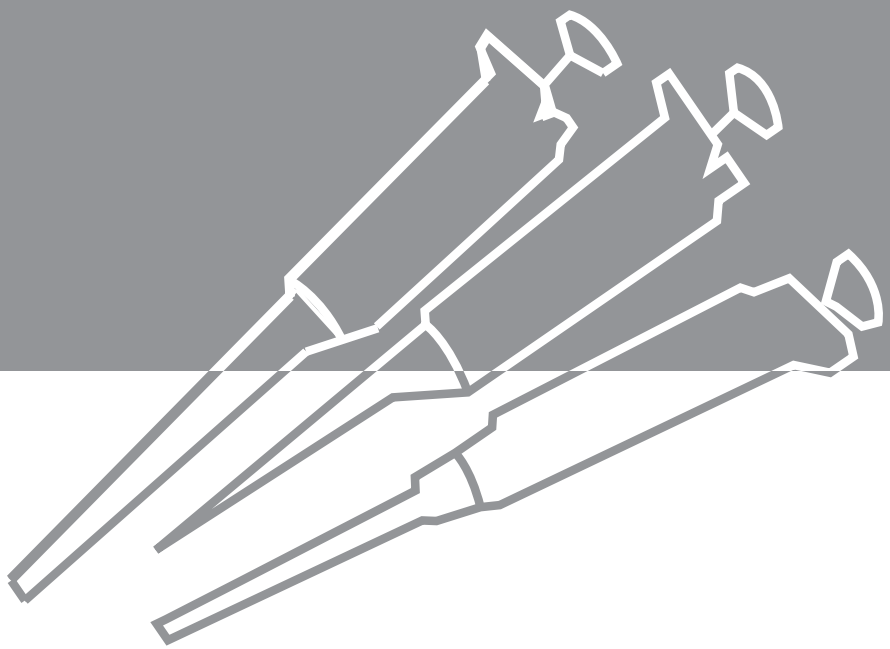


Figure S2. Huwe1 interacts with Dvl and promotes Dvl ubiquitylation. (a) HEK293T cells were transfected with Flag-Dvl3 truncation constructs and HA-ΔN-Huwe1 and co-IP efficiency between Flag-Dvl3 and HA-ΔN-Huwe1 was determined. (b) HEK293T cells were transfected with Flag-Dvl1 truncation constructs and HA-ΔN-Huwe1 and co-IP efficiency between Flag-Dvl1 and HA-ΔN-Huwe1 was determined. (c) HEK293T cells were transfected with Flag-Huwe1 and HA-Dvl2 (upper row) or co-transfected with Flag-Huwe1(CA) and HA-Dvl2 (lower panel). (d) HEK293T cells were transfected with Flag-Dvl1 truncation constructs, His-ubiquitin and HA-ΔN-Huwe1. After pull-down of His-ubiquitin modified proteins, Flag-Dvl1 was detected by Western blotting.



CHAPTER 4

Retromer dependent recycling of the Wnt secretion factor Wls is dispensable for stem cell maintenance in the mammalian intestinal epithelium

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Abstract

In *C. elegans* and *Drosophila*, retromer mediated retrograde transport of Wntless (Wls) from endosomes to the *trans*-Golgi network (TGN) is required for Wnt secretion. When this retrograde transport pathway is blocked, Wls is missorted to lysosomes and degraded, resulting in reduced Wnt secretion and various Wnt related phenotypes. In the mammalian intestine, Wnt signaling is essential to maintain stem cells. This prompted us to ask if retromer mediated Wls recycling is also important for Wnt signaling and stem cell maintenance in this system. To answer this question, we generated a conditional *Vps35*^{fl} allele. As Vps35 is an essential subunit of the retromer complex, this genetic tool allowed us to inducibly interfere with retromer function in the intestinal epithelium.

Using a pan-intestinal epithelial Cre line (*Villin-CreERT2*), we did not observe defects in crypt or villus morphology after deletion of *Vps35* from the intestinal epithelium. Wnt secreted from the mesenchyme of the intestine may compensate for a reduction in epithelial Wnt secretion. To exclude the effect of the mesenchyme, we generated intestinal organoid cultures. Loss of *Vps35* in intestinal organoids did not affect the overall morphology of the organoids. We were able to culture *Vps35*$^{\Delta/\Delta}$ organoids for many passages without Wnt supplementation in the growth medium. However, Wls protein levels were reduced and we observed a subtle growth defect in the *Vps35*$^{\Delta/\Delta}$ organoids. These results confirm the role of retromer in the retrograde trafficking of Wls in the intestine, but show that retromer mediated Wls recycling is not essential to maintain Wnt signaling or stem cell proliferation in the intestinal epithelium.

Introduction

The mammalian intestinal epithelium is a rapidly self-renewing tissue. Stem cells endow the intestine with its proliferative capacity. Intestinal stem cells reside at the bottom of invaginations of the intestinal epithelium; the crypts of Lieberkühn. The intestinal stem cells are characterized by the expression of Lgr5[1], they are actively cycling and give rise to cells that proliferate in the transiently amplifying (TA) compartment of the crypt[2]. Cells move up from the TA compartment and differentiate in the villus domain. The villus epithelium consists of enterocytes, goblet cells and enteroendocrine cells. Paneth cells are differentiated cells that reside at the bottom of the crypt. The Paneth cells are part of the stem cell niche that supports the intestinal stem cells[3]. Various signaling pathways - such as the Wnt, Notch and EGF signaling cascades - are required to maintain intestinal homeostasis[2], but Wnt signaling is of particular importance because it drives proliferation and is essential for stem cell maintenance.

Wnt signaling in intestinal stem cells is activated by Wnt ligands that are expressed in the Paneth cells and cells in the intestinal mesenchyme[4]. Wnt signaling is enhanced by R-spondin, which is the ligand of the stem cell marker Lgr5[5]. It is essential that a fine balance of Wnt pathway activity is maintained in the intestine, as overactivation of Wnt signaling results in adenoma formation and ultimately leads to cancer[6].

Detailed knowledge has accumulated about the mechanism of Wnt signal transduction in Wnt receiving cells, but the mechanism of Wnt secretion has only recently been uncovered (reviewed by Port and Basler and Lorenowicz and Korswagen[7,8]). Wnt protein is produced in the ER and lipid modified by the O-acyltransferase Porcupine[9,10]. Wnt follows the secretory pathway to the Golgi apparatus where it associates with Wntless (Wls), a transmembrane

protein that is essential for Wnt secretion[11–13]. Wls escorts Wnt from the Golgi to the plasma membrane where Wnt is released. Importantly, studies in *C. elegans*, *Drosophila* and mammalian tissue-culture cells have shown that Wls needs to be retrieved back to the *trans*-Golgi network (TGN) to maintain Wnt secretion. This retrieval route involves AP-2 and clathrin mediated endocytosis of Wls from the plasma membrane[14–16] and transport from endosomes to the TGN, a retrograde trafficking step that is mediated by the retromer complex[14,15,17–20]. In the absence of a functional retromer complex, Wls is retained in the endosomal system and degraded in lysosomes. As a result, less Wls is available in the Golgi to mediate Wnt secretion, leading to various Wnt signaling related phenotypes[14,15,18–22].

The retromer complex is a multi-protein complex that mediates transport of membrane proteins from endosomes to the TGN. Retromer cargo proteins include the cation-independent mannose-6-phosphate receptor (CI-MPR), Sortilin, the polarity protein Crumbs and Wls (reviewed by Seaman[23]). Vps35 is the central cargo-binding subunit of the retromer complex and loss of Vps35 strongly reduces Wnt secretion in *C. elegans, Drosophila* and mammalian tissue culture cells[14,18–20,24].

Retromer mediated recycling of Wls is required for Wnt signaling in invertebrate model systems, but the *in vivo* role of this retrieval pathway has not been tested in mammalian Wnt signaling. We generated a floxed allele of *Vps35* to conditionally interfere with retromer function in the murine intestinal epithelium. We investigated the effect of *Vps35* deletion *in vivo,* and in a recently established intestinal organoid culture system. We show that Vps35 is required to maintain Wls protein levels in intestinal cells, but growth of intestinal organoids was only mildly affected. This suggests that retromer mediated recycling of Wls is dispensable in the mammalian intestinal epithelium in steady state conditions.

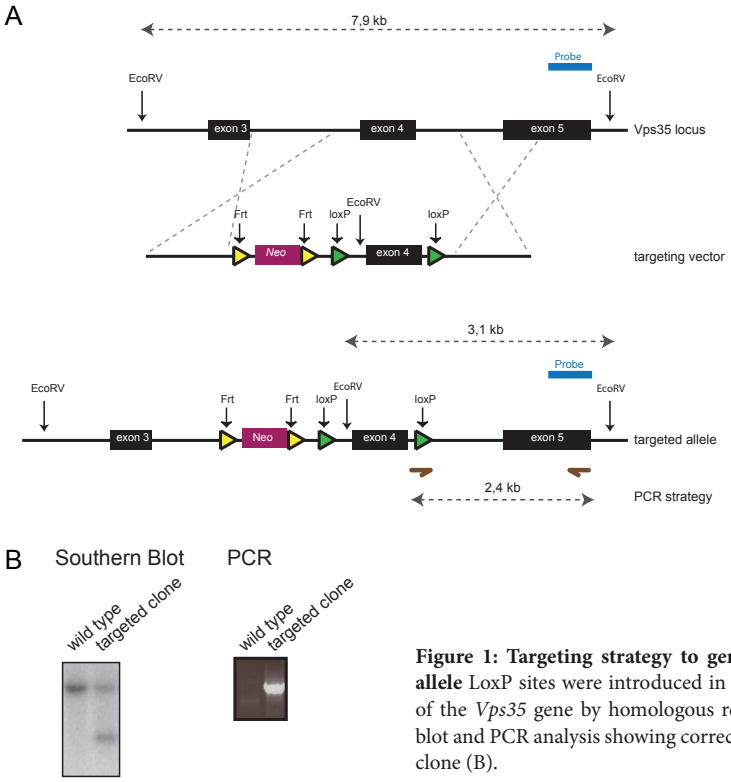


Figure 1: Targeting strategy to generate a conditional *Vps35* allele LoxP sites were introduced in the third and fourth introns of the *Vps35* gene by homologous recombination (A). Southern blot and PCR analysis showing correct targeting of selected ES cell clone (B).

Results

Since loss of retromer function is embryonic lethal[25] and we aimed to specifically investigate the role of *Vps35* in the intestinal epithelium, we generated a conditional *Vps35*^{fl} allele to inducibly delete *Vps35* in the intestinal epithelium of 4 week old mice. We introduced loxP sites in the third and fourth intron of *Vps35* by homologous recombination in mouse embryonic stem cells. We crossed the *Vps35*^{fl} mice to animals carrying a CreERT2 transgene

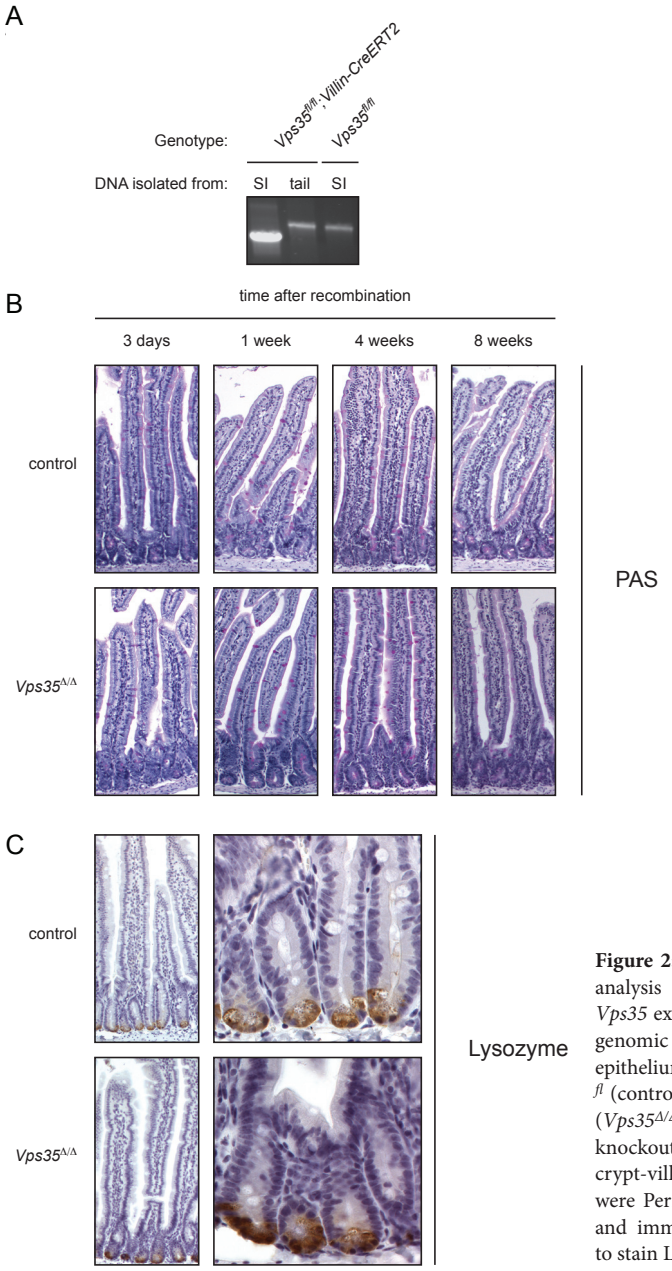
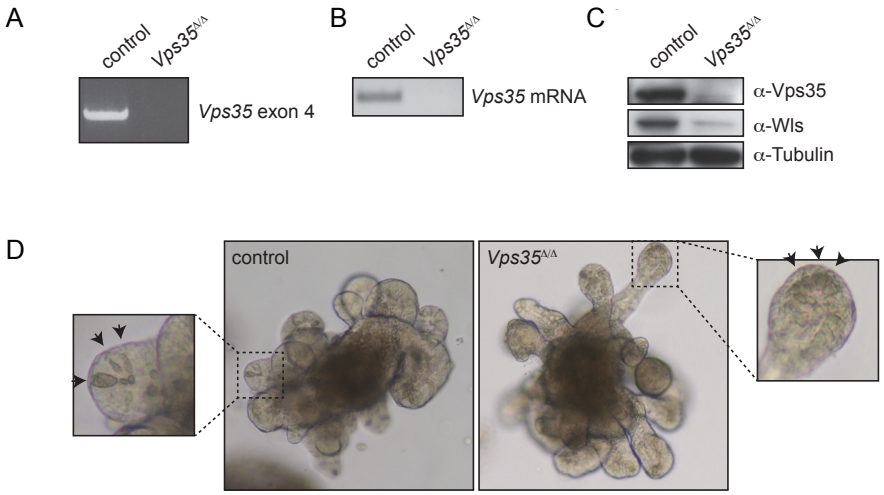


Figure 2: Knockout of *Vps35 in vivo*. PCR analysis using primers that anneal outside *Vps35* exon 4 shows deletion of exon 4 from genomic DNA isolated from small intestinal epithelium (SI) of 4-OHT induced *Vps35*$^{fl/fl}$ (control) or *Vps35*$^{fl/fl}$; *Villin-CreERT2* mice (*Vps35*$^{\Delta/\Delta}$) (A). Histological analysis of *Vps35* knockout intestine showed no defects in crypt-villus morphology. Intestine sections were Periodic Acid Schiff (PAS) stained (B) and immunohistochemistry was performed to stain Lysozyme (C).

driven by the *Villin* promotor (Fig. 1A, B). This allowed us to inducibly delete *Vps35* in the intestinal epithelium using intraperitoneal 4-Hydroxytamoxifen (4-OHT) injections. We confirmed that recombination occurred in the intestinal epithelium by PCR on genomic DNA obtained from intestinal epithelial cells (Fig. 2A). We examined the intestines of the mice 3 days, 1 week, 4 weeks and 8 weeks after 4-OHT injection. We performed periodic acid Schiff (PAS) staining and immunohistochemistry to detect the Paneth cell marker Lysozyme, but we found no qualitative differences in crypt or villus morphology of *Vps35*$^{\Delta/\Delta}$ mice compared to control littermates (Fig. 2B, C). This result indicates that *Vps35* is dispensable for intestinal homeostasis in adult mice.

It was recently shown that Wnt ligands secreted from the mesenchyme of the intestine can compensate for loss of *Wnt3* from the intestinal epithelium[4]. We reasoned that these mesenchyme derived Wnt ligands may also compensate for a reduction in Wnt secretion induced by loss of *Vps35* from epithelial cells. In order to investigate the effect of loss of *Vps35* specifically in the intestinal epithelium, without the influence of the surrounding mesenchyme, we derived intestinal organoids from the *Vps35*$^{fl/fl}$; *Villin-CreERT2* mice. Intestinal organoids can develop form single Lgr5(+) stem cell and consist solely of epithelial cells. Organoids form crypt-like buds that contain stem cells and Paneth cells as well as villus-like domains that contain differentiated cells[26]. We induced recombination *in vitro* by addition of 4-OHT to the culture medium. Using PCR analysis, we found that the recombination of the *Vps35*$^{fl/fl}$ allele



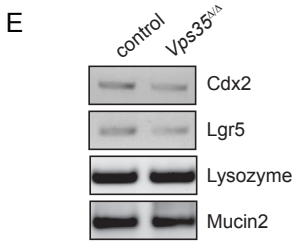


Figure 3: Knockout of *Vps35* in intestinal organoids. Intestinal organoids were obtained from a *Vps35*$^{fl/fl}$; *Villin-CreERT2* mouse and treated with 0.5µM 4-OHT for 12 hours (*Vps35*$^{\Delta/\Delta}$), or control treated (control). PCR analysis of genomic DNA from *Vps35* knockout organoids shows complete deletion of exon 4 of *Vps35 in vitro* (A). RT-PCR shows absence of *Vps35* exon 4 from mRNA of *Vps35*$^{\Delta/\Delta}$ organoids (B). Western blot analysis shows absence of Vps35 protein and reduced Wls protein levels in *Vps35*$^{\Delta/\Delta}$ organoids (C). *Vps35*$^{\Delta/\Delta}$ organoids show normal morphology, Paneth cells are indicated by arrowheads. (D). RT-PCR analysis of molecular markers of differentiated intestinal cells and intestinal stem cells (E).

was complete (Fig. 3A, B). Western blot analysis showed that Vps35 protein was absent form the *Vps35*$^{\Delta/\Delta}$ organoids. Importantly, we found that Wls protein levels were strongly reduced (Fig. 3C), demonstrating that Vps35 is required to maintain Wls levels in the murine intestinal epithelium. These results are consistent with the current model of Wls trafficking and for the first time show that retromer dependent trafficking is required for Wls stability in the mouse.

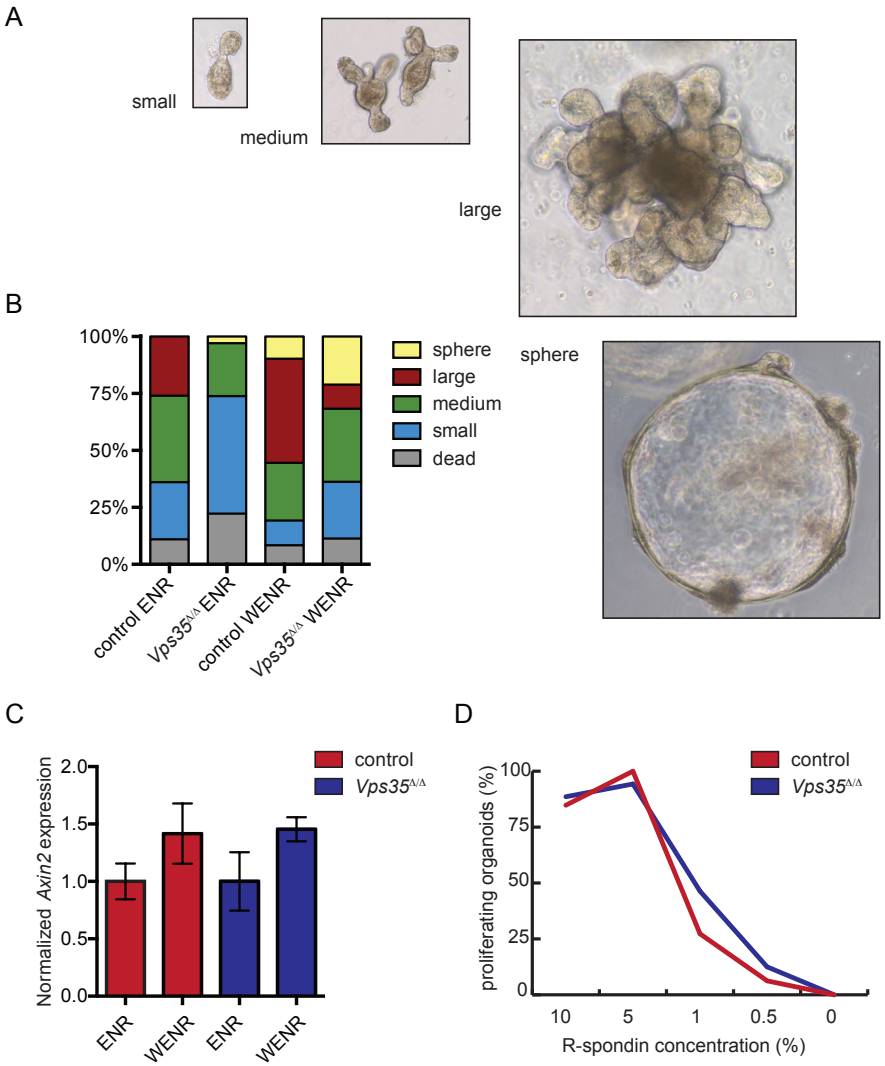


Figure 4: *Vps35*$^{\Delta/\Delta}$ organoids show a growth defect but are competent to respond to Wnt signaling. Growth was quantified by scoring the organoids in categories based on the number of buds the organoids had produced 5 days after passaging (A). Vps35$^{\Delta/\Delta}$ organoids show reduced proliferation compared to control organoids. This could not be completely rescued by Wnt3a supplemented in the medium (ERN: small intestine organoid medium, containing EGF, R-Spondin, Noggin, WENR: ERN medium supplemented with 30% Wnt3a conditioned medium) (B). Vps35$^{\Delta/\Delta}$ organoids can respond to Wnt signaling as assayed by Axin2 qPCR (data are represented as mean ± SD, n=3) (C). Percentage of growing organoids cultured in ENR medium with varying R-spondin concentrations (D).

We did not observe any major morphological changes in the phenotype of the *Vps35*$^{\Delta/\Delta}$ organoids. Paneth cells were visible at the tips of the buds (Fig. 3D) and reverse transcriptase PCR (RT-PCR) analysis showed that the *Vps35*$^{\Delta/\Delta}$ organoids express the intestine marker *Cdx2*, the stem cell marker *Lgr5*, the Paneth cell marker *Lysozyme* and the goblet cell marker *Mucin2* (Fig. 3E). We were able to culture the *Vps35*$^{\Delta/\Delta}$ organoids for many passages (>30) in standard intestinal organoid culture medium that contains EGF, Noggin and R-spondin (ENR medium). In contrast, deletion of *Wnt3* from intestinal organoids causes a characteristic 'pointy crypt' phenotype and a loss of Paneth cells in the buds[4]. Furthermore, *Wnt3*$^{\Delta/\Delta}$ organoids cannot be maintained in regular ENR medium, but need Wnt supplemented in the medium for continuous culturing[4]. We conclude that deletion of *Vps35* does not affect the gross morphology or block differentiation or proliferation of intestinal organoids.

Next, we quantified the growth of *Vps35*$^{\Delta/\Delta}$ organoids and compared it to control organoids. We categorized the organoids based on the number of crypt-like buds that had developed five days after passaging and used this as a proxy for the growth rate (Fig. 4A). We found that *Vps35*$^{\Delta/\Delta}$ organoids consistently had fewer buds compared to control organoids. However, we found that this reduction in growth rate can only be partly attributed to a reduction in Wnt3 secretion because supplementation of exogenous Wnt3a in the medium did not fully rescue this growth defect (Fig. 4B). These results show that expansion of *Vps35*$^{\Delta/\Delta}$ organoids is reduced through both Wnt dependent and Wnt independent effects.

We found that the *Vps35*$^{\Delta/\Delta}$ organoids are competent to respond to Wnt3a, since both control and *Vps35*$^{\Delta/\Delta}$ organoids formed spheroid structures upon Wnt3a stimulation, a phenotype that is characteristic for Wnt pathway activation in intestinal organoids[3]. Furthermore, quantitative PCR (qPCR) analysis showed that Wnt3a stimulation induced a similar upregulation of the Wnt target gene *Axin2* in control and *Vps35*$^{\Delta/\Delta}$ organoids (Fig. 4C).

Finally, we investigated the dependence of *Vps35*$^{\Delta/\Delta}$ organoids on the Wnt signaling agonist R-spondin. R-spondin is the ligand for Lgr5 and Lgr4 and forms an essential component of the culture medium of intestinal organoids[5]. We titrated the R-spondin concentration in the medium, but we found no difference in survival rates between control and *Vps35*$^{\Delta/\Delta}$ organoids cultured in different R-spondin concentrations (Fig. 4D).

Discussion

Retromer dependent recycling of Wls is essential for efficient Wnt secretion in *C. elegans, Drosophila* and mammalian tissue culture cells[14,17–20], but the *in vivo* role of Wls recycling in mammalian Wnt signaling has not been tested. In the murine intestine, Wnt signaling is required for proliferation and stem cell maintenance. We therefore investigated whether the retromer complex is required for stem cell maintenance and tissue homeostasis in the intestine.

We generated a floxed allele to inducibly delete *Vps35* from the intestinal epithelium of adult mice. We did not observe defects in the morphology of the intestine of these mice. To circumvent potential effects of Wnt secretion from the surrounding mesenchyme, we derived *Vps35*$^{fl/fl}$ intestinal organoids. Deletion of *Vps35* in these organoids showed that Vps35 is required to maintain Wls protein levels in the intestinal epithelium. This confirms the role of retromer in retrieving Wls from the endosomal-lysosomal degradative pathway in intestinal cells. Surprisingly, the *Vps35*$^{\Delta/\Delta}$ organoids could be cultured for many passages and showed no morphological defects. Loss of *Vps35* did not affect the ability of the organoids to respond to Wnt signals or the dependence on R-spondin. However, the *Vps35*$^{\Delta/\Delta}$ organoids showed

a reduced growth rate compared to control organoids. This proliferation defect cannot be completely rescued by supplementation of Wnt3a in the medium, indicating that *Vps35* controls organoid growth through both Wnt dependent and Wnt independent effects.

Why does loss of Vps35 and the resulting reduction in Wls protein levels cause only a subtle proliferation defect in the mouse intestine? In *C. elegans*, loss of the retromer complex mainly affects Wnt signaling processes that act over a relatively long distance, such as neuroblast migration and the establishment of neuronal polarity[22,24]. These Wnt signaling processes require the formation of long-range Wnt concentration gradients and are therefore dependent on efficient Wnt secretion. In contrast, loss of retromer function does not significantly affect Wnt signaling processes that take place between neighboring cells[24]. Also in the *Drosophila* wing imaginal disc, loss of retromer mainly affects the expression of high threshold Wnt target genes, while low threshold target genes such as *Distalless* are normally expressed [18–20]. Taken together, these studies show that loss of retromer reduces, but not eliminates Wnt secretion in worms and flies. Stem cells in intestinal organoids require stimulation by Wnt proteins that are secreted from neighboring Paneth cells[3]. In analogy with *C. elegans*, this short range Wnt signaling may be less sensitive to loss of *Vps35*. In addition, Wnt signaling in intestinal stem cells is amplified by R-spondin, a ligand that acts through the Lgr5 receptor[5]. This amplification mechanism may also explain why the intestinal stem cells are relatively insensitive to a reduction in Wnt secretion. Finally, it has been shown that Wls is a Wnt target gene in the mouse[27]. By stimulating the expression of Wls, secretion of mammalian Wnt proteins may be less dependent on retromer mediated recycling of Wls.

The subtle growth defect that we observed could not be fully rescued by supplementation of Wnt3a in the culture medium. Missorting of other retromer cargos such as the CI-MPR, Sortilin or the polarity protein Crumbs, may negatively influence organoid growth[23]. It was recently shown that Lgr5 undergoes retrograde traffic from endosomes to the TGN in Human Embryonic Kidney (HEK) cells. Therefore, Lgr5 itself may be a retromer cargo in these cells[28] and potentially in the intestinal stem cells. However, the fact that the *Vps35*$^{\Delta/\Delta}$ organoids were equally dependent on R-Spondin argues against a critical role of retromer in Lgr5 regulation. We were unable to detect endogenous Lgr5 protein in organoid lysates by Western blot (data not shown), so we could not confirm if Lgr5 is a retromer cargo in intestinal cells.

In our experimental approach, we investigated the role of *Vps35* in Wnt signaling and stem cell maintenance in intestinal homeostasis of juvenile and adult mice. In these conditions, retromer mediated recycling of Wls is dispensable. There may be situations, for example during embryonic development or during regeneration after injury, which require enhanced levels of Wnt secretion, and may therefore be more dependent on retromer mediated retrograde transport of Wls. For example, Wnt5a signaling is essential for intestinal tube elongation during development and regeneration of the colon after tissue injury[29,30]. It will be interesting to determine if *Vps35*$^{\Delta/\Delta}$ mice have defects in recovery from injury, or if deletion of *Vps35* during development of the intestine causes defects.

In summary, we show that the retromer complex is required to maintain high Wls protein levels in intestinal epithelial cells, which is in agreement with the current model of Wls trafficking. Proliferation and the maintenance of stem cells in organoids are however minimally affected by loss of *Vps35*. The mouse strain that carries the floxed *Vps35* allele will be a valuable tool to study retromer function during development and regeneration of the intestine as well as in other tissues and in different biological contexts.

Materials and methods

ES cell targeting and generation of mouse strains

Conditional Vps35 mice were generated by homologous recombination in embryonic stem cells using a targeting strategy that is schematically depicted in Fig. 1A. Exon 4 and flanking homology arms were PCR amplified from 129/Ola derived DNA to generate a targeting construct. The linearized construct was transfected into male 129/Ola derived IB10 embryonic stem cells by electroporation (800V, 3mF). Recombinant targeted ES cell clones were selected in medium supplemented with G418, TK was used as counter selection. Clones were screened by Southern blotting and confirmed by PCR analysis. Positive clones were injected into C57BL/6 blastocysts. The neomycin selection cassette was excised in vivo by crossing the mice to the FLPeR deleter strain. Vps35fl mice were crossed to the Villin-CreERT2 strain[31]. Recombination *in vivo* was induced in 4 week old mice by intraperitoneal 4-OHT injection (5 mg 4-OHT, dissolved in 200 ml sunflower oil). Mice were sacrificed 3 days, 1 week, 4 weeks and 8 weeks after Cre induction. Histology and immunohistochemistry was performed as described[32]. The animal experiments were approved by the Animal Experimentation Committee of the Royal Academy of Arts and Sciences (protocol number HL06.1010).

Intestinal organoid culture

Mouse organoids were derived from isolated crypts of the proximal small intestine of a Vps35$^{fl/fl}$;Villin-CreERT2 mouse as described[26]. The organoids were maintained in ENR culture medium in a drop of Matrigel (BDBiosciences) as described[26]. The ENR culture medium consists of advanced Dulbecco's modified Eagle medium/F12 supplemented with penicillin/streptomycin, 10 mmol/L HEPES, 1xGlutamax, 1xB27 (Life Technologies), 1 mmol/L N-acetylcysteine (Sigma), 50 ng/mL murine recombinant EGF (Peprotech), R-spondin1 (conditioned medium, 10% final volume) and Noggin (conditioned medium, 10% final volume). The conditioned media were produced using HEK293T cells stably transfected with HA-mouse Rspo1-Fc (gift from Calvin Kuo, Stanford University) or after transient transfection with mouse Noggin-Fc expression vector. Advanced Dulbecco's modified Eagle medium/F12 supplemented with penicillin/streptomycin, 10 mmol/L HEPES, and 1xGlutamax was conditioned for 1 week. Wnt3a conditioned medium was produced using stably transfected L cells after 1 week of conditioning in medium containing 10% fetal bovine serum. To induce Vps35 deletion, 4-OHT (Sigma, 0.5 mM/L) was added to the culture medium for 12 hours. Organoid growth was quantified by scoring the number of buds that had developed 5 days after passaging.

RT-PCR and qPCR

Organoids were dissolved in TRIzol (Life technologies), RNA was isolated from organoids using an RNAeasy kit (Qiagen) and cDNA was generated using the RT-II kit (Invitrogen) using oligo dT primers. Primers sequences used for RT-PCR:
Vps35: CTGTTGGCTCTCCTTCATCAG; AACTGCACTACTTGGAGGTC,
Cdx2: GTACACAGACCATCAGCGGC; CCACCCCATCCAGTCTCACT,
Lgr5: TGCCATCTGCTTACCAGTGTTGT; ATTCCGTCTTCCCACCACGC,
Lysozyme: GAGACCGAAGCACCGACTATG; CGGTTTTGACATTGTGTTCGC,
Mucin2: GAACGGGGCCATGGTCAGCA; CATAATTGGTCTGCATGCC.
qPCR was performed using the iQ SYBR green reagent in a MiIQ real-time PCR system (Biorad). Relative expression was calculated using the ΔΔCt method relative to Ywhaz expression.

Primer sequences used for qPCR:
Axin2: TGACTCTCCTTCCAGATCCCA; TGCCCACACTAGGCTGACA,
Ywhaz: TGCAACGATCTACTGTCTCTTTTG; CGGTAGTAGTCACCCTTCATTTTCA.

Western blot analysis of organoid proteins

Organoids were washed twice in ice cold PBS and taken up in Laemli sample buffer. Samples were boiled for 5 minutes prior to SDS-PAGE and Western blotting following standard procedures. The following antibodies were used for detection: anti-Wls (ab72385-500, Abcam), anti-Vps35 (ab10099-100, Abcam), anti-alpha-Tubulin (DM1A, Sigma), anti-mouse-HRP (GE Healthcare), anti-chicken-HRP (Abcam), anti-rabbit-HRP (GE Healthcare).

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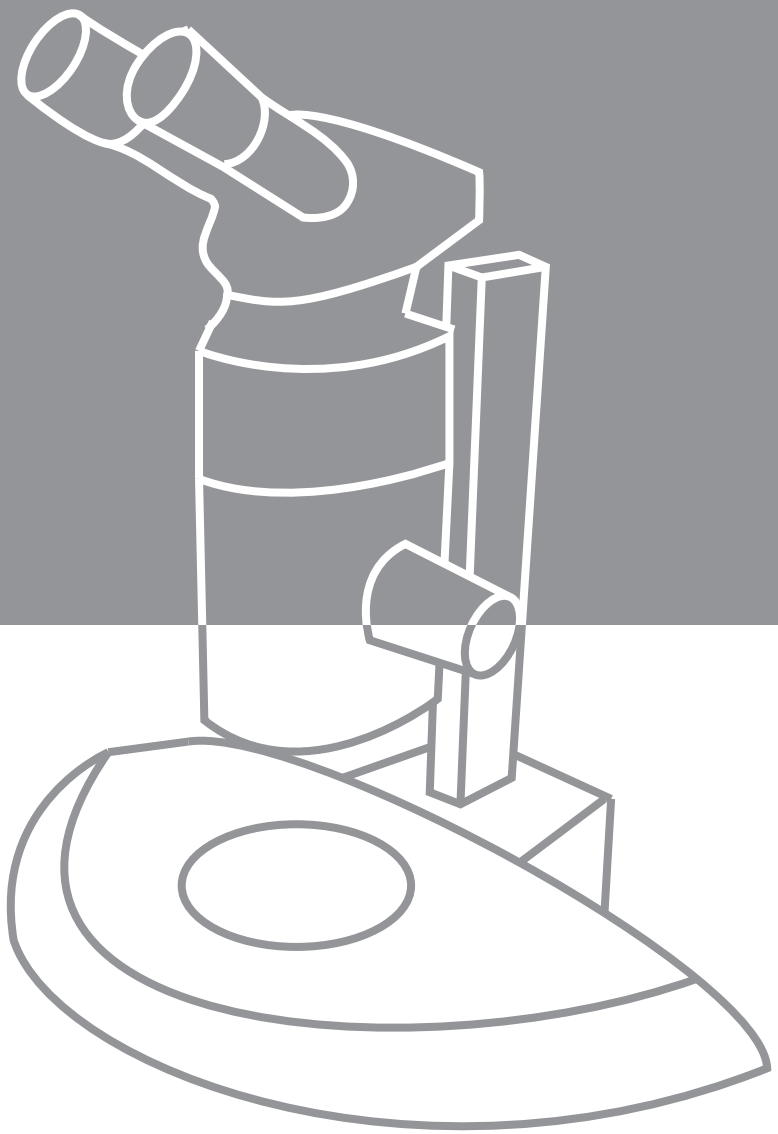

CHAPTER 5

SNX3-retromer requires an evolutionary conserved flippase complex to mediate Wntless sorting and Wnt morphogenic gradient formation

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Abstract

Wntless (Wls) transports Wnt morphogens from the Golgi to the cell surface and is required for Wnt secretion and the establishment of Wnt morphogenic gradients. Recycling of internalized Wls occurs through the SNX3-retromer-dependent endosome-to-Golgi transport pathway[1,2]. SNX3-retromer recycling of Wls does not require the SNX-BAR proteins found in the SNX-BAR-retromer, which via their BAR domain possess membrane remodelling capabilities[3]. This raises the question: how is membrane deformation achieved during the formation of SNX3- retromer-decorated transport carriers? Here we demonstrate that a proposed flippase-containing membrane deformation complex[4], composed of MON2, DOPEY-2 and ATP9A, interacts with SNX3 and is found on sub-domains of the SNX3 retromer labelled endosomes from where SNX3-retromer carriers enriched with Wls are generated. *In vivo* suppression of *C. elegans mon-2, pad-1* or *tat-5*, respective orthologs of MON2, DOPEY-2 and ATP9A, results in enhanced lysosomal degradation of Wls and a Wnt phenotype, the latter also being observed upon overexpression of a flippase inhibited TAT-5(E246Q) mutant. These new mechanistic details of SNX3-retromer mediated transport further our understanding of the diversity of retromer function and the formation of Wnt-morphogenic gradients.

The sorting nexin (SNX) and Bin, Amphiphysin, Rvs (BAR) domain-containing retromer (SNX-BAR-retromer) is composed of two sub-complexes[5]. The first, termed the cargo-selective sub-complex (CSC), is a hetero-trimer of VPS26, VPS29 and VPS35 and functions as a recruitment hub for both cargo and retromer-associated proteins[5,6]. A hetero-dimer of SNX-BAR proteins, SNX1 or SNX2 complexed to either SNX5, SNX6 or SNX32, forms the second subcomplex which is responsible for membrane deformation and subsequent carrier formation[7,8]. It is postulated that the SNX-BAR proteins generate membrane curvature through the insertion of an amphipathic helix into the lipid bilayer resulting in the generation of positive membrane curvature that is stabilised by the concave shaped BAR domain[3,9,10]. SNX3 also engages the CSC, but does so independently of the SNX-BAR proteins, to form the SNX3-retromer[1,2]. SNX3-retromer decorated carriers are morphologically distinct from the SNX-BAR retromer tubular carriers, appearing instead as small vesicular structures[1]. However, the mechanism by which these SNX3-retromer carriers are generated remains poorly understood.

To address this specific issue, we used quantitative Stable Isotope Labelling with Amino acids in Cell culture (SILAC)-based proteomics to identify potential SNX3 interactors responsible for the coupling of this unique component of the SNX3-retromer to the process of membrane carrier formation. To ensure a low level of transgene expression, RPE1 cells were stably transduced with either GFP or GFP-SNX3, and subsequently grown in medium containing unlabelled (R0, K0) or heavy-labelled (R6, K4) amino acids for approximately six doublings, prior to highly efficient GFP trap immunoisolation[11] and identification of co-immunoprecipitating proteins by LC-MS/MS spectrometry. From 1,854 quantified proteins,

at the TGN. HeLa cells transiently transfected with ATP9A-HA were fixed and stained for TGN46 and EEA1 (red). (G) DOPEY2-GFP binds both MON2 and ATP9A-HA. Cell extracts derived from HEK293 cells transiently transfected with GFP and ATP9A-HA or DOPEY2-GFP and ATP9A-HA were subjected to a GFP nanotrap and subsequently blotted with antibodies raised against endogenous MON2 and HA. (H) MON2 can self-associate. Cell extracts derived from HEK293 cells transiently transfected with GFP and MON2-FLAG or GFP–MON2 and MON2-FLAG were subjected to a GFP nanotrap and subsequently blotted with a FLAG antibody. (I) DOPEY2 can self-associate. Cell extracts derived from HEK293 cells transiently transfected with GFP and DOPEY2-FLAG or DOPEY2-GFP and DOPEY2-FLAG were subjected to a GFP nanotrap and subsequently blotted with an anti-FLAG antibody.

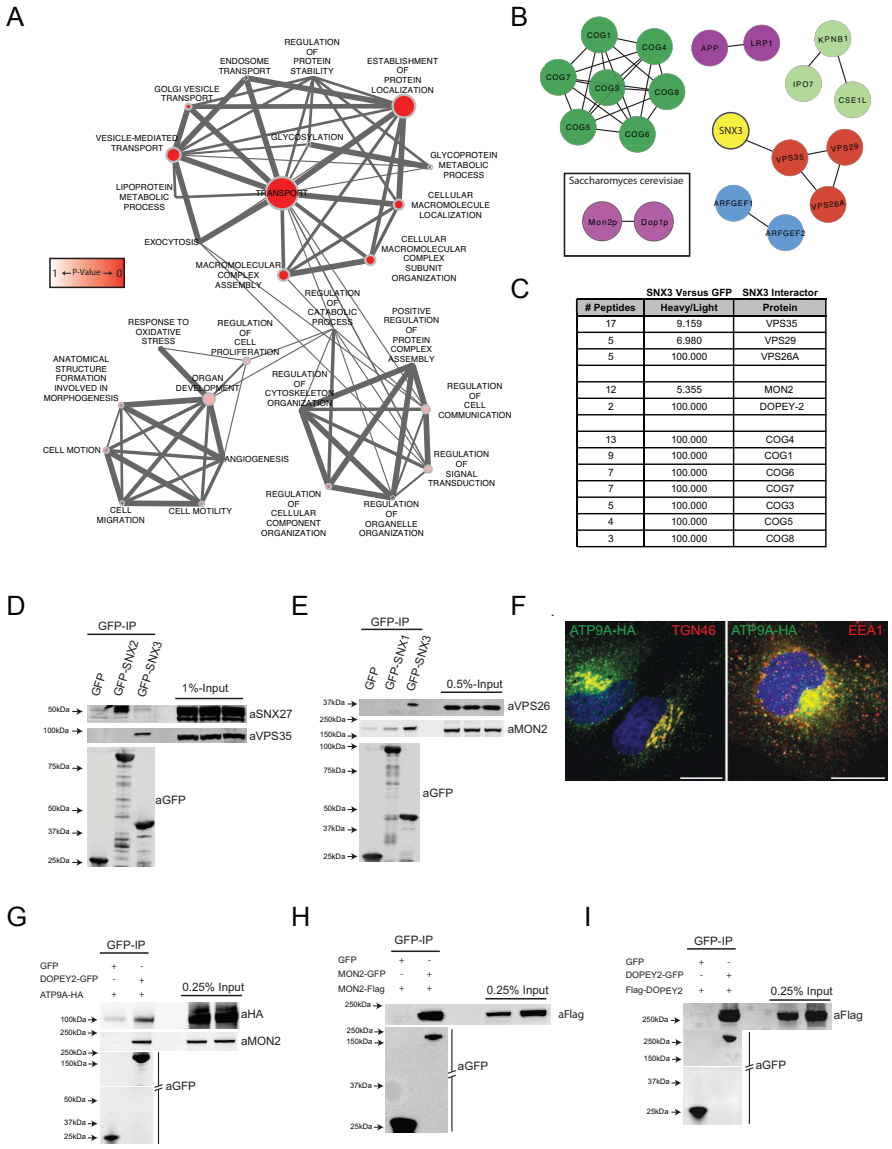

Figure 1: SNX3 engages an evolutionarily conserved flippase complex proposed to regulate membrane deformation. (A) Functional annotation analysis of proteins identified in the SNX3 SILAC proteomics using Gene Ontology annotations with a greater than 3 fold enrichment and with a minimum of two peptides revealed a preponderance of proteins involved in "transport" (47/106), "vesicle mediated transport" (33/106) and "establishment of protein localization" (33/106). The larger the node the greater the number of proteins classified in that category, while the thicker the edge between nodes the greater the overlap of proteins within those classifications. (B) Network analysis of putative SNX3 interactome components classified within the "transport" node using the STRING database. (C) Fold enrichment and number of peptides detected for components of the retromer and COG complexes and MON2 and DOPEY-2 in the SNX3 proteomics. (D) SNX3 does not engage SNX27. HEK293 cells transiently expressing GFP-SNX2 and GFP-SNX3 were immuno-precipitated and analyzed for binding to endogenous SNX27. (E) SNX3 but not SNX1 associates with MON2. Cell extracts derived from RPE1 cells lentivirally transduced with GFP, GFP–SNX3 or GFP–SNX1 were subjected to a GFP nanotrap and subsequently analyzed for binding to MON2. (F) ATP9A-HA is found on early endosomes and

data was filtered based on two criteria: a greater-than 3-fold enrichment in the GFP-SNX3 over GFP interactome; and protein quantification achieved through 2 or more peptides. This defined the resultant 106 enriched proteins as the SNX3 interactome. Functional annotation analysis of these proteins revealed functional clusters annotated as 'vesicle-mediated transport' (33/106), 'transport' (47/106) and 'establishment of protein localisation' (33/106) (Fig. 1A). In addition, to identify functional complexes and possible network hubs we performed a network analysis (Fig. 1B). Confirming previous work[1,2,12], this identified the retromer CSC components VPS26A, VPS29 and VPS35. Importantly, within the SNX3 interactome there was no enrichment in the retromer SNX-BARs, SNX1, SNX2, SNX5, SNX6 or SNX32, nor SNX27, the core component of the SNX27-retromer[13,14]; data that we confirmed by western analysis (Fig. 1D). Moreover, manipulation of *snx-27* did not affect Wnt signalling in *C. elegans* (Supplementary Fig. 1) thereby establishing the *in vitro* and *in vivo* molecular and functional distinction between the SNX3-retromer and the SNX-BAR- and SNX27-retromers[5].

In the context of the mechanism by which SNX3-retromer carriers are generated, it was noteworthy that the SNX3 interactome did not contain any evolutionary conserved proteins with predicted domains known to elicit membrane re-modeling (e.g. BAR domains, ENTH domains). That said, the network analysis did draw our attention to an interaction between *S. cerevisiae* Mon2p and Dop1p, the human equivalents of which, MON2 and DOPEY-2, were both enriched in the SNX3 interactome (Fig. 1B,C). MON2 is a large scaffold protein that is structurally similar to the BIG family of ARF guanine-nucleotide exchange factors (GEFs)[15]. However, MON2 lacks the SEC7 domain that endows this family with GEF activity, making it unlikely that it functions as an ARF-GEF. MON2 can engage the GGA adaptors and control their subcellular distribution[16], and mutants of yeast Mon2p, display defective vacuolar protein sorting[15–17], implicating Mon2p as a protein involved in trafficking. Comparatively little is known about DOPEY-2 and the related DOPEY-1. Yeast have a single DOPEY gene termed DOP1. Dop1p interacts both genetically and physically with Mon2p[17], and together they couple to the endosome and trans-Golgi network (TGN)-resident flippase Neo1p[4]. Neo1p belongs to the P4-type family of ATPases that have proposed aminophospholipid translocase activity that drives the transport of phospholipids from the extracellular or lumenal leaflet to the cytosolic leaflet[18,19]. The net result of such flippase activity is the lateral expansion of one leaflet relative to the other which, if spatially restricted and stabilised by associated coat and affliated accessory proteins, causes lipid asymmetry that supports membrane re-modeling in the form of positive membrane curvature[20]. In the case of Neo1p, the associated Mon2p and Dop1p are suggested to stabilise membrane re-modeling[4]. Given such a precedent, we hypothesized that SNX3 might engage the Neo1p-Mon2p-Dop1p flippase complex to support formation of the observed vesicular SNX3-retromer decorated and Wls-enriched transport carriers[1].

To validate the identification of MON2 we transduced RPE1 cells with GFP, GFP SNX3 or the SNX-BAR-retromer component GFP-SNX1. After GFP-trap immuno-isolation, the resultant immunoprecipitates were probed for the presence of endogenous MON2. This confirmed the association of SNX3 with MON2 and revealed the specific nature of this interaction for the SNX3-retromer over components of SNX-BAR-retromer (Fig. 1E). The Neo1p-Mon2p-Dop1p complex has so far only been shown to exist in yeast[4]. Neo1p has two human orthologs, ATP9A and ATP9B. Transient expression of ATP9A-HA and ATP9B-HA in HeLa cells revealed that, as previously reported[21], ATP9A was localized to endosomes and the TGN as shown by colocalization with the early endosomal marker early endosomal antigen-1 (EEA1) and the TGN marker TGN46 (Fig. 1F), while ATP9B localized exclusively to the Golgi apparatus (data not shown). To determine if endosome-associated ATP9A formed a complex with MON2 and

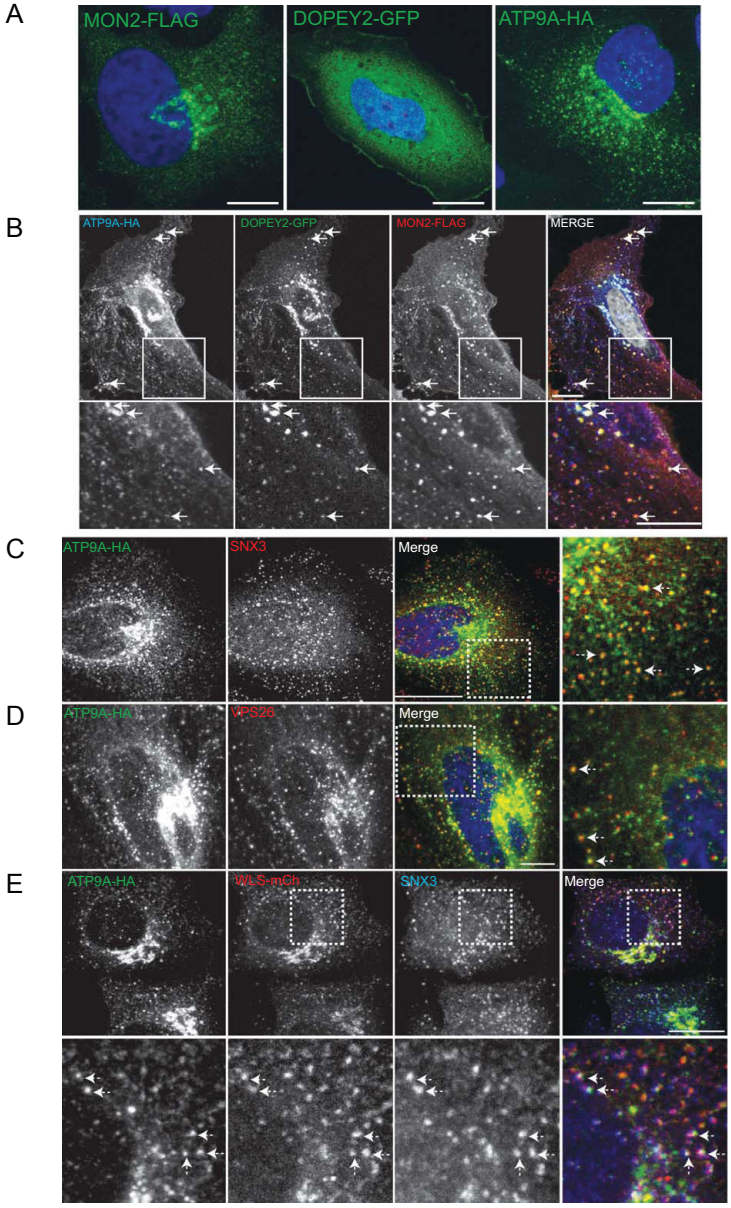

Figure 2: ATP9A recruits MON2 and DOPEY2 to endosomes. (A) HeLa cells were transiently transfected with MON2-Flag, DOPEY2-EGFP or ATP9A-HA, fixed and in the case of MON2-Flag and ATP9A-HA expressing cells stained with anti-FLAG and anti-HA antibodies respectively. Scale bar represents 10 µm. (B) HeLa cells were transiently transfected with ATP9A-HA, DOPEY2- pEGFP and MON2-Flag and fixed and stained with anti-HA and anti-FLAG antibodies. Endosomes labeled with all three are indicated with arrows. Scale bar represents 10 µm. (C,D) ATP9A colocalises with the SNX3-retromer and WLS. HeLa cells were transiently transfected with ATP9A and subsequently fixed and stained for (C) SNX3, (D) VPS26. (E) HeLa cells were transiently transfected with ATP9A and WLS-mCherry and subsequently fixed and stained for SNX3. Arrows indicate endosomes labelled for all three components. Scale bar represents 10 µm.

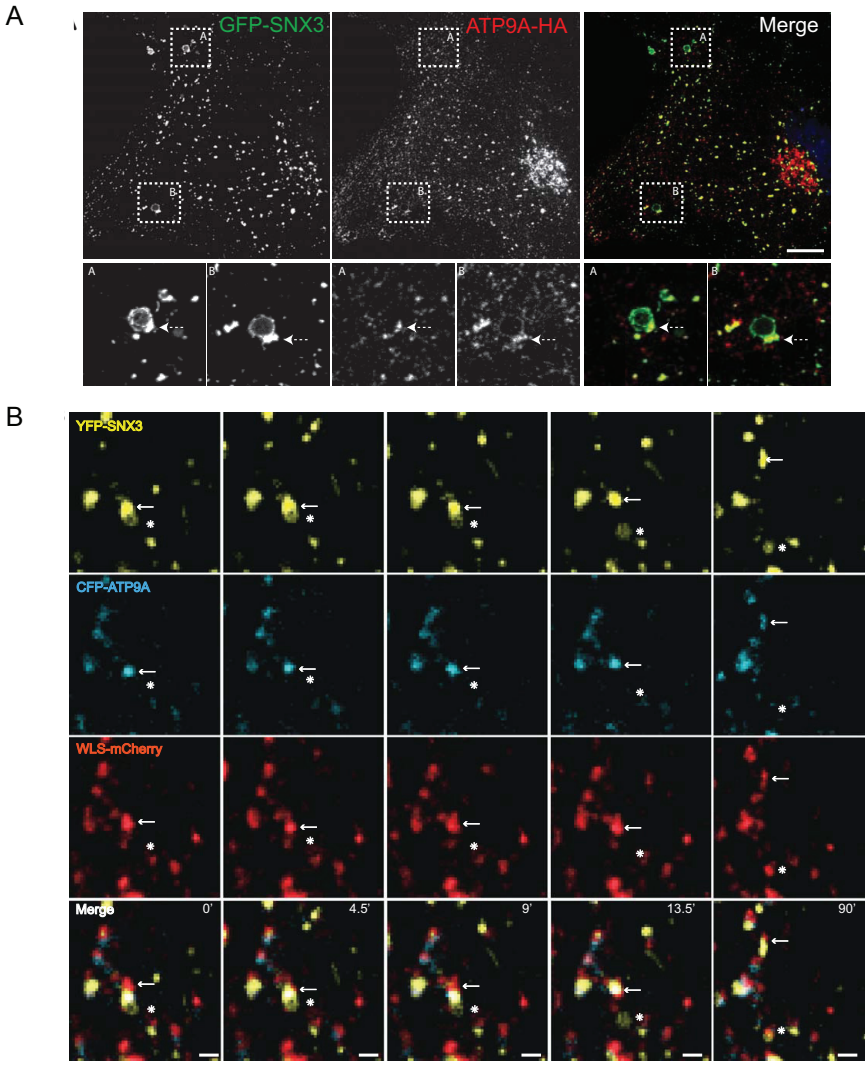

Figure 3: SNX3 carriers enriched with WLS form at ATP9A hotspots. (A) ATP9A accumulates at points of SNX3 enrichment. HeLa cells were transiently transfected with ATP9A-HA and GFP-SNX3 and subsequently fixed and imaged. Arrow indicates SNX3 endosome with ATP9A-HA localizing at the point of greatest SNX3 enrichment. This enrichment at a point on the endosome usually precedes SNX3 carrier formation. Scale bar represents 10 µm. (B) RPE-1 cells were transiently co-transfected with YFP-SNX3, WLS-mCherry and CFP-ATP9A. Cells were subsequently imaged live after a 36 h incubation period. Frames depicting the formation and scission of YFP-SNX3- labelled buds at CFP-ATP9A hotspots from endosomes positive for both YFP-SNX3, CFP-ATP9A and WLS-mCherry are shown. The arrow indicates the CFP-ATP9A hotspot that is enriched with both SNX3 and WLS and the subsequent budded vesicle. The asterisk indicates the endosome. Scale bar 2 µm.

DOPEY-2, HEK293 cells were transiently transfected with ATP9A-HA and DOPEY-2-GFP prior to immunoprecipitation of DOPEY-2-GFP and western blotting with antibodies raised against endogenous MON2 and the HA epitope. This revealed that immunoprecipitation of DOPEY-2-GFP co-precipitated ATP9AHA and endogenous MON2, verifying that the yeast complex also exists in humans (Fig. 1G). Interestingly, both MON2 and DOPEY-2 possessed the ability to self-associate, as shown by GFP-MON2 and GFP-DOPEY-2 coimmunoprecipitating their Flag-tagged equivalents (Fig. 1H and I). Thus, like their yeast counterparts[4,17], and a common characteristic of many coat complexes[22], MON2 and DOPEY-2 have the ability to form higher-ordered assemblies that may aid membrane re-modeling.

We next investigated the subcellular distribution of the ATP9A-MON2-DOPEY-2 complex. GFP or Flag tagged MON2 localized predominantly to the TGN but was also observed on a small number of cytoplasmic punctae (Fig. 2A), while DOPEY-2-GFP was cytoplasmic (Fig. 2A). Consistent with the assembly of the ATP9A-MON2-DOPEY-2 complex, co-expression of MON2-Flag and DOPEY-2-GFP with ATP9A-HA led to the recruitment of both MON2 and DOPEY-2 to ATP9A-labeled early endosomes (Fig. 2B). Together these data establish the evolutionary conserved nature of the ATP9A MON2- DOPEY-2 complex and reveal that interaction of ATP9A with MON2 and DOPEY-2 aids the recruitment of these cytosolic proteins to early endosomes.

To define the spatial relationship between SNX3-retromer and the ATP9A-MON2-DOPEY-2 complex, we transiently transfected ATP9A-HA into HeLa cells, fixed and stained for HA and either endogenous SNX3 or VPS26. ATP9A showed extensive co-localization with SNX3 and VPS26 (Fig. 2C, D). In addition, the flippase was observed on endosomes positive for both SNX3 and the SNX3-retromer cargo Wls (Fig. 2E). Intriguingly, on enlarged SNX3-labelled endosomes, ATP9A-HA localized to 'hot-spots' on the limiting membrane that were also enriched with SNX3 (Fig. 3A). Triple live cell imaging of CFP-ATP9A, YFP-SNX3 and mCherry-Wls revealed that both ATP9A and Wls were most frequently found at the site of SNX3 enriched subdomains prior to the formation of SNX3-labelled carriers and that both ATP9A and Wls were found on subsequent SNX3 carriers as they moved away from the early endosome (Fig. 3B, Supplementary Movie 1). These data place the ATP9A-MON2-DOPEY-2 complex at the precise sub domain from where membrane re-modeling occurs to generate SNX3-retromer decorated and Wls-containing vesicular transport carriers.

SNX3-retromer mediates endosome-to-TGN retrieval of the Wls ortholog MIG-14 in *C. elegans*[1]. In mutants of *snx-3* and the CSC subunit *vps-35*, MIG-14 fails to be retrieved from the endosomal system and is degraded in lysosomes[1,2]. As a consequence, steady state levels of MIG-14 are reduced, leading to defects in Wnt secretion and a range of Wnt related phenotypes[1,23,24]. To investigate whether the ATP9A-MON2-DOPEY-2 complex is required for Wls recycling in *C. elegans*, we knocked down the MON2 ortholog *mon-2* and the DOPEY-2 ortholog *pad-1* using RNA interference (RNAi) and examined whether steady state levels of MIG-14 were changed. Similar to knock down of *vps-35*, depletion of *mon-2* and *pad-1* resulted in a significant reduction in total MIG-14 protein levels (Fig. 4A). Furthermore, knock down of *mon-2* and *pad-1* induced clear co-localization of a functional MIG-14::GFP fusion protein with the late endosomal and lysosomal marker LMP-1::mCherry (Fig. 4B)[1], indicating that MIG-14 is targeted for lysosomal degradation in the absence of *mon-2* and *pad-1*.

Next, we investigated whether knock down of *mon-2* and *pad-1* reduces Wnt secretion. The Wnt protein EGL-20 is expressed by a group of cells in the tail and forms a posterior to anterior concentration gradient which can be visualized using a functional fusion of EGL-20 with the immunoglobulin domain of protein A[1,23]. In control RNAi treated animals, EGL-20::protA was visible as a punctate staining pattern that ranges from the producing cells in

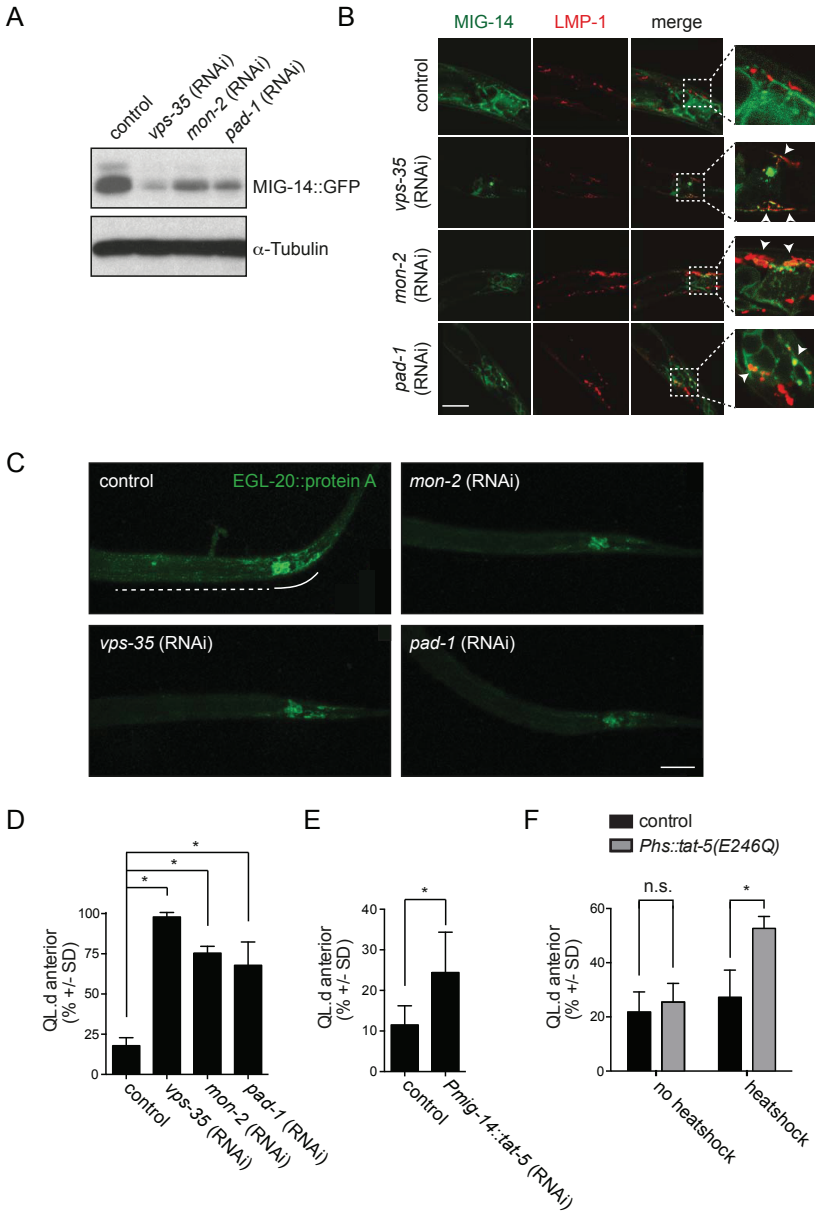

Figure 4: The ATP9A-MON-2-DOPEY-2 complex is required for Wls recycling, Wnt gradient formation and Wnt signaling in *C. elegans*. (A) Western blot quantification of endogenously expressed MIG-14::GFP (huSi2) protein levels in animals treated with control, *vps-35, mon-2* or *pad-1* RNAi. (B) Confocal imaging of MIG-14::GFP (green, huIs72) and the late endosomal and lysosomal marker LMP-1::mCherry (red, huEx149) in L1 larvae. The tail area, which includes the EGL-20 producing cells, is shown. Arrows indicate regions of colocalization. In all images, anterior is left and dorsal is up. Scale bar is 10 µm. (C) Staining of EGL-20::protA with rabbit-anti-goat-Alexa647 in L1 larvae[1,23]. The EGL-20 producing cells are indicated with a solid line, the punctate gradient that is formed by EGL-20::protA is indicated with a dashed line. In all images, anterior is left and dorsal is up. Scale bar is 10 µm. (D) Systemic knock down of *mon-2* or *pad-1* interferes with the EGL-20/Wnt dependent posterior migration of the QL descendants (QL.d) in a *vps-29(tm1320)* sensitized genetic background. The percentage of animals with anteriorly displaced QL.d is shown (data are presented as mean +/- SD

the tail to the mid-body region (Fig. 4C). Knock down of *mon-2* and *pad-1* resulted in a clear reduction in EGL-20::protA staining, indicating that *mon-2* and *pad-1* are required for Wnt secretion *in vivo*.

During the first stage of larval development, EGL-20 controls the posterior migration of the QL neuroblast descendants (QL.d)[25,26]. In the absence of EGL-20 or in mutants that interfere with Wnt secretion, the QL.d migrate in the opposite, anterior direction[1,23,26,27]. To investigate whether *mon-2* and *pad-1* are required for EGL-20 signaling, we asked whether knock down of these genes affects the posterior migration of the QL.d. Because QL.d migration is relatively insensitive to changes in EGL-20 levels[1], we used a sensitized genetic background (a mutation in the retromer subunit gene *vps-29* which partially reduces MIG-14 retrieval and enhances the QL.d migration phenotype of Wnt secretion pathway components[1]). Similar to knock down of *vps-35*, we found that RNAi of *mon-2* and *pad-1* induced a significant increase in the percentage of animals with anteriorly displaced QL.d (Fig. 4D).

Systemic RNAi of the ATP9A ortholog *tat-5* is early embryonic lethal[28]. To examine whether *tat-5* is also required for EGL-20 signaling, we circumvented the essential function of *tat-5* during embryonic development by specifically knocking down *tat-5* in Wnt producing cells using transgene mediated RNAi. As shown in Fig. 4E, knock down of *tat-5* resulted in a significant defect in QL.d migration, supporting the notion that TAT-5/ATP9A functions together with MON-2 and PAD-1 in Wnt secretion. Furthermore, we found that overexpression of a TAT-5(E246Q) mutant interfered with QL.d migration; in the yeast P4-ATPase flippase Drs2p the corresponding mutant blocks the enzymatic cycle at the E2P conformation and hence inhibits flippase activity[29,30] (Fig. 4F). This is in agreement with the model that the TAT-5 ATPase cycle and hence flippase activity is required for Wnt signaling. Taken together, these results are consistent with an evolutionarily conserved function of the ATP9A MON2-DOPEY-2 flippase complex in SNX3-retromer mediated Wls retrieval and Wnt secretion.

In describing the human ATP9A-MON2-DOPEY-2 complex and establishing its interaction with SNX3 our data have revealed a functional role for this evolutionary conserved complex in SNX3-retromer mediated endosome-to-TGN transport of Wls and Wnt signaling in *C. elegans*. Building on evidence from yeast[15–17], we propose a working model in which ATP9A induces the recruitment of MON2 and DOPEY-2 to a sub-domain of the SNX3-labeled early endosome (Fig. 5). Here, the flippase activity of ATP9A generates an asymmetric membrane structure that in expanding the surface area of the cytosolic leaflet over the luminal leaflet within a restricted sub-domain induces initial membrane bending into the cytosol. Assisted by the assembly of coat proteins and other accessory factors, including MON2 and DOPEY-2 oligomers, this aids formation of vesicular transport carriers: events that are co-ordinated with capture and enrichment of cargo (i.e. Wls) through the SNX3-retromer. In yeast and humans Mon2p/MON2 is able to associate with GGA clathrin adaptor coats to support carrier formation[16], an interaction entirely consistent with Wls-containing SNX3-labeled vesicular transport carriers being clathrin decorated[1].

Our network analysis of the SNX3 interactome also revealed enrichment of components

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and include results from 4 experiments, with n≥25 per experiment) *p=1.158 x 10^{-6} for *vps-35* RNAi, *p=1.44 x 10^{-5} for *mon-2* RNAi and *p=0.0024 for *pad-1* RNAi (Student's t-test). (E) Tissue specific RNAi of *tat-5* in Wnt producing cells (*Pmig-14::tat-5* RNAi) in a *vps-29(tm1320)* sensitized mutant background. The percentage of animals with anteriorly displaced QL.d is shown (data are presented as mean +/- SD and include results from 7 experiments, n≥24 per experiment) *p=0.013 (Student's t-test). (F) Overexpression of catalytically inactive TAT-5(E246Q) in a *vps-29(tm1320)* sensitized mutant background. The percentage of animals with anteriorly displaced QL.d is shown (data are presented as mean +/- SD and include results from 3 experiments, n≥30 per experiment) *p=0.0089 (Student's t test)

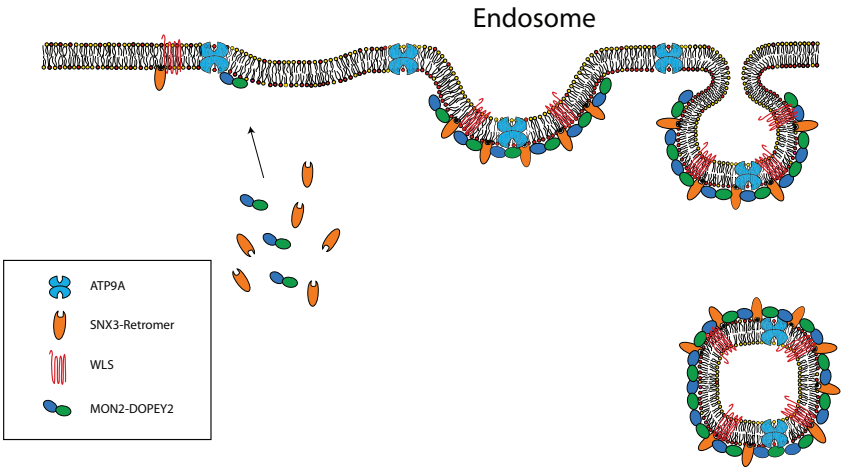

Figure 5: A model of the SNX3-retromer and the proposed ATP9A flippasebased membrane deformation complex in carrier formation. See text for detailed discussion.

of the conserved oligomeric Golgi (COG) complex, a Golgi and TGN-localized multi-subunit tethering complex with established roles in intra-Golgi transport and the capture and organization of endosome-derived transport carriers for fusion at the TGN[31,32] (Fig. 1B, 1C and Supplementary Fig. 2 for biochemical validation). While we have confirmed by western analysis the presence of COG components in the SNX3 interactome and established that this interaction is not observed with retromer SNX-BAR proteins (Supplementary Fig. 2), translating these findings into an *in vivo* context for Wnt signaling in *C. elegans* is compromised by the need of Golgi mediated glycosylation for Wnt secretion and function[33]. Hence, at present we can only speculate as to the likely role of the COG complex in the capture and organization of SNX3-retromer carriers as they arrive at the TGN. Equally, the enrichment of VAC14 and FIG4, two components of a heterotrimeric complex that by regulating PtdIns(3)P-to-PtdIns(3,5)P2 interconversion controls early to late endosomal maturation[34], and Syndecan-1 and Syntenin, proteins that together regulate intraluminal budding of endosomal membranes[35], may provide mechanistic insight into the proposed roles of SNX3 in endosomal maturation[36] and intraluminal vesicle formation[37].

Overall, these data add to the mechanistic diversity between SNX-BAR and SNX3-retromers[5]. Furthermore, in describing the first analysis of an endosome-associated flippase complex in a metazoan model organism, we have provided molecular insight into how SNX3-retromer orchestrates cargo capture with membrane re-modeling during vesicular endosome to TGN transport of Wls, a process required for Wnt-morphogenic gradient formation[1,2].

Materials and Methods

Cell culture, transfection, immunofluorescence and Western blot analysis

HeLa, HEK293T and RPE1 cells were maintained in DMEM (Gibco- Invitrogen) plus 10% (v/v) fetal calf serum (Sigma-Aldrich) and penicillin/streptomycin (PAA). For immunofluorescence

analysis, plasmids containing the stated constructs were transfected using Lipofectamine LTX reagent (Invitrogen). 48 hours after transfection cells were fixed in 0.1 M phosphate buffer containing 4% (w/v) paraformaldehyde for 10 minutes on ice and permeabilized with 0.1% (v/v) Triton X-100 for 5 minutes. Thereafter, cells were incubated with 0.5% (w/v) BSA for 30 minutes followed by incubation with the indicated primary antibodies and subsequent incubation with secondary antibodies (Molecular Probes). For nuclear staining DAPI was used. Images were recorded on a Leica SPE or a Leica AOBS-SP2 confocal microscope. For immunoprecipitation experiments HEK293 cells were grown to 85% confluence in 15 cm dishes prior to transfection with 10 µg of plasmid DNA using PEI (Sigma-Aldrich), and incubated for 48 hours prior to immunoprecipitation using GFP nanotrap beads (Chromotek) as previously described[1]. Western blots were performed using standard procedures. Detection was carried out on a Licor Odyssey Infrared scanning system using fluorescently labelled secondary antibodies.

SILAC interactome analysis

All SILAC reagents were sourced from Thermo Fisher; except for dialysed FBS (Sigma). Lentivirally transduced GFP and GFP-SNX3 RPE1 cells were grown in SILAC DMEM for at least 6 passages to achieve full labelling. GFP expressing control cells were grown in unlabeled medium containing regular arginine and lysine (R0, K0). GFP-SNX3-expressing cells were grown in medium containing 13C6-arginine and 4,4,5,5-D4-lysine (R6, K4). GFP was precipitated with GFP trap beads (Chromotek) for 60 minutes. The beads were pooled, proteins were eluted in sample buffer, separated on Nupage 4- 12% precast gels (Invitrogen) and subjected to LC–MS/MS analysis on an Orbitrap Velos (Thermo) mass spectrometer. The mass spectrometric detection and quantification was performed as previously described[14].

Antibodies and Constructs

The following antibodies were used in this study: polyclonal rabbit anti-SNX3 (Proteinteck); monoclonal mouse anti-EEA1 (BD Transduction Laboratories); monoclonal mouse anti-GFP (mix of clones 7.1 and 13.1, Roche); polyclonal rabbit anti-VPS26 and anti-VPS35 (Epitomics); monoclonal mouse anti-SNX27 (Abcam); monoclonal mouse anti-HA (Santa Cruz); monoclonal mouse anti-Flag (Sigma); the rabbit polyclonal anti-Mon2 antibody and Mon2-Flag construct were kind gifts from Dr Yoshihiro Kawaoka (University of Wisconsin); sheep anti-TGN46 (AbD Serotec); the anti-COG antibodies and constructs were kind gifts from Dr. Sima Lev (Weizmann Institute of Science, Rehovot, Israel); fluorescent Alexa secondary antibodies were obtained from Molecular Probes. The construct encoding ATP9A-HA was a gift from Dr. Hye-Won Shin (Kyoto University, Japan). A cDNA clone encoding DOPEY2 was purchased from Fermentas. Both MON2 and DOPEY2 were subcloned into both pEGFP-N1 (Clonetech) and a modified pcDNA3.1 vector which encodes a Flag tag.

Live-cell imaging

Cells were transferred to CO2-independent medium (Gibco-Invitrogen) supplemented with 10% (v/v) fetal calf serum and imaged at 37°C on a spinning disc confocal system (Perkin-Elmer UltraVIEW ERS 6FE confocal microscope with Yokogawa CSU22 spinning disk) using a 63x lens.

Computational and statistical analysis

Functional gene annotation for the set of SNX3-interacting proteins was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, version 6.7).

The Cytoscape plugin Enrichment Map was used to display overlap between gene ontology terms within Cytoscape (version 2.8.2). Node size was mapped to the number of genes within a GO category and node color was mapped to the p-value for enrichment of a given category. For network analysis, a network interaction map was built using STRING. The network was visualized in Cytoscape.

C. elegans strains and culture

C. elegans strains were cultured at 20°C using standard conditions as described[38]. Mutant alleles and transgenes used were: *vps-29(tm1320), muIs32[Pmec-7::gfp]*[39], *huIs60[Pegl-20::egl-20::protA]*[23], *huIs72[Pmig-14::mig-14::gfp]*[26], *huSi2[Pmig-14::mig-14::gfp]*[1], *huEx149[Pmyo3::lmp-1::mCherry]*[1], *huEx529[Phs::tat-5(E246Q)], huEx430[Pmig-14::tat-5(RNAi)], huEx516[Pmig14::vps 35(RNAi)]* and *huEx518[Pmig-14::gfp(RNAi)]*.

C. elegans RNAi, transgenesis and imaging

Systemic RNAi by feeding and tissue-specific RNAi by transgene mediated expression of double stranded RNA (dsRNA) was performed as described[40,41]. To express *tat-5, vps-35* or gfp dsRNA from the *mig-14* promoter, 500 base pair (bp) fragments of coding sequence were PCR amplified from genomic DNA or plasmid template. After PCR fusion to the *mig-14* promoter (in the sense as well as the antisense orientation), the final PCR products were injected in *vps-29(tm1320); muIs32* animals at a concentration of 7 ng/µl with 7 ng/µl Pmyo2::mCherry injection marker and 150 ng/µl pBluescript plasmid DNA, yielding the transgenes *huEx430[Pmig-14::tat-5(RNAi)], huEx516[Pmig14::vps 35(RNAi)] and huEx518[Pmig-14::gfp(RNAi)]*. To generate *Phs::tat-5(E246Q)*, the *tat-5(E246Q)* coding sequence was PCR amplified from pDONR221_TAT-5(E246Q)29 using primers (TCCCGGGatgggcaaacggaagaagaacgac and TGCTAGCtcagttgactttcgcgtagcttg) that re-introduce a translational start codon at the 5' end of the *tat-5* coding sequence. After sub-cloning of the resulting PCR fragment into pJET (Promega), *tat-5(E246Q)* was inserted into the heat-shock promoter vector pPD49.78 using XmaI and NheI restriction sites. The construct was injected at 25 ng/µl with 7 ng/µl Pmyo2::mCherry injection marker and 150 ng/µl pBluescript plasmid DNA, yielding the transgene *huEx529[Phs::tat-5(E246Q)]*. Synchronized embryos were heat-shocked for 1 hour at 33°C and the QL.d migration phenotype was determined when the animals reached the young adult stage. The final position of the QL descendant PVM was scored relative to the vulva in young adult animals as described[39]. EGL-20::protA staining was performed as described[23]. Antibodies used were anti-goat-Alexa647 (Life Technologies), anti-GFP (BD Livingcolors) and anti-mouse-HRP (GE Healthcare).

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Author Contributions

P.J.C. and H.C.K. concieved the project; I.J.M., C.D., and P.J.C. designed and carried out the cell biological and bioinformatic analysis; K.J.H. performed the proteomics; R.G. and H.C.K. designed and carried out the *C. elegans* experiments, and I.J.M., R.G., H.C.K. and P.J.C. wrote the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

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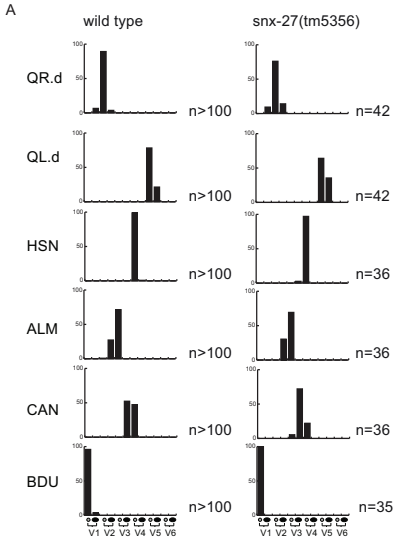
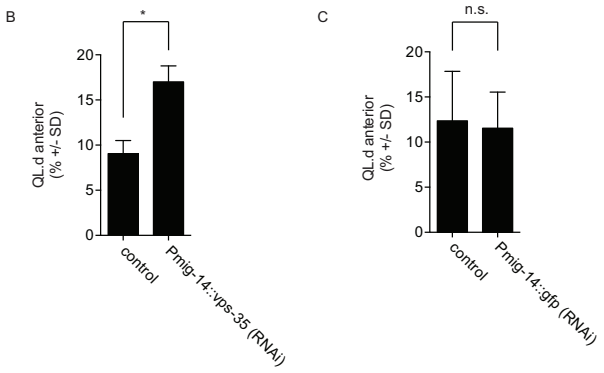
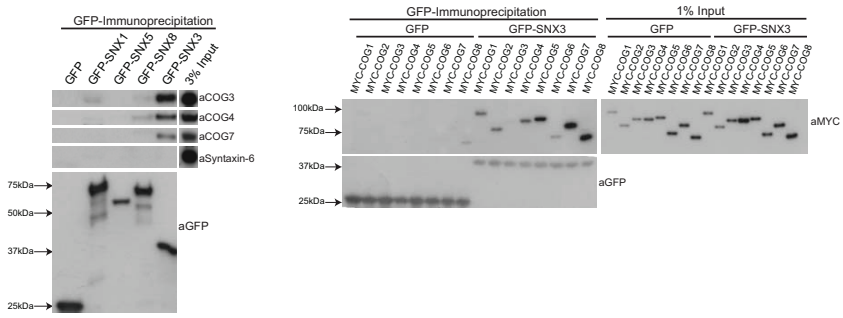


Supplementary Figure S1: *snx27* is not required for Wnt signaling in *C.elegans*. (A) Final positions of the QR descendants (QR.d), the QL descendants (QL.d), the HSN, ALM, CAN and BDU neurons in wild type or *snx-27(tm5356)* mutants. Positions in L1 larvae are indicated relative to the hypodermal seam cells V1 to V6 and were determined as described[23,42,43]. (B) Tissue specific RNAi targeting *vps-35* in Wnt producing cells (*Pmig-14::vps35* RNAi) interferes with the EGL-20/Wnt dependent posterior migration of the QL.d in a *vps-29(tm1320)* sensitized mutant background. The percentage of animals with anteriorly displaced QL.d is shown (data are presented as mean +/- SD and include results from 3 experiments, n≥30 per experiment) *p=0.0064 (Students t-test). C) Tissue specific expression of GFP dsRNA does not influence QL.d migration in the *vps-29(tm1320)* mutant background (data are presented as mean +/- SD and include results from 3 experiments, n≥30 per experiment).





Supplementary Figure S2: Confirmation of the SNX3 COG complex interaction. (A) Cell extracts derived from HeLa cells lentivirally transduced with either GFP, GFP-SNX1, GFPSNX5, GFP-SNX8, or GFP-SNX3 were subjected to a GFP nanotrap and Western blotted with antibodies raised against the specified components of the COG complex and Syntaxin6. (B) Cell extracts derived from HEK293T cells transiently transfected to co-express either GFP or GFP-SNX3 with each of the individual myc tagged COGs were subjected to a GFP nanotrap and Western blotted with antibodies raised against myc and GFP.

CHAPTER 6

A role for the kinase CK2 in Wnt secretion and Wls internalization

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Abstract

Wnt proteins are members of a conserved family of lipid modified signaling molecules that have essential functions in development and adult tissue homeostasis. The secretion of Wnt proteins is mediated by the transmembrane protein Wntless (Wls), which binds Wnt in the Golgi and transports it to the cell surface for release. To maintain efficient Wnt secretion, Wls needs to be recycled back to the Golgi through endocytosis and retromer dependent endosome to Golgi transport. We have previously identified the kinase CK2 in a genome-wide screen for novel regulators of Wnt signaling in *C. elegans*. Here, we show that CK2 function is required in Wnt producing cells for Wnt secretion. This function is evolutionarily conserved, as inhibition of CK2 activity also interferes with Wnt5a secretion from mammalian cells. Mechanistically, we show that knock down of CK2 results in enhanced plasma membrane localization of Wls, consistent with the notion that CK2 is involved in the regulation of Wls internalization.

Introduction

Wnt signaling is an evolutionary conserved signal transduction pathway with important functions during development and adult tissue homeostasis[1]. The mechanism of Wnt signaling has been the focus of intense investigations for over 30 years. This research has yielded a detailed understanding of the molecular mechanisms of Wnt signal transduction[2]. Wnt signaling can be categorized in a β-catenin dependent Wnt signal transduction route that revolves around the regulation of the levels of the β-catenin protein. Next to this 'canonical' Wnt signal transduction cascade, Wnt proteins can induce β-catenin independent signal transduction pathways[3]. The mechanism of Wnt production and secretion is much less well understood.

Wnts are cysteine rich secreted proteins with a molecular mass of approximately 40 kDa. Wnt proteins are lipid modified in the endoplasmic reticulum of Wnt producing cells by the acyl transferase Porcupine[4]. Wnt follows the secretory pathway and requires the membrane protein Wntless (Wls) for secretion[5–7]. When Wls has escorted Wnt from the Golgi to the plasma membrane, Wls undergoes retrograde trafficking from the plasma membrane, via endosomes, to the trans-Golgi network (TGN)[8–13]. Abrogation of retrograde trafficking of Wls in *C. elegans* and *Drosophila* causes defects in Wnt secretion and Wnt signaling. Genetic screens in *C. elegans* have identified genes that are involved in retrograde trafficking of Wls[9,14–16]. Internalization of Wls requires the clathrin adaptor AP2, while transport of Wls from endosomes to the TGN requires the sorting nexin *snx-3* and the cargo selective subcomplex of the retromer complex which consists of subunits encoded by *vps-29, vps-26* and *vps-35*.

We have previously identified the kinase CK2 in a genome-wide RNAi screen for novel regulators of Wnt signaling in *C. elegans*[15]. CK2 is implicated in a plethora of biological processes, regulating the phosphorylation of many substrates[17]. In this study, we show that CK2 also has an evolutionary conserved role in Wnt secretion.

overview of ALM polarity, a read-out for β-catenin independent Wnt signaling in *C. elegans*. (D) Knock down of *kin-10* interferes with polarization of the ALM neuron in a *mig-14(mu71)* sensitized mutant background (data are represented as mean +/- SD and include results from 3 experiments, n>30 per experiment, * p<0.01 (Students t-test)). (E) Knock down of *kin-3* in Wnt producing cells interferes with the β-catenin dependent posterior migration of the QL descendants in a *vps-29(tm1320)* Wnt secretion hypomorph (data are represented as mean +/- SD and include results from 5 experiments, n>30 per experiment, * p<0.02 (Students t-test)).

Results

kin-10 is required for the EGL-20/Wnt dependent posterior migration of the QL descendants

kin-10 was identified in a genome-wide RNAi screen aimed at identifying novel regulators of Wnt secretion and Wnt signaling[15]. *kin-10* is the *C. elegans* ortholog of the regulatory beta-subunit of the serine-threonine protein kinase casein kinase 2 (CK2). To further characterize the function of *kin-10* in Wnt signaling, we analyzed the effect of *kin-10* knock down on the Wnt dependent migration of the QL neuroblast descendants (QL.d). During the first stage of larval development, QL and its three descendants migrate from a position in the mid body to

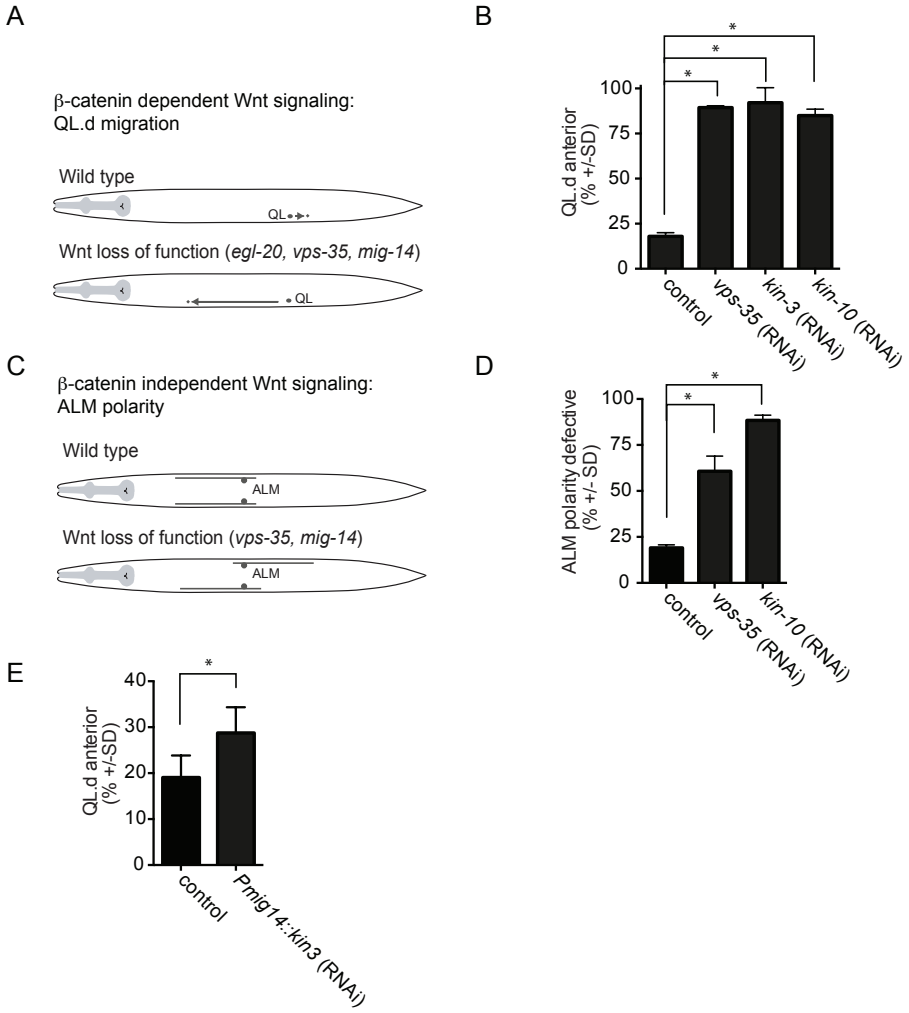

Figure 1: CK2 is required for Wnt signaling in *C. elegans*. (A) Schematic overview of QL descendant migration, a read-out for β-catenin dependent Wnt signaling in *C. elegans*. (B) Knock down of CK2 interferes with the β-catenin dependent posterior migration of the QL descendants in a *vps-29(tm1320)* sensitized mutant background (data are represented as mean +/- SD and include results from 3 experiments, n>30 per experiment, * p<0.01 (Students t-test)). (C) Schematic

distinct positions in the posterior (Fig. 1A). This migration is dependent on the Wnt protein EGL-20, which induces posterior migration by activating the hox gene *mab-5*. When EGL-20 signaling is inhibited, *mab-5* expression fails to be induced and as a consequence, the QL.d migrate in the opposite, anterior direction.

Because *kin-10* is an essential gene[18], we had to rely on partial knock down to study the function of *kin-10* during post-embryonic development. Therefore, we used a sensitized genetic background to enhance Wnt signaling phenotypes. The background that we used is a mutation in the retromer subunit gene *vps-29*. In *vps-29* mutants, secretion of EGL-20 is reduced, resulting in a partially penetrant defect in QL.d migration[11]. Interfering with Wnt pathway components strongly enhances this phenotype[15]. We found that knock down of *kin-10* also resulted in a significant increase in the percentage of animals with anteriorly displaced QL.d (Fig. 1B). Next, we subjected *vps-29* mutants to *kin-3* RNAi, which targets the catalytic alpha-subunit of CK2 and observed a comparable increase in the QL.d migration phenotype (Fig. 1B), suggesting that the CK2 holoenzyme is required for β-catenin dependent Wnt signaling in *vps-29* mutants.

kin-10 is required for the Wnt dependent polarization of the ALM neuron

Next, we asked if knock down of CK2 also inhibits β-catenin independent Wnt signaling in *C. elegans*. To this end, we investigated the polarity of the ALM neurons. The ALM neurons direct a long protrusion towards the anterior and a short protrusion posteriorly (Fig. 1C). This process is regulated by the Wnts EGL-20 and CWN-1 but is independent of β-catenin[19,20]. Using a partial loss of function mutation of *mig-14*/Wls as a sensitized genetic background[11],

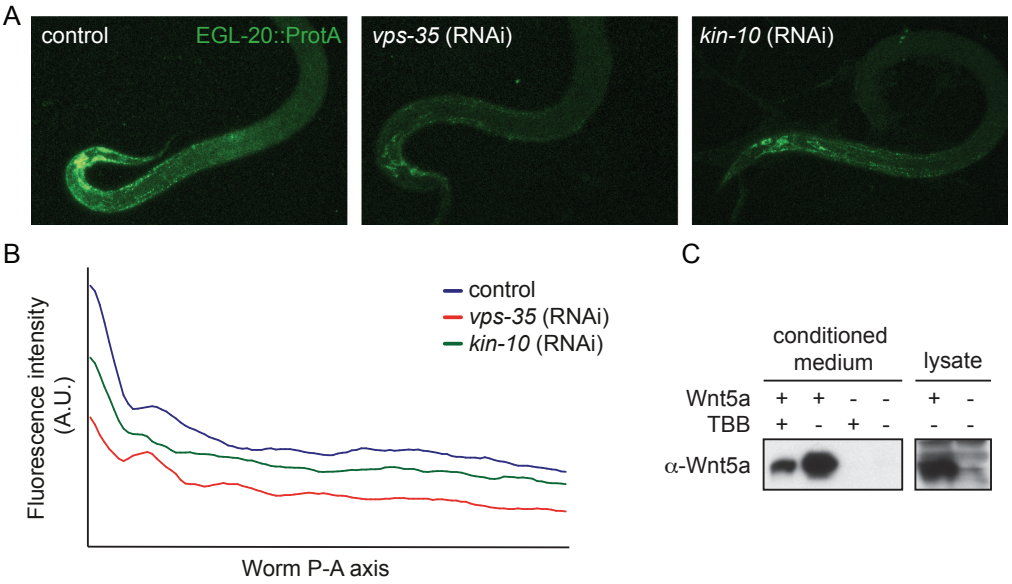

Figure 2: CK2 function is required for EGL-20/Wnt gradient formation in *C. elegans* and Wnt5a secretion from L cells. (A) EGL-20::ProtA gradient stained with rabbit-anti-goat-Alexa647 in L1 larvae14 subjected to control, *vps-35* or *kin-10* RNAi. (B) Quantification of EGL-20::ProtA intensity along the anterior-posterior axis of *C. elegans* L1 larvae subjected to control, *vps-35* or *kin-10* RNAi. (C) Western blot analysis of Wnt5a in the medium of L cells, medium was conditioned for 20 hours in presence of the CK2 inhibitor TBB or DMSO.

we found that *kin-10* RNAi induces a clear increase in the percentage of animals with defects in ALM polarity (Fig. 1D). Taken together, these results show that *kin-10* is required for β-catenin dependent as well as β-catenin independent Wnt signaling. This places *kin-10* either upstream in the Wnt signaling pathway, at the level of the Frizzled receptor or Dishevelled, or in the Wnt secretion pathway.

CK2 is required for secretion of EGL-20 from producing cells

To investigate whether CK2 is required in Wnt producing cells, we specifically knocked down *kin-3* in Wnt producing cells using transgene mediated RNAi[21] and investigated whether this affects the EGL-20 dependent migration of the QL.d in the *vps-29* sensitized mutant background. As shown in Fig. 1E, we observed a significant increase in the percentage of animals with posteriorly displaced QL.d, indicating that *kin-3* is required in Wnt producing cells. To investigate whether the CK2 kinase is required for EGL-20 secretion, we visualized EGL-20 using a fusion of EGL-20 with the immunoglobulin binding region of protein A. Staining with fluorescently tagged IgG revealed a punctate gradient of EGL-20 that ranges from the producing cells in the tail to the mid body region[14]. We found that the intensity of this staining was reduced when *kin-10* was knocked down by RNAi (Fig. 2A, B). Taken together, these results are consistent with a function of CK2 in Wnt producing cells.

The function of CK2 in Wnt secretion is evolutionarily conserved

To investigate if the function of CK2 in Wnt secretion is evolutionary conserved, we treated Wnt5a producing mouse L cells[22] with the specific CK2 inhibitor TBB[23]. We found that inhibition of CK2 resulted in a significant reduction in the amount of Wnt5a that is secreted into the medium (Fig 2C), indicating that CK2 is also required for the secretion of Wnt proteins from mammalian cells.

CK2 is required for Wls internalization

Wnt secretion depends on the continuous shuttling of Wls between the Golgi and the plasma membrane[9–13]. We hypothesized that CK2 may be involved in the regulation of Wls trafficking. Abrogation of retromer mediated Wls transport from endosomes to the TGN causes missorting of Wls to lysosomes and Wls degradation[9–13,15]. We investigated if CK2 is also required for this retrograde trafficking step, but observed no significant decrease in steady state MIG-14/Wls protein levels in animals treated with *kin-10* RNAi (Fig. 3A). An alternative possibility is that *kin-10* is required for the internalization of MIG-14/Wls from the plasma membrane. Endocytosis of MIG-14/Wls requires the AP2 adaptin complex and knock down of the AP2 subunit gene *apa-2* results in increased plasma membrane localization of a functional MIG-14::GFP fusion (Fig. 3C, D)[11]. Interestingly, we observed a similar cell surface accumulation of MIG-14::GFP in animals that were treated with *kin-10* RNAi (Fig. 3D). Taken together, these results support a model in which CK2 controls the internalization of MIG-14/Wls.

Discussion

In this study we have investigated the role of the kinase CK2 in Wnt signaling. We show that CK2 is required for both β-catenin dependent and independent Wnt signaling in *C. elegans* and using tissue-specific RNAi we show that CK2 is required in Wnt producing cells. Furthermore, we show that CK2 has an evolutionarily conserved function in Wnt secretion, most likely by facilitating the internalization of the Wnt secretion factor Wls.

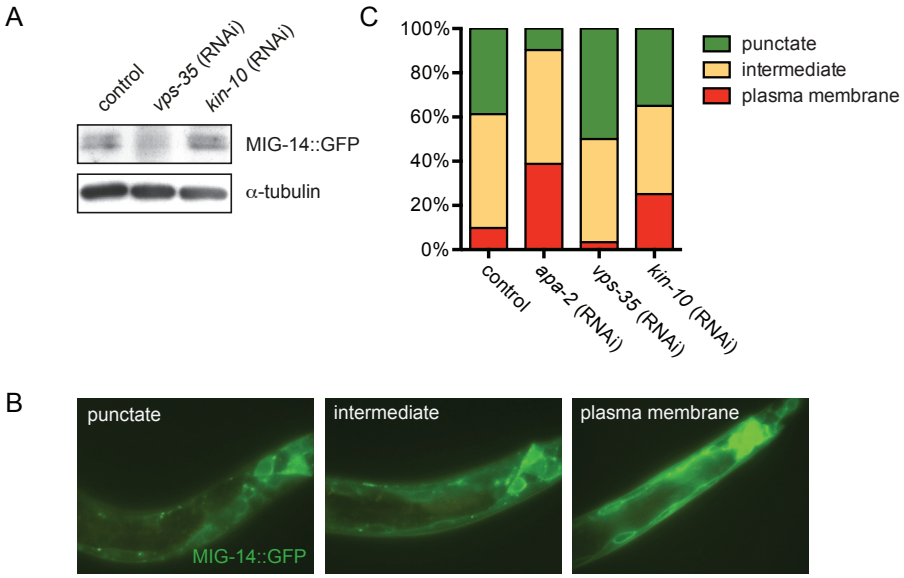

Figure 3: *kin-10* does not control endosome to TGN trafficking of Wls/MIG-14, but controls MIG-14 internalization. (A) MIG-14::GFP proteins levels in L1 larvae subjected to control, *vps-35* or *kin-10* RNAi. (B) MIG-14::GFP localization in *C. elegans* scored in 3 categories: punctate, intermediate, plasma membrane. (C) Quantification of MIG-14::GFP localization in *C. elegans* subjected to control, *apa-2, vps-35* or *kin-10* RNAi.

Although CK2 has previously been implicated in the regulation of the Wnt pathway component Dishevelled[24], our results show that CK2 also functions as part of the Wnt secretion pathway in *C. elegans*. This function is evolutionarily conserved, as CK2 function is also required for the secretion of Wnt5a from mammalian L cells. Wnt secretion requires the retrograde trafficking of Wls from the plasma membrane, via endosomes, to the TGN. CK2 has been implicated in retrograde trafficking of the cation-independent mannose-6-phosphae receptor (CI-MPR). Thus, CK2 is recruited by PACS-1 to phosphorylate the clathrin adapter GGA-3, a phosphorylation event that is necessary for endosome to TGN transport of the CI-MPR[25]. It is however unlikely that CK2 has a similar function in MIG-14/Wls retrograde trafficking, as CK2 knock down did not influence steady state MIG-14/Wls protein levels. Instead, our results indicate that CK2 may regulate Wls internalization. CK2 is known to phosphorylate several components of the endocytosis machinery[26]. For example, CK2 has been shown to phosphorylate the clathrin light chain, myosin light chain and dynamin. Furthermore, CK2 is known to regulate internalization of the EGFR though phosphorylation of N-WASP and the regulation of actin dynamics[27]. Perhaps CK2 plays a similar role in the regulation of Wls internalization. Alternatively, CK2 may phosphorylate Wls directly and in this way regulate Wls internalization, but so far we have been unable to show a robust interaction between CK2 and Wls or CK2 mediated phosphorylation of Wls.

Materials and Methods

C. elegans strains and culture

C. elegans strains were cultured at 20°C using standard conditions as described[28]. Mutant alleles and transgenes used were: *vps-29(tm1320), mig-14(mu71), muIs32[Pmec-7::gfp]*[29], *huIs60[Pegl-20::egl-20::protA]*[14], *huIs72[Pmig-14::mig-14::gfp]*[11], *huSi2[Pmig-14::mig-14::gfp]*[15], *huEx442[Pmig-14::kin-3(RNAi)]*

C. elegans RNAi, transgenesis and imaging

Systemic RNAi by feeding and tissue-specific RNAi by transgene mediated expression of double-stranded RNA (dsRNA) was performed as described[21,30,31]. To express *kin-3* dsRNA from the *mig-14* promoter, 500 base pair (bp) fragments of coding sequence were PCR amplified from genomic DNA. After PCR fusion to the *mig-14* promoter (in the sense as well as the antisense orientation), the final PCR products were injected in *vps-29(tm1320); muIs32* animals at a concentration of 7 ng/µl with 7 ng/µl Pmyo2::mCherry injection marker and 150 ng/µl pBluescript plasmid DNA, yielding the transgene *huEx442[Pmig-14::kin-3(RNAi)]*.

The QL.d migration phenotype was determined when the animals reached the young adult stage. The final position of the QL descendant PVM was scored relative to the vulva in young adult animals as described[29]. ALM polarity was determined in L4 larvae, animals that displayed at least one ALM with reversed polarity were scored as defective. EGL-20::protA staining was performed as described[14]. MIG-14 protein levels were determined as described[15]. MIG-14 localization was imaged in young adult animals[11]. The subcellular localization of MIG-14 was scored blind by 5 lab members and the results were qualitatively consistent among the different scorings. Antibodies used were anti-goat-Alexa647 (Life Technologies), anti-GFP (BD Livingcolors), anti-alpha-tubulin (Sigma) and anti-mouse-HRP (GE Healthcare).

Wnt5a secretion

Wnt5a expressing L cells and control L cells were cultured in DMEM with 10% FBS, 5% L-glutamine and 5% penicillin/streptomycin and grown to confluence in 12 well plates. Cells were washed with PBS and culture medium before incubation in culture medium supplemented 50 µM TBB (Sigma) or DMSO for 20 hours. Conditioned medium was collected and centrifuged for 4 minutes at 2400 rpm before analysis by standard Western blotting techniques. Antibodies used: anti-Wnt5a (Cell signaling), anti-rabbit-HRP (GE Healthcare)

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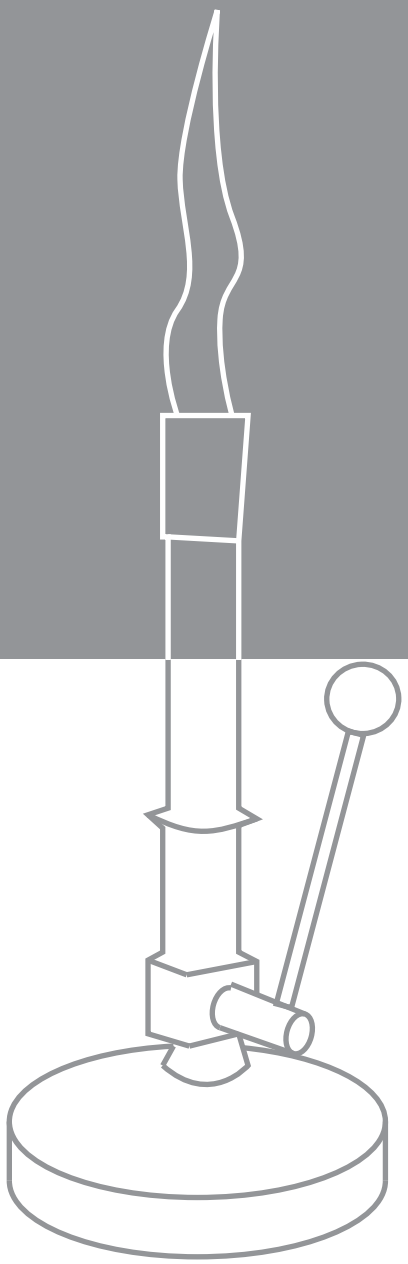

CHAPTER 7

Summarizing discussion

Wnt signaling is the focus of intense investigations for over 30 years. Production and secretion of Wnt protein is of key importance for the physiological activation of the Wnt pathway. In order to maintain efficient Wnt secretion, Wntless (Wls) must transport Wnt from the Golgi apparatus to the plasma membrane. Subsequently, Wls is endocytosed and transported from endosomes back to the Golgi[1]. The goal of my PhD studies was to gain further insight into the molecular mechanisms of Wls trafficking.

Ubiquitylation is a well-known mechanism to regulate endocytosis and intracellular trafficking[2]. Furthermore, it was hypothesized, and later shown, that Wnt can be secreted on exosomes[3–5]. Exosomes are extracellular vesicles that form when multivesicular bodies fuse with the plasma membrane and release their intraluminal vesicles (ILVs). Sorting of proteins onto ILVs requires the ESCRT machinery, which sort ubiquitylated cargo onto ILVs[6]. We hypothesized that ubiquitylation would be important for the regulation of EGL-20/Wnt secretion in *C. elegans*, either by regulating Wls trafficking, or by regulating exosomal secretion of EGL-20/Wnt. We set out to identify an ubiquitin ligase that would be involved in this process. To this end, we performed an RNAi screen to identify ubiquitin ligases or DUBs that influence the EGL-20/Wnt dependent posterior QL.d migration in a sensitized genetic background. We identified several genes in our screen, and performed some additional experiments to further characterize the role of these genes in Wnt signaling. This work, described in chapter 2 of this thesis, provides a basis for further investigations. In my opinion, the membrane bound RING domain E3 ligase *d2089.2* will be particularly interesting to characterize further because similar genes were recently shown to regulate plasma membrane localization of the Wnt receptor Frizzled[7,8]. An interesting possibility is that *d2089.2* regulates trafficking of a membrane protein that is involved in Wnt secretion, potentially Wls. However, at this time, a role for ubiquitylation in the regulation of Wnt secretion remains to be established.

A novel negative regulator of Wnt signaling: *eel*-1/Huwe1

Our screen for novel regulators of Wnt signaling identified a negative regulatory role for *eel-1* in Wnt signaling. In parallel, our collaborators discovered that the mammalian homologue of *eel-1*, Huwe1, is a negative regulator of Wnt signaling in human cells. Furthermore, our collaborators identified Huwe1 as a Dvl binding partner. This immediately raised the question if Huwe1 is a Dvl ubiquitin ligase. Satisfyingly, we discovered that it is.

The most straightforward way in which a Dvl ubiquitin ligase can function as a negative regulator of Wnt signaling is to target Dvl for degradation. However, we did not find indications that this is the function of Huwe1. In contrast, Huwe1 seems to form K63 linked polyubiquitin chains on the DIX domain of Dvl and influence Dvl polymerization. K63 linked ubiquitylation of the DIX domain is known to positively influence Wnt signaling[9]. Our results indicate that K63 linked ubiquitylation of the DIX domain also negatively regulates Wnt signal transduction. Perhaps, the dynamic regulation of K63 linked ubiquitylation of the DIX domain, and subsequent effects on Dvl polymerization need to be dynamically controlled. In this way, both too little and too much ubiquitylation can interfere with Wnt signal transduction. Still, the exact mechanisms of the regulation of Dvl polymerization, and the way this influences Wnt signaling are incompletely comprehended. Elucidation of these mechanisms may be helped by detailed cell biological investigations of Dvl complexes coupled with detailed structural analysis of Dvl molecules.

The role of retrograde traffic in Wnt secretion.

Wls is an essential component of the Wnt secretion machinery. In the absence of Wls, Wnt is trapped in the Golgi apparatus of Wnt producing cells[10–12]. To maintain Wnt secretion, Wls

needs to cycle between the Golgi apparatus and the plasma membrane. The retrograde route from plasma membrane to the Golgi involves AP-2 and clathrin mediated endocytosis from the plasma membrane followed by retromer mediated transport from endosomes to the Golgi apparatus[13–18]. Screens that were performed in the lab identified components of this retrograde trafficking route such as *vps-35* and *snx-3*[19,20].

In my studies, I have extended investigations into the role of Vps35 in Wnt signaling beyond tissue culture cells and the invertebrate model systems that have been used before. We characterized the role of Vps35 in Wnt signaling and stem cell maintenance in the mammalian intestine. We discovered that the role of Vps35 in stem cell maintenance in the intestinal epithelium is relatively minor. We describe these results in chapter 4. In chapter 5, we describe a novel molecular mechanism to form Wls containing retrograde carriers at the endosome. Lastly, we have identified, and started to characterize, the role for CK2 in Wnt secretion, which is the subject of chapter 6 of this thesis.

Insight into the role of Vps35 in Wnt signaling and stem cell maintenance in the mammalian intestine.

The mammalian intestine is a rapidly self-renewing tissue. Stem cells are essential for self-renewal and homeostasis in the intestine. Wnt signals are required to maintain intestinal stem cells[21]. For this reason, we decided to use the mammalian intestine as a model system to study the role of Vps35 mediated retrograde trafficking for Wnt signaling and stem cell maintenance.

When we deleted Vps35 from the intestinal epithelium *in vivo,* we did not observe any defects in the morphology of the intestinal epithelium, or differentiation of intestinal cells. This result was not unexpected because Wnts that are produced by the intestinal mesenchyme can compensate for a loss of Wnt secretion from the epithelium[22]. We were surprised to find that the loss of Vps35 from intestinal organoids only had subtle effects. The organoids displayed normal morphology, but had a reduced growth rate compared to control organoids. We observed a clear reduction in Wls protein levels in the *Vps35*$^{\Delta/\Delta}$ organoids. This result confirms the role of retromer in maintaining Wls protein levels in intestinal cells. We tried to rescue the effect of the deletion of Vps35 by adding exogenous Wnt to the organoid culture medium. In this way, we aimed to compensate the reduction in Wnt secretion that results from the deletion of Vps35. We found that the growth defect of the *Vps35*$^{\Delta/\Delta}$ organoids could not be completely rescued by exogenous Wnt. This result indicates that the reduced growth rate of *Vps35*$^{\Delta/\Delta}$ organoids is the result of Wnt dependent and Wnt independent effects. We did not investigate Wnt unrelated effects of Vps35 deletion because we lacked appropriate tools to address these points.

The conditional Vps35 allele that we generated may be employed to further investigate the role of Vps35 in mouse development, homeostasis and disease. The conditional Vps35 allele may be particularly valuable in the study of Alzheimers disease (AD). Vps35 mRNA and protein levels are reduced in AD patients[23] and single nucleotide polymorphisms in genes that regulate retromer function are associated with AD[24]. In a mouse model of AD, Vps35 haploinsufficiency enhances Alzheimer's disease neuropathology[25]. Wen *et al.* propose that missorting of BACE1 is causal for the enhanced AD pathology observed in Vps35 heterozygous mice, but defects in the intracellular transport of the amyloid precursor protein may also contribute to AD pathology[26]. Our conditional allele may be employed to conditionally interfere with Vps35 function in a murine model of AD and in this way further study the relationship between loss of Vps35 function and Alzheimer disease.

Novel insights into retrograde trafficking of Wls; the role of the Mon2-Dopey2-ATP9A complex.

In chapter 5 we investigated the mechanism of Wls carrier formation at the endosomal membrane. We describe an evolutionary conserved lipid flippase complex that is required for retrograde Wls trafficking and Wnt secretion. According to our model, SNX3 coordinates retromer mediated Wls sorting with the recruitment of a lipid flippase complex that includes Mon2 and Dopey2 and the P4-type ATPase ATP9A. Flipping of lipids from the luminal to the cytoplasmic leaflet of the endosomal membrane by this complex results in the generation of membrane curvature, which is required for retrograde carrier formation.

Our work identified this complex and showed its requirement for Wnt secretion *in vivo*. It will be interesting to further characterize this retrograde trafficking step. Key questions are: how is the generation of membrane curvature regulated? Is the regulation centered on the recruitment of the flippase complex, or is the activity of the flippase complex regulated by additional mechanisms? How is the membrane curvature stabilized? We know that Wls carriers are clathrin coated. Furthermore, the clathrin adaptor GGA is involved in a retrograde trafficking route in yeast which also requires a lipid flippase complex[27]. However, we do not know if GGAs are important for retrograde Wls trafficking. It will be interesting to reconstitute the formation of Wls carriers in a cell-free system, as this will open new avenues to study this retrograde trafficking route.

Retrograde trafficking of the CI-MPR is well studied and many molecular players that mediate its retrograde trafficking route have been identified (as summarized in the introduction). A lot of work will still be required in order to understand retrograde trafficking of Wls at a similar level of detail. We now know that SNX3 is required for cargo sorting and recruitment of the cargo specific retromer subcomplex and the membrane deforming Mon2-Dopey2-ATP9A complex. Open questions that remain are: is there a role for dynamic actin in retrograde trafficking of Wls? How are the Wls carriers abscised from the endosome? How are the Wls carriers transported to the TGN? How are Wls carriers tethered at the TGN (is the COG complex involved in this process)? Which SNARE complexes are involved in the fusion of Wls carriers with the TGN membrane? Further cell biological and genetic studies may help to fill in these blank spots.

A novel regulator of Wnt secretion: CK2

In chapter 6 we describe a role for the protein kinase CK2 in Wnt secretion. CK2 function is required for Wnt gradient formation in *C. elegans* and for Wnt secretion from L-cells. Our results indicate that CK2 regulates Wls internalization.

Internalization of G-protein coupled receptors (GPCRs) is regulated by phosphorylation. Phosphorylated GPCRs bind arrestins, which results in the recruitment of the GPCRs to clathrin coated pits[28]. Wls resembles GPCRs to some extend, both are 7 pass transmembrane proteins and Wls was initially annotated as a GPCR, its name being Gpr177. Therefore, an interesting hypothesis is that Wls itself is phosphorylated by CK2 and subsequently internalized. We were not able to confirm an interaction between Wls and CK2 in tissue culture cells, neither were we able to detect Wls phosphorylation, but this remains an interesting hypothesis. Alternatively, CK2 may regulate the phosphorylation of some other factor that is involved in Wls internalization. In the case of the EGF receptor, CK2 regulates f-actin formation, which is involved in EGFR internalization[29]. A similar mechanism may be involved in CK2 mediated regulation of Wls internalization.

It will be important to identify the relevant CK2 substrate in order to further elucidate the mechanism of CK2 mediated regulation of Wnt secretion. Perhaps phospho-mass-

spectrometry approaches can be employed to identify differentially phosphorylated proteins in worms that were subjected to CK2 RNAi versus control worms. Subsequently, CK2 substrates can be tested for a role in Wnt secretion.

Future perspectives

Like most research, my work answered some questions, but it raised many more. Here, I will discuss and speculate about avenues for future research.

Genetic screens are a fruitful approach to identify the genes that are involved in a particular biological process. When genetic screens were performed for genes that are involved in Wnt secretion, many regulators of retrograde Wls trafficking were identified[19,20]. Genetic screens in *C. elegans* have identified only a few genes that act further upstream (at the level of Wnt production and transport to the plasma membrane): Wnt itself (*mom-2*/Wnt and *egl-20*/Wnt), *mom-1*/porcupine, and Wls/*mig-14* (also identified as *mom-3*)[30]. It has puzzled me why we did not identify genes that are involved in anterograde trafficking of Wls from the Golgi to the plasma membrane. A possible explanation is that there is redundancy in trafficking routes from the Golgi to the plasma membrane. Alternatively, mutations in the required genes may be lethal and have escaped detection in the screens that were performed up to this point. Further genetic screening approaches may identify genes and proteins that are involved in anterograde Wls transport. In addition, proteomic approaches - such as the one that led to the identification of the Mon2-Dopey3-ATP9A complex - may help to further elucidate the mechanisms of Wnt secretion.

Other open questions that remain are: what is the *in vivo* role of exosomal release of Wnt? How is sorting of Wls and Wnt to ILVs achieved? How is sorting of Wls to exosomes differentiated from retrograde trafficking of Wls? How is Wnt released from Wls? How is Wnt transported from the ER to the Golgi, is Wls involved in this transport step? What is the *in vivo* relevance of Wnt glycosylation? These are all basic biological questions, which are of interest to cell- and developmental biologists.

A big challenge lies in using the knowledge about the Wnt secretory pathway for the therapeutic benefit of patients. A this time, the selective porcupine inhibitor LGK974 is being evaluated in a phase 1 clinical trial[31]. Hopefully, inhibiting Wnt secretion will be an efficacious way to treat cancers that depend on active Wnt signaling.

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ADDENDUM

Samenvatting in het Nederlands

Communicatie tussen cellen is van essentieel belang tijdens de ontwikkeling van organismen. Dieren beschikken over een aantal verschillende intercellulaire communicatiemechanismen, ook wel signaaltransductieroutes genoemd. Een van die signaaltransductieroutes is de Wnt signalering route. Wnt signalering is erg belangrijk tijdens de ontwikkeling van alle dieren. Wanneer Wnt signalering verstoord is, door mutaties bijvoorbeeld, zorgt dat voor embryonale letaliteit. Ook in volwassen dieren is Wnt signalering van cruciaal belang. In de darm van zoogdieren is Wnt signalering belangrijk om stamcellen te behouden. Zonder Wnt signaal verdwijnen de stamcellen, wat resulteert in het verdwijnen van de binnenbekleding van de darm. Hyperactivatie van Wnt signalering zorgt voor over proliferatie in de darm. De adenomen die hiervan het gevolg zijn vormen een voorloperstadium van darmkanker.

Het onderzoek naar Wnt signalering begon ruim 30 jaar geleden. In de tussentijd zijn de moleculaire mechanismen van de Wnt signaal transductieroute bijna volledig opgehelderd. De manier waarop Wnt geproduceerd wordt is echter veel minder uitgebreid bestudeerd, dit was het doel van mijn promotieonderzoek. Voordat ik met mijn onderzoek begon was het volgende bekend: Wnt wordt door specifieke cellen gemaakt. Wnt is een eiwit dat gemodificeerd is met een vetzuurstaart, dit is de reden dat Wnt een hydrofoob karakter heeft. Ook is dit waarschijnlijk de reden dat er gespecialiseerde secretie machinerie aanwezig is om Wnt uit de producerende cellen te transporteren. Het membraaneiwit Wntless (Wls) transporteert Wnt van het Golgi apparaat naar het celopppervlak. Vervolgens wordt Wntless teruggebracht naar het Golgi apparaat. Deze retrograde route begint met endocytose van het celoppervlak, vervolgens wordt Wntless via vroege endosomen terug naar het Golgi getransporteerd.

Ik ben mijn onderzoek begonnen door de mogelijkheid te onderzoeken dat ubiquitylering betrokken is bij Wnt secretie. Ubiquitylering is een post-translationele modificatie waarbij het eiwit ubiquitine aan andere eiwitten wordt geplakt. Dit kan voor allerlei veranderingen van het doeleiwit zorgen. Ubiquitylering kan bijvoorbeeld een signaal zijn voor endocytose. Ik heb een RNAi screen gedaan om genen te vinden die betrokken zijn bij ubiquitylering en een rol hebben in Wnt signalering. Deze screen, en de geïdentificeerde genen bespreek ik in hoofdstuk 2. In hoofdstuk 3 ga ik verder in op een van de geïdentificeerde genen, *eel-1*/Huwe1. Deze ubiquitine ligase is niet betrokken bij Wnt secretie of Wls transport, maar werkt als een negatieve regulator van Wnt signalering in de Wnt signaal ontvangende cellen. *eel-1*/Huwe1 ubiquityleert Dvl, een belangrijk eiwit dat nodig is om Wnt signalen van het celoppervlak door te geven naar de celkern. De karakterisatie van *eel-1*/Huwe1 bespreek ik in hoofdstuk 3.

De laatste 3 hoofdstukken van mijn proefschrift gaan over het retrograde transport van Wntless van de plasma membraan terug naar het Golgi. In hoofdstuk 4 beschrijf ik de gevolgen van het uitschakelen van Vps35 in de darm van de muis. Vps35 is essentieel voor transport van Wls tussen endosomen en het Golgi. Verrassend genoeg is het effect van het uitschakelen van Vps35 erg mild. We zien dat Wntless niet meer wordt teruggebracht van endosomen naar het Golgi, zoals verwacht. Dit heeft echter nauwelijks effect op groei of Wnt signalering in de darm.

In hoofdstuk 4 beschrijven we een evolutionair geconserveerd lipide flippase complex dat nodig is om Wntless tussen endosomen en het Golgi te transporteren. Dit transport verloopt via intracellulaire membraan blaasjes. Om deze blaasjes te vormen moet de membraan vervormd worden. Normaal gesproken kunnen eiwitten de membraan vervormen, maar we

konden geen eiwitten vinden die bij dit specifieke transportproces betrokken zijn. Verrassend genoeg vonden we een ander eiwitcomplex dat nodig is. Dit eiwitcomplex verplaatst lipiden van het buitenste naar het binnenste blad van de lipiden dubbellaag van de membraan van het endosoom. Hierdoor ontstaat kromming in de membraan en kan een transportblaasje worden gevormd.

In hoofdstuk 5 bespreken we een eiwit kinase die betrokken is bij Wnt secretie. We hebben aanwijzingen dat deze kinase betrokken is bij endocytose van Wntless.

Het onderzoek dat ik in dit proefschrift beschrijf draagt bij aan het verder begrijpen van de mechanismen van Wntless transport en Wnt signalering. Bovendien is voor de eerste keer aangetoond dat een lipide flippase betrokken is bij retrograde transport in dierlijke cellen en dat dit belangrijk is voor Wnt secretie. Misschien zullen deze inzichten er in de toekomst aan bijdragen dat we Wnt secretie kunnen remmen of versterken om ziektes te behandelen.

Dankwoord

Inmiddels zijn u, de lezer, en ikzelf, de schrijver, bij het laatste - en volgens sommigen het belangrijkste – deel van dit proefschrift gekomen. Ik denk niet dat er velen onder u zijn die het volledige boek van α tot ω hebben doorgelezen, ik verwacht daarom dat deze alinea's het meest gelezen deel van mijn proefschrift zullen zijn.

Voor het schrijven van het dankwoord deed ik inspiratie op door de dankwoorden van oudere proefschriften te lezen, onder het motto 'beter goed gejat, dan slecht bedacht'. Het eerste proefschrift waar ik naar greep was vanzelfsprekend 'Genetic dissection of G protein-coupled signal transduction in *C. elegans*', niet toevallig geschreven door Rik Korswagen, mijn begeleider. In zijn proefschrift vond ik een elegant dankwoord, bestaande uit een enkele regel waarin de erkentelijkheid naar iedereen die bijgedragen had tot de totstandkoming van het proefschrift verwoord werd. Ik moet u bekennen dat mijn vingers jeuken om mijn dankwoord op een zelfde manier in te vullen. Echter, ik zou dan een persoonlijke boodschap in ieder boekje dat ik aan bekenden geef moeten schrijven, wat gezien mijn handschrift misschien geen goed idee is (ik benijd degene niet die over enkele jaren wijs zal proberen te worden uit mijn lab-aantekeningen).

Hoe moet ik mijn dankwoord dan invullen? Een idee dat me aangereikt werd (wederom door Rik) was het schrijven van een ondankswoord. Een aantrekkelijk idee, maar risicovol en daarom een plan dat ik niet zal uitvoeren. Daarom neem ik me voor een tamelijk klassiek dankwoord te schrijven.

Voordat ik aan het feitelijke dankwoord begin wil ik het volgende zeggen. Het lijkt erop dat een belangrijk prioriteit van veel promovendi is om vooral niemand te vergeten die, op welke wijze ook, tot steun is geweest voor de schrijver. Dit gaat soms zo ver dat zelfs de man die de koffieautomaat bijvult een alinea in het dankwoord toegeschreven krijgt (een goed functionerende koffiemachine is immers onontbeerlijk is voor het doen van wetenschappelijk onderzoek). Daarom benadruk ik allereerst dat ik iedereen die, direct of indirect, heeft bijgedragen aan de totstandkoming van dit proefschrift wil bedanken voor hun hulp!

Welnu, het dankwoord:
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Verder was het mooi dat we, buiten wetenschap, nog andere interesses deelden. Ik zal het winterkamperen op de Utrechtse heuvelrug niet snel vergeten; helaas was de isolatiewaarde van mijn matje aan de magere kant, maar het was mooi om in de sneeuw bij Leersum te kamperen en een flesje Jantje Wandelaar te ledigen.

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Curriculum Vitae

Reinoud de Groot was born on july 1st 1983 in Mill, The Netherlands. He attended the Stedelijk Gymnasium Nijmegen from which he obtained his VWO diploma in 2001. After a 2-year stint studying physics, Reinoud switched to study biology at Utrecht University. He obtained his B.Sc. degree in 2006. In 2009 he obtained his M.Sc degree in biomedical sciences. Within the framework of the Cancer Genomics and Developmental Biology masters program, Reinoud performed a 6 months internship in the lab of dr. Leo Klomp studying copper transporting proteins and a 9 months internship in the lab of dr. Rik Korswagen. Following this last internship, Reinoud started his PhD studies with Rik Korswagen at the Hubrecht Institute. The fruits of his studies are described in this thesis.

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* equal contribution

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* equal contribution