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Analýza úlohy vybraných regulátorů endocytózy v signální dráze proteinu Wnt
Study of the role of selected endocytosis regulators in the Wnt signalling pathway

Diplomová práce

Vedoucí závěrečné práce: Mgr. Marie Macůrková, Ph.D

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 30.4.2016

Filip Knop

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Content

Abstract

Wnt signalling pathway is indispensable for a proper development and homeostasis in most of the multicellular organisms. Its evolutionary conservation and wide spectrum of diseases caused by its improper regulation only underscores its importance. Wnt signalling pathway can be conveniently divided into two halves. The first one covers processes that take place in Wnt ligand producing cells and the second one includes Wnt signal transduction in Wnt receiving cells. One of the vital steps in both types of cells is the process of endocytosis. Via regulation of this process, outcome of Wnt signalling itself is regulated.

In this work, nematode *Caenorhabditis elegans* was used as a model organism to characterize new regulator of the Wnt signalling pathway which acts at the level of endocytosis. This new regulator described here is SEL-5 protein kinase. SEL-5 protein kinase belongs to a NAK kinase family and its function seems to be conserved throughout evolution. Its role in Wnt signalling of *C. elegans* was estimated by mining database information about its human homolog AAK1 and based on these information experiments were designed that would allow detailed investigation of its involvement in this pathway.

Genetic interaction of *sel-5* with components of the retromer complex was uncovered and it was established that *sel-5* is not required for maintenance of MIG-14/Wls levels. Results of this work helped to draw preliminary hypothesis of SEL-5 function specifically in Wnt receiving cells. Even in nematode *C. elegans* that is much less complex compared to mammals, Wnt signalling regulators and effectors create a very complicated network. This work tried to explain at least some aspects of this network.

Keywords:

C. elegans, Wnt signalling pathway, endocytosis, neuronal development, SEL-5

Abstrakt

Signální dráha proteinu Wnt se značnou měrou podílí na ontogenetickém vývoji a homeostázi ve většině mnohobuněčných organismů. Její důležitost je podtržena její konzervovaností v evoluci a také širokým spektrem chorob způsobených její deregulací. Signální dráhu Wnt lze pro názornost rozdělit na dvě poloviny. V první jsou popsány procesy, důležité pro vznik a sekreci Wnt ligandu ve Wnt produkujících buňkách a v druhé procesy, které se odehrávají při transdukci Wnt signálu v buňkách přijímacích. Důležitým procesem v obou typech buněk je endocytóza. Regulací procesu endocytózy je zprostředkovaně regulován výsledek signalizace proteinu Wnt.

V této práci byla pro charakterizaci nového regulátoru signální dráhy Wnt na úrovni endocytózy použita jako modelový organismus hlístice *Caenorhabditis elegans* (háďátko obecné). Tímto novým regulátorem je protein kináza SEL-5.. SEL-5 kináza patří do rodiny NAK kináz a její funkce se zdá být konzervována napříč evolucí. S využitím informací o lidském SEL-5 homologu AAK1 z dostupných databází byla předběžně popsána role SEL-5 v regulaci signalizace Wnt u háďátka a navrženy experimenty, které by nám umožnily podrobněji prozkoumat jeho roli v této signální dráze.

Popsali jsme genetické interakce *sel-5* s některými komponenty retromerového komplexu a zjistili, že *sel-5* není nutný pro udržování hladiny MIG-14/Wls. Získané výsledky nám umožnili navrhnout předběžnou hypotézu, ve které je místem působení SEL-5 kinázy především Wnt přijímající buňka. Ve srovnání s evolučně mladšími savci je háďátko mnohem méně komplexní. Přesto je i jeho signální dráha Wnt, tvořená jednotlivými regulátory a efektory, velice složitá. V této práci jsme se pokusily popsat alespoň některé aspekty spojené s regulací signalizace Wnt.

Klíčová slova:

háďátko obecné, signální dráha proteinu Wnt, endocytóza, vývoj nervové soustavy, SEL-5

List of abbreviations

AAK1	AP2 associated kinase
ALM	anterior lateral mictrotubule cell
AP	adaptor protein complex
CamKII	calmoduline-dependent kinase II
CCP	clathrin-coated pit
CCV	clathrin-coated vesicle
CK1α	casein kinase 1α
CME	clathrin-mediated endocytosis
CRD	cystein-rich domain
Dkk	Dickkopf-related proteins
Dvl	Disheveled
EGL	egg laying defective
ER	Endoplasmic reticulum
Fz	Frizzled
GA	Golgi aparatus
GAK	cyclin G-associated kinase
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
GSK3β	glycogen synthase kinase 3β
HCV	hepatitis C virus
HMG	high mobility group
IP3	inositol triphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Lef	Lymphoid enhancer-binding factor
LRP5/6	low-density lipoprotein receptor-related protein 5/6
MIG	abnormal cell migration
MVB	multi-vesicular bodies
NAK	Numb-associated kinase
NGM	nematode growth medium
PCP	planar cell polarity
PCR	polymerase chain reaction

PH	Plecstrin homology domain
PKC	protein kinase C
PLC	phospholipase C
PLM	posterior lateral microtubule cell
PM	plasma membrane
Porc	Porcupine
PtdIns	phosphatidylinositol
PTM	post-translational modifications
PVM	posterior ventral microtubule cell
PX	Phox homology domain
QL	left Q neuroblast
QR	right Q neuroblast
RCC	retromer complex core proteins
RME	retromer-mediated endocytosis
ROR	receptor tyrosine kinase-like orphan receptor
RYK	receptor tyrosine kinase
SEL-5	suppresor/enhancer of LIN-12
SFRP	secreted Frizzled related protein
SNX	sorting nexins
SWIM	secreted Wingless-interacting molecule
Tcf	T-cell -specific transcription factor
TF	transcription factor
TGN	trans-Golgi network
TOCA-1	transducer of Cdc-42 dependent actin assembly
TRN	touch receptor neuron
VPS	vacuolar protein sorting
Wg	Wingless
Wls	Wntless
WT	wild type
β-TrCP	β-transducin repeat-containing protein

1. Theoretical background

1.1 Wnt signalling origin and role in organism

Wnt signalling pathway is an example of a conserved mean of communication between cells in multicellular organisms. It is found generally in all metazoans and its role is indispensable for their viability. Complete Wnt signalling cascade can be found even in pre-bilaterian animals such as *Hydra vulgaris.* In this primitive metazoan, Wnt is responsible for creation of head organizer (which is localized to the posterior site of animal). Head organizer is involved in *de novo* creation of the hypostome as well as in regeneration of the hypostome after its removal (Hobmayer et al., 2000). This enables establishing of the oral-aboral axis in *Hydra.* Similar role of the Wnt signalling pathway in axis determination seems to be conserved from radially symmetrical metazoa to bilateria. The origin of some Wnt signalling modules can be traced to unicellular protozoans. For example, the slime mold *Dictyostelium discoideum* genome codes for a few Wnt ligand receptors and other vital members of the Wnt signalling cascade. Nevertheless, no Wnt ligands have been identified so far (reviewed in Holstein et al., 2014). In different protozoans, different Wnt signalling modules were found (reviewed in Holstein et al., 2014). However, complete functional Wnt pathway seems to be restricted to metazoans.

Apart from involvement in axis formation there are other functions attributed to Wnt signalling. Early development is a fast process accompanied by rapid proliferation, differentiation and asymmetrical divisions of cells. All these processes enable formation of an organism from gastrulation in early developmental stages to organogenesis in later stages. They are tightly controlled and Wnt signalling is directly involved in their regulation (reviewed in Wang et al., 2012). The importance of Wnt is not limited to embryogenesis only. It participates in adult organism homeostasis and stem cell regulation, which are both important for tissue regeneration and renewal (reviewed in Baron and Kneissel, 2013; Clevers et al., 2014).

The significance of this signalling pathway is best underscored by the amount of hereditary diseases that are caused by its improper function (reviewed in Clevers and Nusse, 2012). Wnt signalling cascade is also studied in relation with several types of cancer – most prominently but not limited to, the colorectal cancer (reviewed in Reya and Clevers, 2005).

1.2 Wnt canonical vs non-canonical pathways

Wnt signalling can be divided into β-catenin dependent pathway, which is also referred to as the Wnt canonical pathway (reviewed in Clevers, 2006), and to β-catenin independent pathways, that are also collectively referred to as the Wnt non-canonical pathways. Canonical Wnt signalling has been studied for longer time and more details of regulation and signal transduction are known today compared to non-canonical pathways. Non-canonical branch includes, among others, the planar cell polarity (Wnt/PCP) and calcium (Wnt/Ca^{2+}) pathways (reviewed in Veeman et al., 2003). Such division is of course oversimplification of the complex Wnt signalling network. However, this terminology is still used for historical reasons and will be applied throughout this work.

1.2.1 Canonical Wnt signalling

As mentioned above, canonical Wnt pathway is dependent on β-catenin. Armadillo (*Drosophila melanogaster* β-catenin) was first discovered as one of segment polarity genes (Riggleman et al., 1989). Gradually, protein product of this segment polarity gene was described in more details and its role in wingless (Wg) pathway (*D. melanogaster* Wnt signalling) was identified (Peifer et al., 1991).

When Wnt signalling is off, β-catenin is continually destroyed (processes in Wnt receiving cell are described in more detail in chapter 1.3.2). Once there is a Wnt ligand bound to a receptor, β-catenin destruction complex is inhibited and β-catenin accumulates in cytosol and translocates to the cell nucleus (reviewed in Clevers, 2006). Here it interacts with and activates one of T-cell-specific transcription factor/Lymphoid enhancer-binding factor (Tcf/Lef) protein family. Complex containing β-catenin and Tcf/Lef then targets DNA through its high mobility group (HMG) domain and causes transcription of selected genes (Vleminckx et al., 1999). Apart from its role in Wnt signalling, β-catenin is also involved in cell-cell adhesion mediated through E-cadherins. Complex of E-cadherins, β-catenin and α-catenin facilitates interaction between adherens junctions and actin cytoskeleton of cells (Drees et al., 2005).

As an example of many tasks dependent on Wnt/β-catenin signalling, its role in invertebrate organism development and in adult mammal homeostasis is given in this paragraph. Stabilization of β-catenin is important for primary establishment of anterio-posterior axis in early insect development. In *D. melanogaster* Wg/Wnt signalling causes accumulation of nuclear β-catenin and this leads to an expression of hedgehog and engrailed signalling

proteins. These proteins signal to surrounding cells and cause transcription of *wg* coding gene (Dassow et al., 2000, reviewed in Swarup and Verheyen, 2012) Through this feedback signalling, polarity of future segments is established along anterio-posterior axis of the fly. In adult mammals, small intestine is subjected to regular and fast recycling of epithelial cells. These cells need to be replaced by differentiation of intestinal stem cells. Among others, stem cell niche is maintained via Wnt/β-catenin signalling (Korinek et al., 1998).

Among genes regulated by Tcf/Lef there are those needed for positive or negative feedback regulation of Wnt canonical pathway itself, such as Tcf1 or Axin2 (Roose et al., 1999; Yan et al., 2001). Furthermore, large amount of other genes were identified as targets of Wnt signalling. To name just a few there is CyclinD, which is important for regulation of cell cycle progression, or c-Myc, which is itself a transcription factor (TF) involved in cell proliferation (He et al., 1998; Tetsu and McCormick, 1999).

1.2.2 Non-canonical Wnt signalling

Canonical and non-canonical Wnt signalling use some common proteins in their pathways. They can both signal through Frizzled receptors (Fz) (Bhanot, 1996; Medina et al., 2000), however co-receptor low-density lipoprotein receptor-related protein 5/6 (LRP5/6) seems not to be involved in non-canonical branch. Instead, there are multiple co-receptors whose participation specifies the type of non-canonical pathway activated (reviewed in Niehrs, 2012). Another protein used in both branches of Wnt signalling is Disheveled (Dvl). It is involved in at least some of the non-canonical Wnt pathways (Axelrod et al., 1998).

Wnt/PCP signalling is conserved throughout evolution as much as canonical Wnt pathway and plays important roles in early embryogenesis and development generally (reviewed in Veeman et al., 2003). It is necessary for establishment of uniform polarity of *D. melanogaster* body and wing hairs as well as for polarity of hair follicles in vertebrates (Devenport and Fuchs, 2008). Apart from that it participates in proper stereocilia orientation in mechanosensory hair cells of mammalian cochlea (Dabdoub et al., 2003). PCP signalling enables cells to be oriented in direction perpendicular to their apical-basolateral axis. Such an orientation is achieved by asymmetrical localization of proteins within cells and by activation of actin cytoskeleton regulators (Axelrod, 2001; Winter et al., 2001).

Wnt/Ca^{2+} pathway can regulate influx of Ca^{2+} into the cell and this in turn regulates downstream effectors sensitive to Ca^{2+} levels. Among those effectors are kinases such as protein kinase C (PKC) and calmoduline-dependent kinase II (CamKII) or transcription

factor NF-AT (reviewed in Veeman et al., 2003). Similarly to other described Wnt pathways, Wnt/Ca^{2+} is also essential in development (Kühl et al., 2013). Ca^{2+} influx into a cell is caused by Wnt ligand stimulation of Fz receptor, which leads to activation of phospholipase C (PLC) and production of inositol triphosphate (IP_3). IP_3 then binds and opens Ca^{2+} ion channels (reviewed in Michell et al., 1981).

Since common subset of proteins is used in both canonical and non-canonical Wnt signalling, it is not surprising that crosstalk exists between these two pathways. Some of the Wnt ligands typical for non-canonical Wnt signalling were discovered to inhibit β-catenin dependent responses. Competition for proteins used collectively in all Wnt pathways can partially explain this (reviewed in Veeman et al., 2003). Regulation and activation of an alternative β-catenin destruction complex by Wnt5A was described *in vitro* in a colon cancer cell line (Topol et al., 2003). This leads to a reduction of β-catenin levels in cytoplasm and subsequently to downregulation of β-catenin dependent transcription. *In vivo* function of this regulation system was confirmed in limb development of mouse embryos (Topol et al., 2003). Wnt5a and Wnt11 (another Wnt ligand associated with the non-canonical signalling) were also described in cardiomyocyte development where they inhibit β-catenin signalling via activation of cysteine proteases – caspases (Bisson et al., 2015).

This chapter is just to give a hint of large Wnt signalling network. Complexity of Wnt pathway is not caused by canonical and non-canonical pathways crosstalk only. It is further deepened by crosstalk with other signalling pathways as well and surpasses the scale of this thesis exceedingly.

1.3 Process of Wnt signalling

Wnt ligands are conserved cysteine-rich glycoproteins, which are indispensable throughout the lifecycle of metazoan organisms (reviewed in Logan and Nusse, 2004). They are secreted from producing cells and signal in an autocrine and paracrine manner (Bafico et al., 2004; Logan and Nusse, 2004). In the following chapters, Wnt pathway will be divided in two parts to better describe the signalling process. The first part addresses production and intracellular transport of the Wnt ligand in the producing cells. The second one is focused on the Wnt ligand action at the plasma membrane (PM) of receiving cells and the way in which Wnt signal is transduced into the cell. Special attention is paid to the role of endocytosis and intracellular transport in the signalling process.

1.3.1 Wnt producing cells

1.3.1.1 Birth of the Wnt ligand and its PTM

Wnts, as most proteins destined for secretion, are co-translationally translocated to the endoplasmic reticulum (ER). In this organelle, series of PTM is carried out which are necessary for physiological functionality of the Wnt ligand (reviewed in Willert and Nusse, 2015). The most studied are glycosylation and acylation. There are two known acyl modifications present at conserved sites of Wnt. Firstly, palmitoleic acid is attached to the serine residue at the position 209 of murine Wnt3a (Takada et al., 2006). This serine residue and the adjacent region are highly conserved among Wnts from *C. elegans* to human (Figure 1). Mutation of this serine to alanine, which cannot be acyl-modified, leads to an impairment of Wnt transport and its aggregation in the ER (Takada et al., 2006). Membrane-bound O-acyl transferase protein Porcupine (Porc) was identified as the enzyme that is responsible for palmitoleic group addition. Mutants of Porc show the same phenotypes as organisms with mutated Wnts (Kadowaki et al., 1996). Moreover, Wnts are unable to leave ER when Porc is not working properly (Takada et al., 2006).

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Celeg 214 ETAQFDKT---KNVAHLVRRHNNFVGREAIAQNIRRQCRCHGVSGSCEFKTCWLQMQKF 269
Dmela 204 ERGR--------NLREKMNLHNNEAGRAHVQAEMRQECKCHGMSGSCTVKTCWMRLANF 254
Xlaev 201 EREKIHQKGSYESSRIMMNLHNNEAGRRAVSTLADVACKCHGVSGSCSLKTCWLQLADF 259
Mmusc 189 EKGR--------DLRFLMNLHNNEAGRTTVFSEMRQECKCHGMSGSCTVRTCWMRLPTL 239
Hsapi 177 ENRP--------DARSAMNKHNNEAGRTTILDHMHLKCKCHGLSGSCEVKTCWWAQPDF 227
          *           .    :. *** .**  :       *:***:**** .:***     :
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Figure 1: **Alignment of several Wnt ligands in the region of conserved PTM**. Conserved region (in grey) of Wnt molecules around serine modified with palmitoleic acid (in light blue). Celeg, *C. elegans*; Dmela, *D. melanogaster*; Xlaev, *X. leavis*; Mmusc, *M. musculus* and Hsapi, *H. sapiens*.

The existence and role of a second lipid modification is somewhat controversial. It is supposed to be a palmitate group attached to cysteine 77 of murine Wnt3a or similarly located cysteins in other Wnts (Willert et al., 2003). It does not seem to be important for intracellular trafficking and secretion of Wnt as its extracellular levels are unchanged when cysteine 77 is mutated to alanine (Komekado et al., 2007; Willert et al., 2003). Rather its role was thought to be important for Wnt signalling activity (Komekado et al., 2007; Kurayoshi et al., 2007). Contrary to these observations, crystal structure of *Xenopus laevis* Wnt8 bound to its receptor Fz8, revealed that the cysteine, which is supposed to be

palmitoylated, is involved in a disulfide bond with another cysteine instead (Janda et al., 2013).

Another type of PTM performed in ER is glycosylation. This modification is more variable among different Wnts. When Wg/Wnt is isolated from *D. melanogaster* S2 cell line, there are three variants distinguishable according to N-acyl modifications (none, one or two N-glycan chains) (Tanaka, 2002). The role of glycosylation modification was determined as important for signalling activity of Wnts (Komekado et al., 2007; Kurayoshi et al., 2007). Apart from this, Wnts mutated in such a way that they could not be glycosylated had lower level of lipid adducts (Komekado et al., 2007). However, when Porc knock-down was carried out, glycosylation did not seem to be perturbed by the lack of acyl modifications (Doubravska et al., 2011). It thus seems that glycosylation precedes lipidation in the maturation process of Wnt (Doubravska et al., 2011; Komekado et al., 2007).

1.3.1.2 Intracellular transport and secretion of Wnt

While still in the ER, Wnt ligands are bound by a seven-transmembrane domain protein Wntless (Wls) (Yu et al., 2014) (Fig. 2). Without this protein, Wnts are unable to be transported to the PM and accumulate inside the cell (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Some of the previously described PTM seems to be important for Wls binding of Wnt. Palmitoleic acid modification of the conserved serine appears to be particularly vital for the physical interaction of Wls and Wnt (Coombs et al., 2010; Herr and Basler, 2012). The exact rout within the producing cell used for Wls bound Wnt transport to the PM has not been elucidated in full detail yet. Vesicular transport from the Golgi apparatus (GA) to the PM and release of Wnt would be the most direct way. The main problem of straightforward Wnt ligand release from Wls and its secretion to the extracellular space is its hydrophobicity. The existence of lipid modified Wnt in its free form in aqueous extracellular environment does not seem to be likely. The way of transport of Wnts to the PM would be dependent on the way of transport between producing and receiving cells. Several models of Wnt delivery to the PM and subsequently to the receiving cell have been suggested and described *in vitro* and/or *in vivo*.

Removing the lipid moiety would solve the hydrophobicity problem. Extracellularly active carboxylesterase Notum was indeed identified as a regulator of Wnt signalling pathway (Kakugawa et al., 2015). However, as mentioned previously, fatty acids adducts are indispensable for subsequent signalling activity of the Wnt ligand. Notum probably serves

as another extracellular regulator of the Wnt pathway, similarly to Wnt inhibitors Dickkopf-related proteins (Dkk) or secreted Frizzled-related proteins (SFRP) (reviewed in Cruciat and Niehrs, 2012).

Another possibility would be to engage a protein able to bind and shield the hydrophobic regions of Wnt from aqueous surroundings. Secreted Wingless-interacting molecule (SWIM) with those exact abilities was described to play a role in a long-range Wg/Wnt signalling in *D. melanogaster* (Mulligan et al., 2011). SWIM interacts with the Wnt molecule through its palmitate modification and the lack of this PTM leads to the formation of Wg/Wnt aggregates which are unable to signal properly (Mulligan et al., 2011).

Transport on lipoprotein particles or exosomes would allow Wnt to reach receiving cells located further from producing cells. Lipoprotein particles consist of a hydrophobic lipid core covered by a phospholipid monolayer. Lipid modifications of Wnts would allow for their loading to lipoproteins particles. Structures fitting the description of lipoprotein particles were described as carriers of Wnt in *D. melanogaster* and in cultured mammalian cells (Neumann et al., 2009; Panáková et al., 2005). In the models of Wnt secretion on exosomes, Wls-Wnt complex is first delivered to the PM. Here it is endocytosed and transported to the multi-vesicular bodies (MVB) where Wnt is loaded to a newly-emerged exosome (Gross et al., 2012). Wnts attached to exosomes were shown to be active in Wnt signalling (Gross et al., 2012). Signalling relevance of such exosomes was, however, disputed in another paper (Beckett et al., 2013).

The last possible way of Wnt ligand delivery mentioned in this overview are cytonema. These cellular structures are sometimes called signalling filopodia which describes their shape and purpose perfectly. Reports of cytonema role in signalling generally are increasing in numbers lately. It can be either the producing cell that is sending out filopodia with the ligand or the receiving cell stretching filopodia carrying the ligand receptors (reiewed in Pröls et al., 2015). In the context of Wnt signalling both possibilities were described. Wnt8a was shown to be carried on cytonemes to control processes in *Danio rerio* gastrulation (Stanganello et al., 2015). Signalling filopodia reaching towards Wnt producing cells were also found to contain Fz7 in chicken embryos (Sagar et al., 2015).

1.3.1.3 Wls recycling

Whichever way of Wnt secretion is actually used, intracellular Wnt binding partner Wls needs to be recycled from the PM back to the GA and then to the ER (Yu et al., 2014) (Fig.

2). To get inside the cell, Wls is first internalized from the PM through clathrin-mediated endocytosis (CME) and transported to the early endosome (more on this in section 3.1.4). In the early endosomes (sometimes termed sorting endosomes), all endocytosed proteins are sorted to be directed to their prospective locations (reviewed in Huotari and Helenius, 2011). Some proteins are meant for degradation and are transported to late endosomes, which ultimately fuse with lysosomes. Others are recycled back to the PM or to the GA.

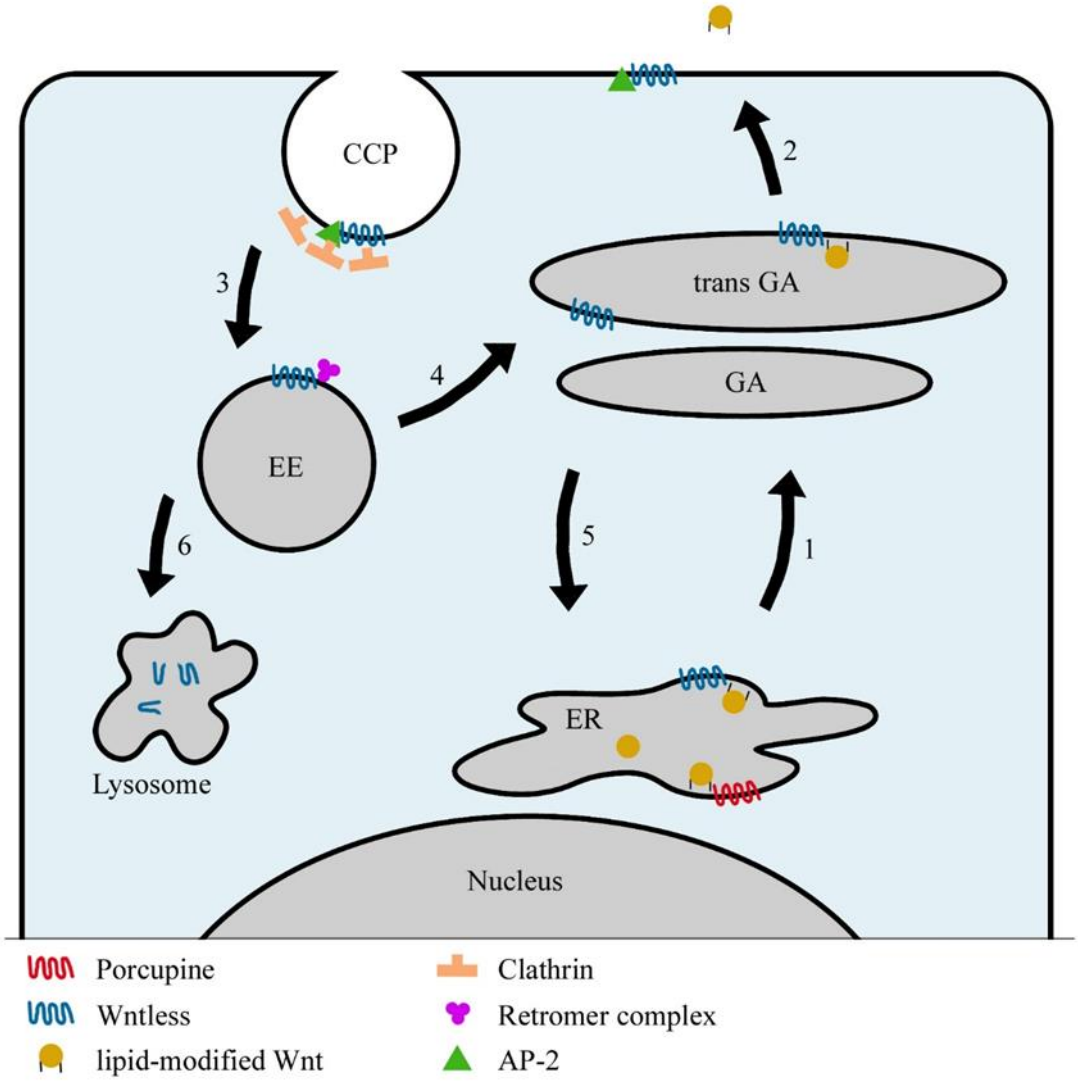


Figure 2: Processes in Wnt producing cells. In this picture a scheme is shown that depicts processes taking part in Wnt producing cells. Wnt synthesis, PTM and association with Wls protein are depicted in the ER. Then Wnt-Wls complex is transported via the GA (1) to the PM (2). From the PM, Wls is internalized via CME into the EE (3) and recycled with the help of retromer complex to the GA (4). Wls is then transported back to the ER (5) for another round of Wnt transport. In the case of improper function of retromer complex, Wls is transported to the lysosome and degraded (6).

Wls is recycled to the GA through its interaction with the retromer complex (Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008) (Fig 2). Retromer consists of three core proteins, VPS26, VPS29, VPS35 and at least one protein of the sorting nexin family (SNX) (reviewed in Gallon and Cullen, 2015). For Wls recycling SNX3 is required rather than other SNXs (Harterink et al., 2011). SNX3 contains a Phox homology domain (PX) which enables its association with phosphatidylinositol 3-phosphate (PtdIns(3)*P*) typically presented in the early endosome membrane (Yu and Lemmon, 2001). Of the three retromer complex core proteins (RCC), it is VPS35 that recognizes and directly binds Wls (Belenkaya et al., 2008). Upon VPS35 disruption, Wls no longer co-localizes with the GA and is shifted to the MVB for subsequent degradation in lysosomal compartment (Franch-Marro et al., 2008; Harterink et al., 2011) (Fig. 2). SNX3 itself does not seem to contribute to Wls recognition. Instead, it helps RCC to associate with the early endosomes and thus enables the retromer interaction with cargo proteins (such as Wls) on the early endosome membrane (Harrison et al., 2014; Harterink et al., 2011).

Other SNXs are also used for retromer cargo sorting and transport to the PM or the GA. A family of SNX-BAR proteins in cooperation with RCC is involved in recycling of lysosomal hydrolases receptors from early endosomes to the trans-Golgi network (TGN) (reviewed in Gallon and Cullen, 2015). BAR domains of those SNXs are able of dimerization. Created concave shape drives and stabilizes endosomal membrane tubules formation (Frost et al., 2008). Lysosomal hydrolases receptors and other cargo proteins are transported to the TGN via tubular-shaped membraneous structures (van Weering et al., 2012). SNX3 protein contains only PX domain and lacks any BAR domains. A mechanism of Wls-containing retromer budding and pinching off early endosome membrane is currently unknown. An article has been recently published, describing the role of TOCA-1 protein (transducer of Cdc-42 dependent actin assembly) in Wls recycling (Bai and Grant, 2015). TOCA-1 belongs among BAR domain containing proteins; however, its role was described later in recycling process in location distinct from early endosome (Bai and Grant, 2015).

For a long time it has been assumed that Wls is recycled only to the GA where it binds another Wnt molecule and enters the Wnt secretion cycle again. While this may be true for *D. melanogaster* Wg/Wnt signalling (Port et al., 2008; Zhang et al., 2016), it turned out that Wls is recycled all the way to the ER in human cell lines (Yu et al., 2014). For the last step of Wls recycling its C-terminal target motif is necessary. Retrograde transport of Wls from the GA to the ER is mediated by the COPI system and passes through an ER-Golgi intermediate compartment similarly to exotoxins such as *Shiga* toxin (Yu et al., 2014).

1.3.1.4 Endocytosis in Wnt producing cells

Endocytosis is a very important process, enabling cells to take up extracellular molecules and to recycle transmembrane proteins and lipids of the PM. It is a very dynamic process with substantial portions of the PM endocytosed every hour. In some specialized cells, this number is estimated to reach up to twice of the overall PM area per hour (reviewed in Steinman et al., 1983). Multiple ways of endocytosis has been discovered and described so far. Apart from specialized forms of endocytosis such as pinocytosis or phagocytosis, there are clathrin-mediated (CME) and caveolin-dependent endocytosis. The latter two, termed collectively receptor-mediated endocytosis (RME), are most relevant for Wnt signalling.

As previously described, first step in Wls recycling is its endocytosis from the PM and transport to early endosome (figure 2). For this process functional clathrin and dynamin, both participating in CME, are indispensable (Gasnereau et al., 2011; Pan et al., 2008). Clathrin itself is unable to bind to the PM, or any other membrane, as it does not contain suitable binding domains. How does it contact cell surface structures then? Clathrin membrane binding is dependent on adaptor protein complexes (APs) that are able to bind clathrin as well as components of the membrane sheet. Multiple APs were identified to fulfill this role (reviewed in Owen et al., 2004); some of the most studied are given as an example in Table 1.

Clathrin adaptor	Cargo motif recognized	Ubiquitin binding	Membrane component bound
AP2 complex	ΦxxYxxΦ [DE]xxxL[LI]	Yes	PtdIns(4,5)P_2
AP1 complex	ΦxxYxxΦ [DE]xxxL[LI]	Yes	PtdIns4P
GGA	DxxLL	Yes	ArfGTP
Epsin		Yes	PtdIns(4,5)P_2
HRS/Vps27		Yes	PtdIns3P
DAB2/ARH	[FY]xNPxY	Yes	PtdIns(4,5)P_2
Arrestin	GPCRs	Yes	PtdIns(4,5)P_2

Table 1: **Selected adaptor protein complexes (APs) specifically interacting with clathrin**. First column-APs name, second column-cargo motif recognized by a given AP, third column-ability to bind ubiquitin modification, fourth column-molecule of membrane bound by a given AP. (Adapted from Owen et al., 2004).

Of the APs in Table 1, AP2 was found out to have a vital role for Wls endocytosis and Wnt secretion from producing cells (Pan et al., 2008; Port et al., 2008; Yang et al., 2008). AP2 is a complex composed of four proteins – two large subunits (α and β), one medium size subunit (μ2) and one small subunit (σ). Interaction between α subunit of AP2 and phosphatidylinositol *4,5*-bisphosphate (PtdIns(4,5)*P*), a phosphoinositide typically present in the PM, is what drives AP2 localization to the PM. Wls and other cargo recognition is the task of the μ2 subunit and clathrin is bound to the β subunit of AP2 complex (reviewed in Owen et al., 2004).

Cargo specific motif recognized by the μ2 subunit of AP2 consists of a short amino acid sequence YxxΦ (Table 1). Such a motif is indeed localized to the third intracellular loop of Wls protein (Gasnereau et al., 2011). Mostly YxxΦ cargo recognition motifs are found in the C-terminus of transmembrane proteins. Its localization in Wls is thus rather unusual, however, it is a *bona fide* recognition motif as its mutation leads to an accumulation of Wls at the PM and to lowered levels in the GA (Gasnereau et al., 2011).

Recognition of Wls (or cargo protein generally) by AP2 at the PM leads to an accumulation of clathrin light and heavy chain subunits. This is in turn followed by creation of a clathrin-coated pit (CCP), an invaginated structure surrounded by membrane and covered with clathrin lattice (Cocucci et al., 2012). This structure is still attached to the PM. To separate Wls proteins containing CCP from the PM a catalytic activity of GTPase enzyme dynamin is necessary (Meinecke et al., 2013). Dynamin is recruited to the site of CCP scission through its Pleckstrin homology domain (PH) and through binding of the AP2 α subunit (Achiriloaie et al., 1999; Wang et al., 1995). Similarly to PX domain, PH domain enables proteins to interact with members of the phosphoinositide (PIs) lipid family (Harlan et al., 1994).

Other APs and CCP associated proteins are known to regulate CME. However, their role specifically in Wls endocytosis have not been studied so far. Actin participation in the process of CME is well defined in unicellular eukaryote *Saccharomyces cerevisiae* (Hay et al., 1998). Its role in multicellular organisms is, nevertheless somewhat controversial. In the adherent cell lines, dynamic actin network was required, whereas for cells grown in suspension it seems to be dispensable (Cheng et al., 2012).

After CCP pinching off the PM, clathrin-coated vesicle (CCV) is formed. Before it is able to fuse with other endocytosed vesicles into early endosome its clathrin coat and associated proteins need to be disassembled. Molecules such as chaperon protein Hsc70 and its cofactor auxilin mediate this disintegration of clathrin lattice (Hirst et al., 2008; Ungewickell et al., 1995). When uncoated, endocytosed Wls-containing vesicles fusion can progress and early

endosomes emerge. Then Wls is ready to be bound by retromer complex and recycled to the ER (see chapter 1.3.1.3).

1.3.2 Wnt receiving cells

Above, I described processes of Wnt signalling taking part in Wnt producing cells. Now I am going to focus on series of events happening at and in Wnt receiving cells. Wnt signal transduction and its regulation is main theme of following chapters.

1.3.2.1 Receptors of Wnt ligands

Multiple receptors are able to bind Wnt molecules at the surface of Wnt receiving cells (reviewed in Niehrs, 2012). One group of them, Frizzled proteins (Fz), is commonly used in both, canonical and non-canonical branch of Wnt signalling (Bhanot, 1996; Medina et al., 2000). Fz belongs to a large family of G-protein coupled receptors (GPCR) and consists of seven hydrophobic regions that intersect the PM. All Fz proteins contain cysteine rich domain (CRD) in their extracellular N-terminal region, which is responsible for Wnt ligand binding (Janda et al., 2013). For a proper receptor-ligand interaction, PTM of Wnt molecules are also important (described in chapter 1.3.1.1).

For functional β-catenin dependent Wnt signalling, cooperation of Fz protein with another family of Wnt receptors, low-density lipoprotein receptor-related protein (LRP), is indispensable. Namely LRP5 or LRP6 (referred to as LRP5/6 in further text) whose interaction with both Fz receptor and Wnt ligand enables Wnt signal transduction (Tamai et al., 2000). Unlike Fz protein, LRP5/6 is a single-span transmembrane receptor and is utilized only by the β-catenin dependent pathway, although a Wnt-independent function has also been reported for LRP5 (Chin et al., 2015). Extracellular region of LRP5/6 is able to bind Wnt ligands and Fz receptors simultaneously (Bourhis et al., 2010).

Series of specific receptors have been found to be involved in the β-catenin independent Wnt signalling, nevertheless there is an overlap between their function in both Wnt pathways (reviewed in Niehrs, 2012). Single-pass transmembrane proteins, receptor tyrosine kinase-like orphan receptor (ROR) and receptor tyrosine kinase (RYK) are an example of such receptors. They both need Fz for their activity in Wnt signalling. Similarly to Fz, ROR contains CRD in its extracellular domain which enables interaction with Wnt ligand. Different domain, WIF in the N-terminal region, serves the same purpose in RYK receptor. Despite containing intracellularly located tyrosine kinase domains, these are not always

indispensable for functional Wnt signalling (Forrester et al., 2004; Hikasa et al., 2002). In fact, RYK tyrosine kinase is completely inactive (Katso et al., 1999).

1.3.2.2 Wnt signal transduction

After Wnt ligand is bound to a proper receptor, a cascade of events is triggered at the PM of Wnt receiving cells and in its cytoplasm. In further text, only the Wnt/β-catenin signalling pathway will be described.

Without ligand bound to a receptor, Wnt/β-catenin signalling is constitutively turned off (Clevers, 2006). To keep this pathway off, it is necessary for β-catenin protein to be continually degraded. Complex of proteins, termed β-catenin destruction complex, binds its target and marks it for degradation. This complex consists of scaffolding proteins, adenomatous polyposis coli (APC) and Axin and protein kinases, casein kinase-1α (CK1α) and glycogen synthase kinase 3β (GSK3β). CK1α and GSK3β sequentially phosphorylate β-catenin. Phosphate modifications are recognized by β-transducin repeat-containing protein (β-TrCP), and through its activity, β-catenin is ubiquitinated. This ubiquitination serves as a signal for elimination of β-catenin in proteasomal complex (Clevers, 2006). Intracellular levels of β-catenin are thus kept low and no transcription of target genes should occur.

As soon as Wnt ligand is bound to its receptor Fz and co-receptor LRP5/6, situation starts to change. β-catenin protein accumulates in cell cytoplasm and translocates to the nucleus where it directs target genes expression. For this to happen, it is necessary that β-catenin destruction complex is disrupted or its activity is inhibited. Fz receptor with bound Wnt ligand recruits Dvl protein to its intracellular domain (Bilić et al., 2013). Dvl contains three domains important for it roles in Wnt signalling: PDZ, DEP and DIX. DIX domain in the N-terminal region is vital for polymerization of Dvl with itself and with Axin (Schwarz-Romond et al., 2007). A complex of several proteins, termed LRP5/6 signalosome, is thus created upon Wnt signalling. Axin brought to this complex is associated with GSK3β which is able to phosphorylate LRP5/6 prime-phosphorylated by membrane bound CK1γ (Davidson et al., 2005). Several PPPSP motives present in LRP5/6 are phosphorylated in this way and in turn, modified LRP5/6 binds Axin with higher affinity (Tamai et al., 2004). This feedforward positive loop promotes creation and stability of LRP5/6 signalosome at the PM. As part of β-catenin destruction complex proteins are relocated to the LRP5/6 signalosome, the complex is not efficient in degrading β-catenin any more.

There is an ongoing discussion as to whether endocytosis plays any role in Wnt signal transduction. Several papers have been published so far regarding this topic. However, data presented in these papers seem to be conflicting to a certain degree and few of them are contradictory. Overview of endocytotic processes in Wnt receiving cells are described in the following chapter.

1.3.2.3 Endocytosis in Wnt receiving cells

Similarly to its role in Wnt producing cells, CME has also been suggested to act in Wnt receiving cells. Apart from CME, multiple articles published results, hinting to participation of caveolin-mediated endocytosis along or instead of CME.

Wg/Wnt ligand, produced at dorso-ventral boundary of *D. melanogaster* imaginal disc, binds receptors on Wg/Wnt receiving cells and is endocytosed (Piddini et al., 2005). This internalization is necessary for proper canonical Wg/Wnt signalling (Seto and Bellen, 2006). When dynamin is knocked down, endocytosis as well as Wg/Wnt signalling are reduced. However, as dynamin is involved in both CME and caveolin-dependent endocytosis, it is not possible to say which one is involved here.

Dynamin together with clathrin were shown to be important for internalization of Wnt3a and canonical Wnt signalling in murine L cells (Blitzer and Nusse, 2006). Clathrin was found out to be important in Wnt signalling regulation in HEK293T cells as well. Nevertheless, opposite to what was observed in murine L cells, here it was involved in inhibition of canonical Wnt signalling rather than in its stimulation (Yamamoto et al., 2008). In a process involving Wnt inhibitor Dkk binding to Wnt co-receptor, LRP5/6 is endocytosed via CME. The level of LRP5/6 is thus reduced and unable to participate in β-catenin stabilization (Yamamoto et al., 2008). In the same article, caveolin-dependent endocytosis was shown to participate in internalization of LRP5/6 signalosome and β-catenin stabilization.

Yet another research group proposed that neither CME nor caveolin-mediated endocytosis were necessary for Wnt3a activation of Wnt/β-catenin signalling (Kim et al., 2013). Interestingly enough, AP2 and clathrin itself were found to be indispensable for LRP5/6 signalosome formation and stability (Kim et al., 2013). In *D. rerio* embryos, μ2 subunit of AP2 was also identified as essential for LRP5/6 signalosome stability and Wnt/β-catenin signalling activity (Hagemann et al., 2014). Here, however, CME of LRP5/6 was observed to support these processes (Hagemann et al., 2014). An overview of selected articles and their findings regarding endocytosis role in Wnt signalling are listed in Table 2.

Table 2

cell line/organism	internalization after Wnt/Wnt inhibitor stimulation	involved proteins	role in Wnt/β-catenin signalling	reference
HEK293 MEF BS-C1	no Wnt-3a induced LRP-5/6 internalization	AP-2, clathrin	LRP-5/6 signalosome formation and β-catenin stabilization	Kim et al., 2013
D. rerio embryo	Wnt-8 induced LRP-5/6 internalization	AP-2	LRP-5/6 signalosome formation and β-catenin stabilization	Hagemann et al., 2014
murine L cells	Wnt-3a internalization	clathrin, dynamin	Wnt-3a internalization and β-catenin stabilization	Blitzer et al., 2006
HEK293T HeLa	Wnt-3a induced LRP-5/6 phosphorylation and internalization	caveolin	LRP-5/6 internalization and β-catenin stabilization	Yamamoto et al., 2008
HEK293T HeLa	Dkk induced LRP-5/6 internalization	clathrin	LRP-5/6 internalization and attenuation of Wnt/β-catenin signaling	Yamamoto et al., 2008
HEK293T	Wnt-3a induced LRP-5/6 internalization	caveolin	LRP-5/6 internalization and β-catenin stabilization	Demir et al., 2013
Hep-G2	Wnt-3a induced caveolin-mediated endocytosis	caveolin	β-catenin stabilization	Glinka et al., 2011
F9	Wnt-3a induced LRP-6 internalization	caveolin	β-catenin stabilization and activation of Wnt/β-catenin signalling	Jiang et al., 2012
MDC	Wtl knockdown stimulates LRP-6 internalization	caveolin	Wnt/β-catenin signalling activation	Jing et al., 2015

Table 2: **Overview of different endocytosis regulators in Wnt signalling.** (See the next page)

Table 2: **Overview of different endocytosis regulators in Wnt signalling.** Importance of endocytosis for Wnt/β-catenin signalling in selected cell lines or organisms. For every cell line/organism, consequence of Wnt or Wnt inhibitor stimulation, type of endocytosis and participating proteins are indicated. HEK293; human embryonic kidney cells, MEF; mouse embryonic fibroblast, BS-C1; monkey kidney epithelial cell, HeLa; human cervical cancer cell line, Hep-G2; human hepatocellular carcinoma cell line, F9; murine embryonal testis carcinoma cell line, MDC; mouse podocyte cells.

Apart from LRP5/6 internalization and degradation through its interaction with Wnt inhibitor Dkk, another mechanism using receptor endocytosis and subsequent degradation exists. This time it is Wnt receptor Fz, which is ubiqitinated by activity of two PM localized ubiquitin ligases, Rnf43 and Znrf3, and afterwards endocytosed and degraded (Koo et al., 2012). It is not known so far what type of endocytosis is involved here. However, Wnt signalling activity is accompanied by Rnf43 and Znrf3 endocytosis via CME.

Whether for signal transduction or receptors level regulation, endocytosis seems to be an important process taking part in Wnt receiving cells. However, more research is needed for complete elucidation of individual endocytosis pathways in positive and negative regulation of Wnt signalling. On the basis of the latest articles, caveolin and CME role in Wnt signalling are pictured in Fig 3.

1.4 Wnt signalling in *C. elegans*

Nematode *C. elegans* was used as a model organism to gain experimental data presented in this diploma thesis. Therefore, a short overview describing the role of Wnt signalling in worm and differences between this and other organisms (predominantly vertebrates) will be given in this chapter.

As in other multicellular organisms, *C. elegans* Wnt signalling participates in processes from early embryogenesis to homeostasis of adult animals. It directs cell asymmetrical divisions, their proliferation, polarization and migration. As a model system, *C. elegans* nervous apparatus was used in most experiments described in result section of this thesis. Adult worm consists of roughly 1000 cells of which slightly over 300 are neurons of all kinds (Sulston and Horvitz, 1977). Canonical as well as non-canonical Wnt signalling pathways were described instructing correct migration and polarization in some of these neurons (Pan et al., 2006; Whangbo and Kenyon, 1999).

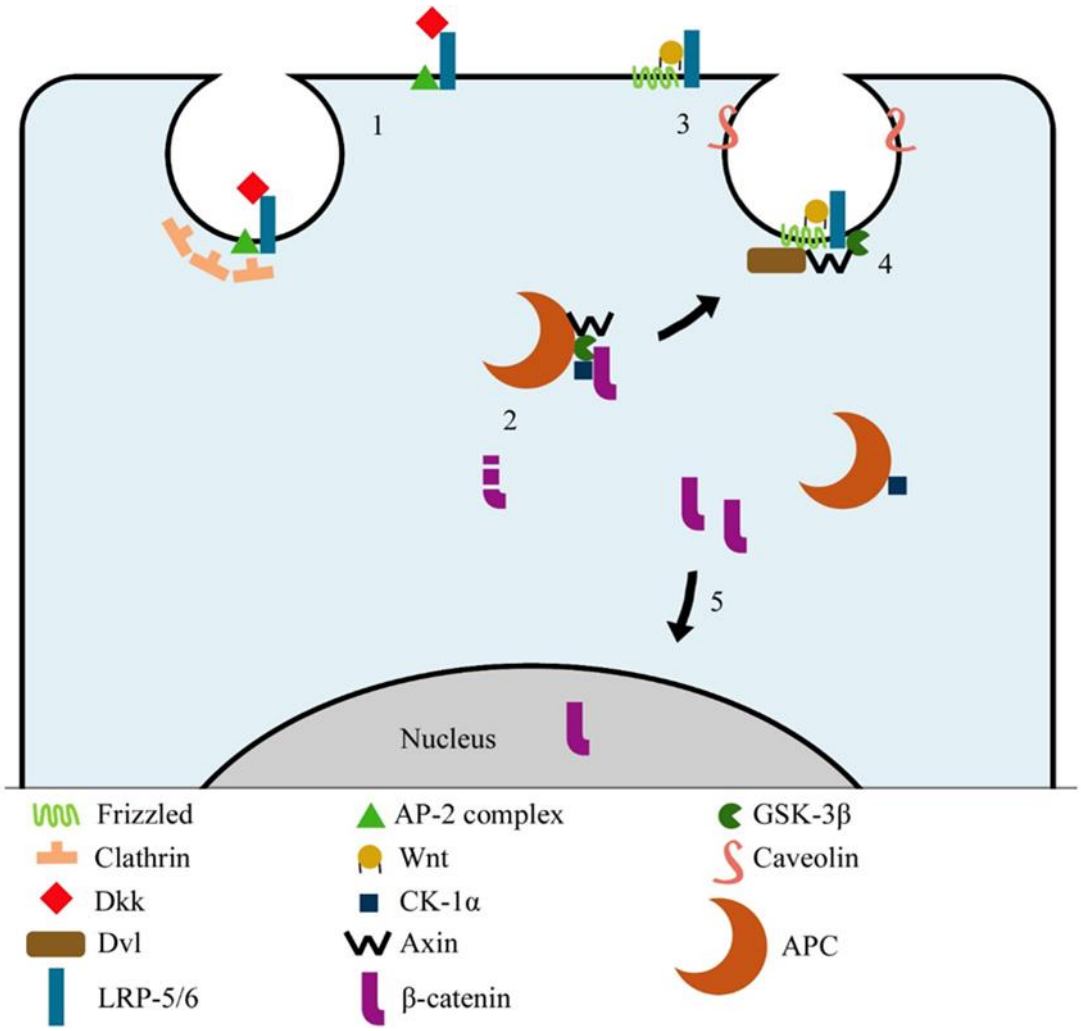


Figure 3: Processes taking part in Wnt receiving cells. In the case of Wnt signalling inhibitor Dkk binding to LRP5/6 co-receptor, this complex is internalized via CME and Wnt signalling is attenuated (1). If that is the case, β-catenin is phosphorylated by destruction complex and degraded (2). In the case of Wnt ligand interaction with Frizzled receptor and LRP5/6 co-receptor, this complex interacts with Dvl and is internalized via caveolin-mediated endocytosis (3). The signalosome further recruits some of β-catenin destruction complex (4) which leads to its inactivation, stabilization of β-catenin and its translocation to the nucleus (5).

Most components of Wnt signalling present in higher organisms were identified in worms as well. However, some variation exists and need to be mentioned. The most striking disparity is the lack of LRP5/6 co-receptor or its homologs in *C. elegans*. Up to date, it is not known whether LRP5/6 is just elusive or another protein receptor takes up its role. Apart from LRP5/6, components of Wnt/β-catenin pathway seem to be involved in signal transduction comparably with other organisms.

Another difference is the use of β-catenin or TCF/LEF in non-canonical Wnt signalling. This is not observed in higher organisms and seems to be specific for *C. elegans* (Shin et al., 1999). Despite the above mentioned variability between worm and vertebrate Wnt signalling, *C. elegans* is still very useful organism for studying this pathway. It is generally less complex in worm, compared to higher organisms, and thus provides better chances of comprehensibly mapping the Wnt signalling network.

Most of the neurons used in this work belong to touch receptor neurons (TRN). Some of the neurons whose position or polarity was used as a marker of functional Wnt signalling are descendant cells of QL neuroblast. QL neuroblast originates from a region slightly posterior to the centre region of an animal. As it matures and divides, its descendants (PQR, SDQL and PVM) migrate posteriorly to their final positions. This migration is positively affected by Wnt/β-catenin signalling mediated through one of *C. elegans* Wnt ligand, EGL-20. This is an example of Wnt/β-catenin signalling in *C. elegans* (Fig. 4). Homologs of all members of this pathway were described in previous chapters and their mutations were shown to affect, more or less, a proper QL descendants migration. Erroneous anterior position of PVM neuron is termed QL phenotype in this thesis.

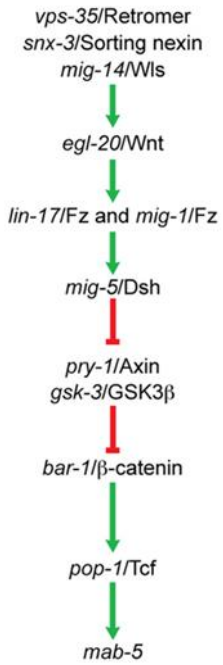


Figure 4: Wnt signalling cascade of β-catenin dependent pathway in *C. elegans*. This schematic picture of Wnt signalling pathway shows worm homologs of mammalian Wnt/β-catenin pathway that are involved in proper localization of QL neuroblast descendants along anterio-posterior axis. The last link in the scheme is *mab-5*, a transcription factor coding gene whose expression ultimately leads to a proper Q descendants migration. (adapted from Sawa H., Korswagen H. C. Wnt signaling in *C. elegans* at http://www.wormbook.org.)

Apart from QL migration other TRN developmental features are dependent on Wnt signalling. ALML and ALMR are two TRN located in anterior half of an animal. In WT animals, each possess one long neurite protruding towards nerve ring located in a head region and no (or very short) posterior process (Fig. 5). In animals carrying mutation in some Wnt signalling pathway components, these TRN can have either reversed or bipolar orientation. Proper orientation is dependent on a combination of Wnt ligands and mutation of one could be compensated for by others. However, when Wnt ligand secretion is affected globally, as is the case of *mig-14/wls* mutation, then incorrect ALM polarization occurs (Yang et al., 2008).

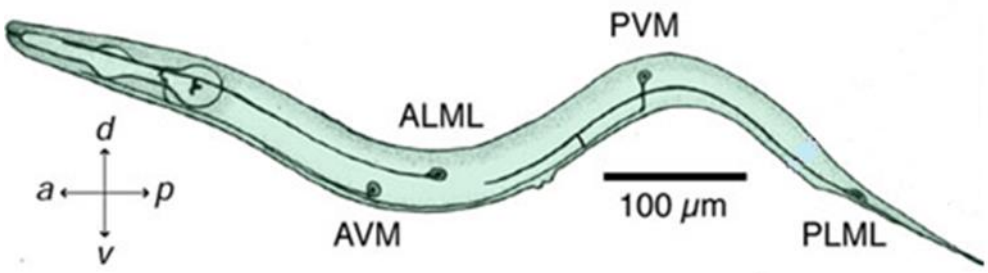


Figure 5: Touch receptor neurons position and polarity. A group of neurons that can sense soft touch is depicted here in their WT localization and polarity. There is symmetrical pair of ALM and PLM neurons, however, only one of each pair is shown in this lateral view. (Adapted from Goodman, M.B. Mechanosensation, WormBook at http://www.wormbook.org.

The last TRN affected by components of the Wnt pathway are two symmetrical PLM neurons located in the posterior tip of an animal (Fig. 5). They have one long neurite heading anteriorly to the central position of a worm and one short heading in the opposite way. This polarity can be reversed in Wnt signalling mutants. The long neurite grows posteriorly instead and when it reaches the tip of the tail it turns around and continues growing anteriorly. Similarly to ALM neurons, PLM orientation is also affected by several Wnt ligands (Hilliard and Bargmann, 2006).

2. Objectives

Endocytosis plays role in multiple cellular processes and is influenced by many regulatory proteins. We wanted to scan through those regulators and determine which of them are specifically important for Wnt signalling pathway. To this end, we exploited general and specialized molecular biology methods applicable to a model organism, *C. elegans*. For a better clarity, we divided objectives into three main themes listed below:

I) Identify new endocytosis regulators involved in controlling Wnt signalling in the nematode *C. elegans*.

II) Identify the site of action of the potential new regulators either in the Wnt producing or Wnt receiving cell.

III) Characterize the function of new regulators in Wnt signalling to expand our knowledge about the role of endocytosis in the Wnt pathway.

3. Materials and methods

3.1 Bacterial and *C. elegans* strains

Bacterial strains:

OP50 *E. coli* uracil auxotroph used for *C. elegans* standard cultivation

HT115 *E. coli* containing IPTG-inducible T7 polymerase used for *C. elegans* RNAi feeding

DH5α *E. coli* competent cells used for plasmid cloning

***C. elegans* strains, mutants and transgenes:**

Standard N2 Bristol strain was used as a wild type. Following mutant alleles and transgenes were used:

mutant alleles

LGII	*mig-14(mu71)*
LGIII	*mtm-6(ok330)*
	sel-5(ok363)
	vps-29(tm1320)
LGIV	*dyf-3(m185)*

transgenes

LGII	*muIs32 [mec-7p::GFP; lin-15(+)]*(Ch'ng et al., 2003)
	huSi2 [mig-14p::mig-14::GFP] (Silhankova et al., 2010a)
LGV	*muIs35 [mec-7p::GFP; lin-15(+)]*(Ch'ng et al., 2003)
Extrachromosomal	*mamEx5 [egl-20p::sel-5(RNAi); myo-2p::tdTomato]*
	mamEx6 [egl-17p::sel-5(RNAi); myo-2p::tdTomato]
	mamEx9 [egl-20p::sel-5::gfp; myo-2p::tdTomato]
	mamEx10 [egl-17p::sel-5::gfp; myo-2p::tdTomato]

3.2 Cultivation media and *C. elegans* culturing

3.2.1 Nematode growth medium (NGM) agar plates preparation

Per 1 litre of agar NGM following ingredients were mixed:

NaCl……3 g

tryptone……...2,5 g

agar……17 g

H_2O…….975 ml

Autoclaved at 120°C and let stand to cool down to 55°C. Then the following ingredients were added:

1M CaCl……...1 ml

cholesterol (5mg/ml in EtOH)..1 ml

1M $MgSO_4$……....1 ml

KPO_4 buffer……...25 ml

NGM was poured to plates (10 ml for 60mm plate) and let stand overnight (o/n) at room temperature. Next day OP50 bacterial strain cultured o/n at 37°C in LB was pipetted and spread over NGM plate (approx. 30µl for 60mm plate) and let stand to grow o/n at room temperature. Next day plates were ready to be used for worm cultivation. Alternatively, they were stored at 4°C for later use.

NGM plates with worms were kept at 20°C. If slower worm growth and life cycle was required, they were kept at 15°C.

Per 500 ml of KPO_4 buffer solution following ingredients were mixed:

KH_2PO_4……54,2 g

K_2HPO_4……17,8 g

Water was added to 500 ml and pH adjusted to 6, then the solution was autoclaved.

3.2.2 NGM for RNAi feeding of *C. elegans*

For this application, NGM was prepared in the same way as for common *C. elegans* culturing. However, when NGM was removed from autoclave and cooled down to 55°C, additional ingredients were added – ampicillin (final concentration 50 µg/ml), tetracyclin (final concentration 12,5 µg/ml), and IPTG (final concentration 1mM). We used Ahringer lab library of HT115 bacterial strains (Kamath et al., 2000) carrying L4440 plasmid with a coding region DNA fragment of selected gene inserted. Antibiotics prevented growth of

bacteria other than resistant HT115 strain. IPTG induced expression of T7 polymerase, which in turn transcribed inserted DNA coding region fragment into a future dsRNA. *C. elegans* RNAi feeding was carried out as described in (Kamath et al., 2000).

3.2.3 Cleaning of contaminated *C. elegans* plates (bleaching)

For cleaning of contaminated *C. elegans* plates, solution of 5M KOH and household bleach (Savo) (in ration 1:2) was used. Gravid worms from contaminated plates were washed with M9 and transferred to 1,5ml tube. The tube was centrifuged for 30 seconds at low speed (500xg) and worm pellet with 350 µl of M9 were transferred to a fresh tube. 150 µl of KOH and household bleach mixture was pipetted to the worm solution and mixed vigorously. Tubes were checked continually under a dissecting microscope and when approximately 50% of worms were lysed, they were centrifuged and supernatant was removed. Pellet was washed three times with fresh M9. After final washing, pellet was transferred to a fresh NGM plate. Bacteria and other biological contaminants should be killed by this treatment, only embryos from gravid worms survive bleaching.

3.2.4 Bacteria growth medium

Per 1 litre of Luria-Bertani broth (LB) following ingredients were mixed:

tryptone……10 g
yeast extract……..5 g
NaCl…….10 g

Water was added to 1 litre and then autoclaved. For agar LB plates, 15 g of agar was added. For selective LB, appropriate antibiotics were included in LB.

3.2.5 *C. elegans* crossing procedures

To create double-mutant animals or to introduce a transgene into mutant animals it was necessary to carry out a genetic cross. Females are non-existent in nematode *C. elegans.* In a wild, these animals are mostly hermaphrodites, which are able to fertilise their own eggs without help of other gender. Males exist, however, their occurrence is very rare outside laboratory conditions. When males are introduced to hermaphrodite containing plates, they manifest typical mating behaviour. Hermaphrodites seems to prefer male sperm to their own and such animals tend to have higher numbers of progeny. Up to 50% of this progeny is of male gender. These properties were used for creating double mutant or transgenic animals.

As an example, introduction of transgene *muIs35* (*[mec-7p::GFP; lin-15(+)]*) to a mutant strain RB638 (deletion in *sel-5* gene) is described here:

1) *muIs35* ♀ x N2 ♂……four *muIs35* hermaphrodites (L4 stage) transferred to 35mm NGM plate with six N2 wild type males

→ progeny of successful crossing was roughly 1:1 (male:hermaphrodite ratio)

all progeny of this cross should have been heterozygous for *muIs35* ($\frac{muIs35}{+}$)

(*muIs35* animals express GFP in touch receptor neurons (TRN) and could be easily checked under fluorescent dissecting microscope)

2) $\frac{muIs35}{+}$ ♂ x *sel-5(ok363)* …….. six $\frac{muIs35}{+}$ males transferred to 35mm NGM plate with four *sel-5* hermaphrodites (L4)

→progeny of successful crossing was roughly 1:1 (male:hermaphrodite ratio) all animals of F1 generation should have been heterozygous for *sel-5(ok363)* ($\frac{ok363}{+}$), also part of F1 generation had GFP expressed in TRN

3) $\frac{ok363}{+};\frac{muIs35}{+}$ ♀ transferred to a fresh NGM plate and let there for three days (at 20°C) to lay embryos. F2 generation was a mixture of WT and *sel-5(ok363)* homozygotes and heterozygotes (according to Mendelian genetic rules the ratio was (1:2:1)

4) 12 ♂ worms positive for *muIs35* from the F2 generation were transferred to fresh 35mm NGM plates (one animal per one plate) and let there for three days (at 20°C) to lay embryos. After three days, parent animals were picked, their DNA isolated and PCR genotyping for *ok363* was conducted (see chapter 3.2.6 for details)

5) Only plates which contained parents homozygous for *sel-5(ok363)* were used further. These plates were checked for animals expressing GFP in their TRN. Required strain was found at plates were all animals showed GFP fluorescence (those plates carried worms with a genotype $\frac{ok363}{ok363};\frac{muIs35}{muIs35}$).

3.2.6 PCR genotyping of *C. elegans*

After worm crossing procedures, PCR genotyping was used (where possible) to detect whether animal with correct genotype was obtained. The following procedure applied to strains where mutation was caused by a longer deletion in the gene coding sequence. Substitutions or very short deletions needed to be detected through sequencing of the gene in question.

For the purpose of genotyping, special primers were designed. Two forward primers (one of which pairs with region of gene which was deleted in mutant animal) and one reverse primer. Distribution of primers across the gene in question was as showed in Fig. 6. When PCR was conducted and the resulting mixtures were loaded on an electrophoretic gel, it was then possible to decide whether animal, from which DNA was extracted, was mutant or WT.

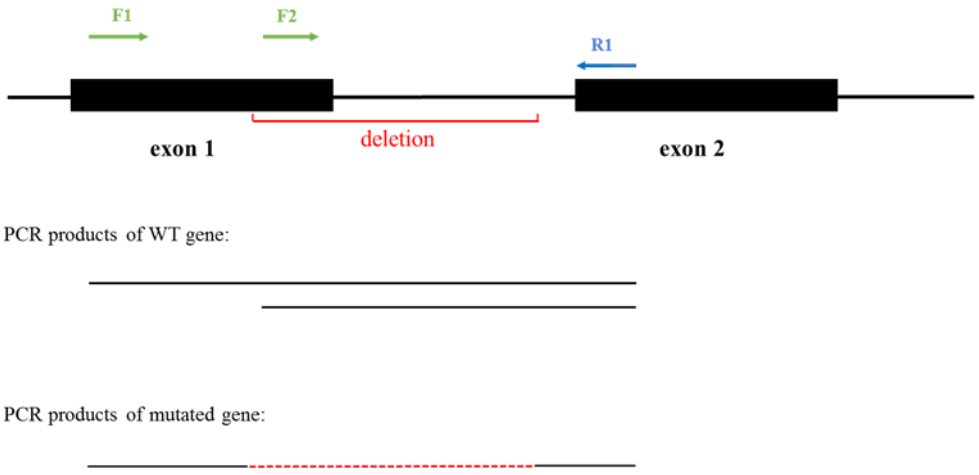


Figure 6: Depiction of PCR genotyping principle. This pictures shows a fictive gene with two exons (exon 1 and 2) and a deletion in the middle of the gene (deletion in red). To decide whether it is a WT or mutated allele , three primers were designed, two forward primers (F1 and F2 in green) and one reverse primer (R1 in blue). One of the forward primers is located to the deleted region. Two PCR mixtures are prepared, one containing primers F1,R1 and another one with primers F2,R1. When the allele is WT then there is one product (full length) in both reactions after PCR. When the allele is mutated, there is a product in the first reaction (shorter because of the deletion) and no product in the second reaction.

Isolation of worm genomic DNA:

Worms are picked from NGM plate and transferred to a tube containing 10 µl of worm lysis buffer and proteinase K mixture (recipe in chapter 3.3). Tubes are then placed in PCR cycler

with the following settings: 60 min at 65°C (worm lysis) followed by 15 min at 95°C (proteinase K inactivation).

PCR reactions were prepared by mixing following ingredients:

DreamTaq buffer 10x…….....2,0 µl
dNTPs 10mM……..0,5 µl
primer forward 10µM……0,5 µl
primer reverse 10µM…….0,5 µl
Taq polymerase (homemade)….0,3 µl
ddH_2O……..……..15,5 µl
worm lysate…….…..1,0 µl

for genotyping PCR cycler was set as follows:

initial denaturation…….2 min……..94°C
denaturation……...20 sec…..…94°C
primer annealing……40 sec……..58°C
Taq polymerase elongation……1 min/kb….72°C
} 35x
final elongation……..5 min……...72°C

(for colony-PCR adjusted PCR cycler setting was used: initial denaturation 3 min 30 sec and only 33 cycles instead of 35)

Separation of DNA fragments after PCR:

For DNA fragments separation agarose gel with Tris/Borate/EDTA buffer (TBE) was used. Concentration of agarose gel was usually 1%. For proper separation of larger fragments 0,7% agarose gel was used. To correctly determine the size of DNA fragments, DNA ladders were used (1kb DNA ladder or DNA ladder mix from ThermoFisher). To visualise DNA fragments under UV light, ethidium bromide (EtBr) was added to agarose gel to a final concentration of 1 µg/ml. All DNA samples were mixed with 6x loading buffer (3 ml of glycerol, 25 mg of bromophenol blue and ddH_2O to 10 ml).

3.3 Creating extrachromosomal arrays by injecting *C. elegans*

To explore the tissue specific function of a gene in the *C. elegans* Wnt signalling pathway, a tissue specific expression of this gene from a transgenic construct could be exploited (Mello and Fire, 1995). To generate transgenic animals, worm gonad was injected with a mixture of plasmids. The distal gonad to which plasmid mixture was injected contained syncytial tissue containing future eggs nuclei. The injected plasmids recombined with each other and formed a large DNA structure called an extrachromosomal array. The array could be then incorporated into a nucleus of newly created egg with certain probability. Stable line was established.

The injection mixture consisted of three types of plasmids. Firstly, it was a marker plasmid which told us whether injection was successful. We used plasmid carrying tdTomato expressed from *myo-2* promotor. *myo-2* is a gene coding for myosin subtype which is predominantly expressed in the *C. elegans* pharynx. After successful injection, a portion of progeny of injected animal showed red-glowing pharynx under a fluorescent microscope. Secondly, plasmid carrying a gene, which we wanted to be expressed, regulated by a tissue specific promoter, was added to the mixture. For the purpose of this diploma thesis, I created plasmid constructs (see chapter 3.5.3) expressing *sel-5* cDNA. To ensure its tissue specific expression, *egl-20* and *egl-17* promoters active in Wnt producing and Wnt receiving tissue respectively, were used. To monitor the correct expression of SEL-5 and to assess its subcellular localization, *sel-5* was also tagged with GFP. As high concentration of either the marker plasmid or the expression plasmid could be toxic, a third plasmid was included in the mixture to keep the overall DNA amount sufficient for the formation of the extrachromosomal array. For this purpose, we used pBluescript.

The final concentration of plasmids used for the mixture was as follows:

	pBluescript …….	185 ng/µl
	myo-2::tdTomato …	5 ng/µl
	egl-17p::sel-5::gfp…..	5 ng/µl
or	*egl-20p::sel-5::gfp*…..	5 ng/µl

This mixture was then injected into the distal gonad of young adult hermaphrodite animals with the use of inverted DIC microscope (IX70, Olympus) and injection station (Transjector 5246, Eppendorf). To immobilize injected animals we used a thin dried agarose pad on a cover glass. A drop of mineral oil was placed on this pad and a worm was transported to this

drop. A worm immediately adhered to the dried pad. After injection, the worm was transferred to a fresh NGM plate and let there to lay embryos. F1 progeny was then checked under a fluorescent dissecting microscope for animals with red fluorescence in their pharynx. Those worms were then checked for SEL-5::GFP expression in Wnt producing or receiving tissues. For further cultivation, only animals with whole pharynx tdTomato expression and with SEL-5::GFP visible in proper tissue were selected and their progeny checked to select animals with stably inheritable extrachromosomal arrays.

3.4 Scoring of *C. elegans* touch receptor neurons (TRN) position and polarity

3.4.1 Preparation of *C. elegans* for microscopy

For observing worms under a microscope, they needed to be immobilized, as their constant movement would prevent proper quantification of TRN position and polarity. To do this, a thin agarose pad attached to a glass slide was created. Sodium azide (NaN_3) was mixed with 2% solution of melted agarose to a final concentration of 30mM. NaN_3 anesthetized worms and for up to one hour animals were intact and ready for observation. Right before use a droplet of the mixture was transferred to a glass slide and immediately another glass slide was laid over the droplet. Droplet spread out and cooled down and the top glass slide could be then removed.

Worms that were about to be observed, were washed down from a NGM plate with M9 buffer and transferred to a microtube. It was centrifuged and pelleted worms were pipetted onto the agarose pad. A cover glass was placed over the mounted animals and the glass slide was ready for microscopy.

3.4.2 QL descendant cells scoring

Strains containing transgenes *muIs32* or *muIs35* were used for scoring of *C. elegans* TRN position along the anterio-posterior body axis and their polarity. Both transgenes consists of *mec-7* promotor, which normally directs transcription of a β-tubulin subtype typical for TRN. This promotor is followed by GFP coding DNA sequence. TRN of transgenic animals carrying *muIs32* or *muIs35* were thus easily observable under the fluorescence microscope when excitation light with wavelength from blue spectrum of visible light. Apart from TRN cell bodies, their neurites were visible as well. Of the three QL descendant cells (PQR, SDQL, PVM), only PVM belongs among TRN and was visible in *muIs32* and *muIs35*

animals. When members of Wnt signalling pathway were mutated, QL neuroblast migrated anteriorly instead and PVM was located ectopically in the anterior half of the animal (Fig. 7). Such a phenotype is termed as QL phenotype.

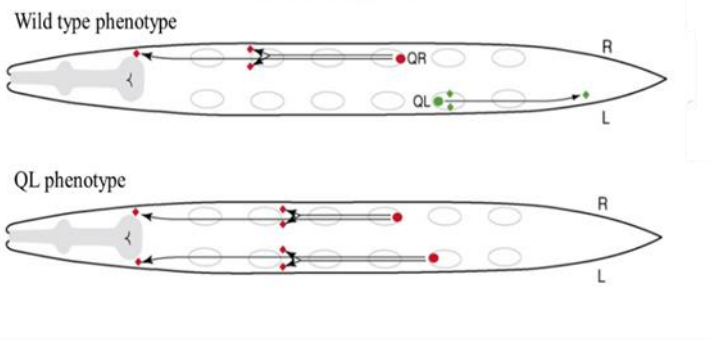


Figure 7: Schematic picture of WT and QL localization of some TRN. *C. elegans* QL neuroblast descendant cells pictured here (in green) in its WT position (upper picture). When Wnt signalling is impaired, QL descendant cells migrate similarly to QR neuroblast descendants (in red) and are located in posterior half of a worm. Anterior is to the left. (adapted from Silhankova et al., 2007).

To distinguish between WT and displaced PVM a vulva position was set as a reference. When PVM was located anteriorly to the vulva then the animal was marked as having QL phenotype.

3.4.3 Scoring of TRN polarity

ALM and PLM neurons also express the mec-7p::gfp marker and thus are readily visible in *muIs32* and *muIs35* animals. Polarity of the ALM and PLM neurons was scored in wild type and mutant animals. Polarity could be either reversed or bipolar. Animals were said to have bipolar ALML/R phenotype when posterior protrusion had length of at least one quarter of a WT anterior neurite. In case of PLM, posteriorly directed neurite was considered to be defective when its length was shorter than one length of the PLM cell body. For both ALM and PLM, left and right neurons were scored independently. In each experiment, at least 60 animals were scored.

3.5 Materials used for cloning

3.5.1 Cloning vectors and primers

Vectors:

pJET1.2……..cloneJET PCR Cloning kit from ThermoFisher

pDC48……used as a source of *egl-20* promoter

pPD95.81…...used for expression of GFP tagged *sel-5* from *egl-20* and *egl-17* promotor in *C. elegans*

pKN136……..source of *egl-17* promotor region

Primers:

pJet1.2fw-new	CTGCTTTAACACTTGTGCCTG
pJet1.2rev-new	CTACAACGGTTCCTGATGAGG
egl17p-FSphI	GCATGCCAGATGGATGTTTACTGCCAAC
egl17p-RBamHI	GGATCCAGCTCACATTTCGGGCACCTG
egl17pseq-F	CATCCATCTTTGATTTCCGAC
egl20seq1	CAATCGTCTTCTTAACCAGG
mig14mu71-F	CTCAAATCTATCAAACGAGCG
mig14mu71-R	GAGCTTCTCGTGAGTTGAG
mtm6-intR	CACGAAGAGGTTGCCATTT
mtm6ex4-F1	ATGTGCACAGCCTCTGACG
mtm6ex4-F2	TCTCATCGAGAAGGATTGGC
pPD95-GFP	TCTTGTAGTTCCCGTCATC
sel5ex1-F	AGATCTATGCCTCTAGGGCTTTTCAGC
sel5ex11-R	GTAGAGATACTGGTTGTGCAG
sel5ex12-F	CTACTGACGAGGACGATG
sel5ex17-R	TGGCCACAAGTCGGTTGGATCATCATG
sel5ex5-F	CTCTGCGATTTTGGAAGTGC
sel5ex8-F	TTGGTCTGCCACATTTGAGG
sel5ok363ins-F	GAAACCAGCAGCTGAAGCTG
sel5ok363out-F	CCACAACCTATCATTCATCGC
vps29-3UTR-R	ACTTGCTCTGTAGGCCATCG
vps29ex1-F	CTTCAACCTGCCTCATCGAG
vps29ex3-F	CTACGAATGCAGTGCTGTCG

3.5.2 Plasmid isolation, purification and digestion

Plasmid isolation was carried out with commercial kits (High pure plasmid isolation kit-Roche or High speed plasmid mini kit-Geneaid) according to manufacturer's instructions or with homemade alkaline lysis plasmid isolation solutions:

Solution I: 50mM glucose	*Solution II*: 0,2M NaOH	*Solution III*: 3M KOAc
10mM EDTA	1% SDS	
25mM Tris		
pH to 8,0		

Overnight grown bacteria (in LB at 37°C) carrying plasmid of interest were centrifuged and pellet was resuspended in 200 µl of cold solution I for up to 5 minutes. 200 µl of solution II was added and lysate was mixed by inverting the tube few times. 300 µl of solution III was added and the lysate was centrifuged (12000xg for 10 minutes). Supernatant was transferred to a fresh tube and 3 volumes of 100% EtOH were added. Precipitated DNA was centrifuged (15000g for 10 minutes at 4°C). Supernatant was removed and pellet was washed with 200 µl of 70% EtOH. Pellet was centrifuged (15000xg for 5 minutes) and supernatant removed. Pellet was dried and resuspended in appropriate amount of ddH_2O.

Plasmid purification was carried out through agarose gel (with subsequent clearance via Gel/PCR DNA fragments extraction kit from Geneaid or QIAquick gel extraction kit from QIAGEN). All digestions were carried out by restriction enzymes from ThermoFisher.

3.5.3 Creation of plasmids for *C. elegans* injection

For *C. elegans* injection, a plasmid vector pPD95.81 carrying ampicillin resistance and coding for GFP tag was used (Fig. 8). First, a proper promotor was amplified and inserted into a linearized pPD95.81. Promotor *egl-17* was originally carried on pKN136 plasmid. To amplify *egl-17p* region with proper overhangs, special pair of primers was designed with restriction site for SphI at the 5´ end of one primer and restriction site for BamHI at the 5´ end of a second primer. A standard PCR reaction was then performed with those two primers and plasmid pKN136 carrying *egl-17p*. Instead of Taq polymerase, HF Phusion DNA polymerase (NEB) was used. Product of PCR reaction was purified via agarose gel and isolated with a kit (see 3.5.2). Purified *egl-17p* was then ligated into pJET1.2 cloning vector

with the help of CloneJET PCR cloning kit (ThermoFisher).

Ligated plasmid was then transformed to competent *E. coli* strain DH5α via following protocol:

1) DH5α cells let to thaw on ice
2) 8 µl of ligation mixture was added to 100 µl DH5α and mixed gently
3) mixture was left for 30 minutes on ice and then heat shocked for 80 seconds at 42°C
4) after heat shock, mixture was left for 5 minutes on ice and then 600 µl of LB was added
5) mixture was kept for 1 hour at 37°C and then inoculated onto LB agar plates with selective antibiotic (ampicillin) and plates were placed in thermal box (o/n at 37°C)

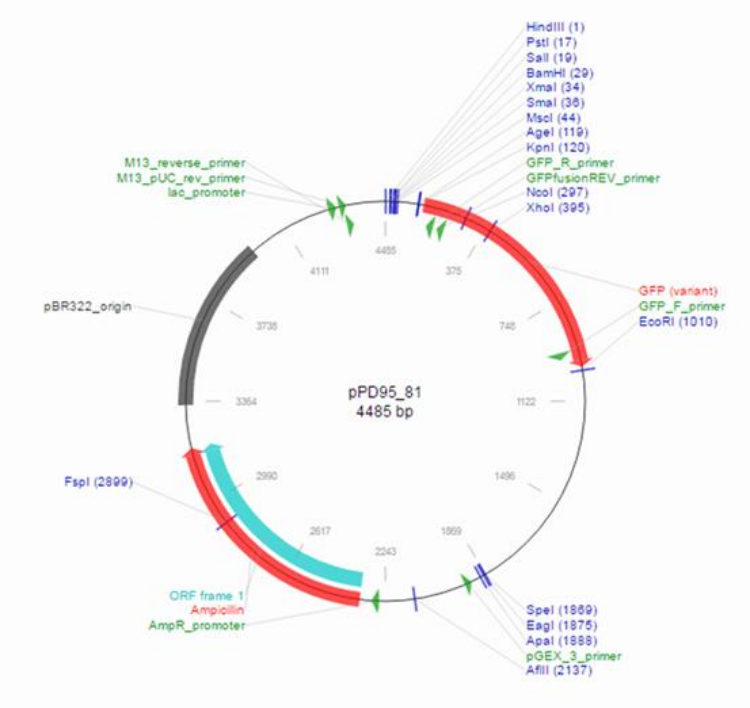


Figure 8: Map of a plasmid pPD95.81 used for injection process of *C. elegans*. Promotor sequence *egl-17p* and cDNA coding for SEL-5 were ligated sequentially into this plasmid. The ligation was carried out in such a way that GFP coding region of the plasmid was in frame with SEL-5 coding region.

Next day, LB plate was checked for colonies. Ten colonies were picked randomly and checked for presence of the correct plasmid construct by colony PCR. For colony PCR a pair of primers, pJET-forward and *egl-17p*-seq were used (for details on PCR cycler settings see chapter 3.2.6). Only bacterial colonies carrying pJET plasmid with *egl-17p* inserted gave 150 base DNA fragment after PCR reaction (Fig. 9).

One colony with pJET-*egl-17p* positive bacteria was inoculated in LB medium with ampicillin and grown o/n at 37°C. Next day, portion of the bacterial culture was stored as a glycerol conserve in -80°C. The rest of the culture was used for plasmid isolation (see 3.5.2).

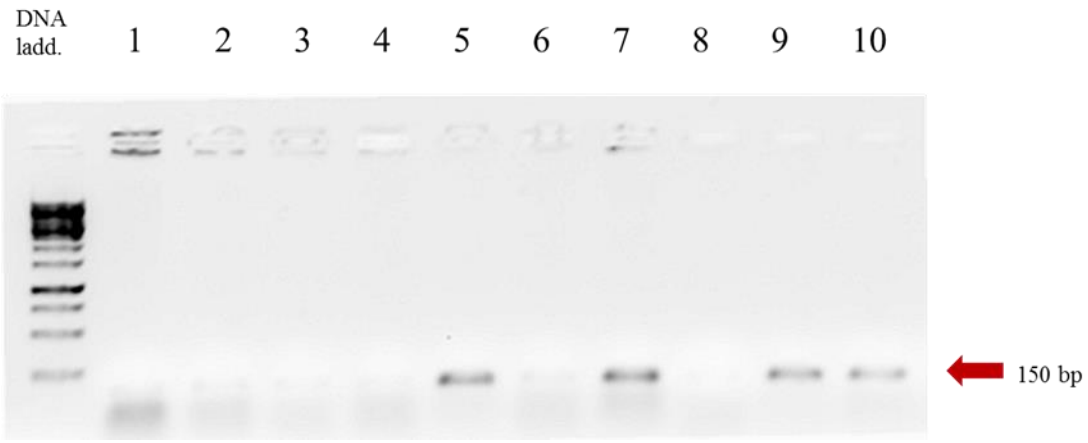


Figure 9: Picture of 1% agarose gel used for detection of *egl-17p* inserted in plasmid pJET1.2. Only colonies carrying such plasmid gave rise to approximately 150 nucleotides long DNA fragment after PCR reaction (red arrow). 1 kb DNA ladder from ThermoFisher used to determine DNA fragment size. Plasmids with correctly inserted *egl-17p* were detected in lines 5, 7, 9 and 10.

Isolated pJET-*egl-17p* plasmid was used for restriction enzyme (RE) digestion with SphI and BamHI. Thus *egl-17p* with proper overhangs were created. Plasmid pPD95.81 was treated with BamHI and SphI to create corresponding overhangs. Ligation reaction was carried out overnight at 15°C with T4 DNA ligase (from ThermoFisher). Next day ligation mixture was transformed to DH5α as described above. To select pPD95.81-*egl17p* positive colonies, colony-PCR was carried out as described above (with primers pPD95-gfp-R and *egl-17p*-seq).

Plasmid was isolated from positive colonies and linearized through RE digestion with enzymes BamHI and MscI to create overhangs corresponding to cDNA of *sel-5*. Gene for *sel-5* was isolated from pJET1.2 with RE BglII and MscI (see chapter 3.7 for more detail on *sel-5* cDNA).

Linearized plasmid pPD95.81-egl17p and insert *sel-5* were ligated o/n at 15°C and the mixture was transformed to DH5α as described above. Colonies positive for plasmid pPD95.81-*egl17p-sel5* were selected via colony-PCR (with primers sel5ex12-F and pPD95-gfp).

Correct insertion of *sel-5* was then confirmed via DNA sequencing. Plasmid construct pPD95.81-*egl20p-sel5* was created in a similar way to pPD95.81-*egl17p-sel5*. Both plasmids were then used for *C. elegans* injection (see chapter 3.3).

3.6 Isolation of *C. elegans sel-5* cDNA

SEL-5 protein was find out to play role in Wnt signalling in *C. elegans*. To further analyse this protein, its cDNA was isolated via techniques described in this chapter.

3.6.1 Total RNA isolation from worms

1) Bristol wild type strain N2 was used as a source of total worm RNA
2) worms were collected to 1,5ml tube and washed three times with M9 buffer
3) after last wash worm pellet was left in 30 µl of M9 and 300 µl of Trizol reagent (ThermoFisher) was added
4) immediately, the tube was frozen in liquid nitrogen
5) the tube was thawed quickly and incubated at 65°C for 15 min
6) 60 µl of chloroform was added, the tube was vortexed and left at RT for 3 min
7) centrifugation for 15 min was carried out (4°C/16000xg)
8) upper fraction was collected and 0,8 volume of isopropanol was added, the tube was vortexed and left for 10 min at RT
9) centrifugation for 10 min was carried out (4°C/16000xg)
10) supernatant was discarded and pellet was washed with 500 µl of 75% EtOH
11) supernatant was discarded and pellet air dried and resuspended in DEPC-treated H_2O

3.6.2 First-Strand cDNA synthesis

1) following ingredients were mixed together:

Oligo(dT) 50µM……..1 µl
Total RNA (1300 ng/µl)…..1 µl
dNTPs 10mM……..1 µl
ddH_2O……..10 µl

2) mixture was heated to 65°C for 5 min and then incubated on ice for 2 min
3) following ingredients were then added to the mixture:

5x First-Strand buffer……4 µl
DTT 0,1M……..1 µl
RNase inhibitor…….1 µl
SuperScript III RT…… 1 µl

4) mixture was pipetted up and down gently and incubated at 55°C for 60 min
5) reaction inactivation was carried out by heating mixture to 70°C for 15 min
6) to remove RNAs from the mixture, 20 min incubation with RNase H at 37°C was carried out (all ingredients used were from kit SuperScript III Reverse Transcriptase from Invitrogen)

3.6.3 *sel-5* cDNA synthesis

For *sel-5* cDNA synthesis, a pair of *sel-5* specific primers was used for PCR reaction. Unlike in standard PCR reaction (see 3.2.6), HF Phusion DNA polymerase and HF buffer were used (from NEB), also annealing temperature was raised to 62°C. Amplification of *sel-5* cDNA was checked through agarose gel electrophoresis. A band of proper size was excised from agarose gel and isolated with a kit (see 3.6.2).

Isolated *sel-5* cDNA was then ligated into pJET1.2 cloning vector and ligation mixture was used for transformation of DH5α competent cells (as described in chapter 3.5.3) and for further experiments. Cloned cDNA was completely sequenced before it was used for further experiments.

3.7 Materials and methods used for protein isolation

3.7.1 Materials used for worm protein isolation and work with protein

Homogenization buffer (HB) (500ml):
Hepes 0,5M (pH 7,6)…….15 ml
KCl 2M……..2,5 ml
$MgCl_2$ 1M……..0,75 ml
EDTA 0,5M……...……100 µl
Sucrose 50%.....14,7 ml
DTT 1M…….0,5 ml
before use, a protease inhibitor added according to attached ROCHE documentation (cOMPLETE^{TM}, EDTA free, Protease Inhibitor Cocktail tablets from ROCHE)

Blotting buffer:

$B_4Na_2O_7.H_2O$…….5,72 g

added H_2O to 1 litre and filtered

Running buffer:

Tris…….15 g

glycin……..72 g

SDS…….5 g

H_2O added to 5 litres all ingredients dissolved and mixture filtered

Tris-buffered saline and Tween 20 (TBST):

Tris-Cl…….1,01 g

NaCl…….8,76 g

Tween 20…….0,50 g

H_2O to 1 litre added, pH adjusted to 7,6 and solution filtered

Laemmli sample buffer:

10% SDS……4,0 ml

glycerol……..2,0 ml

1M Tris-Cl pH 6,8…….1,2 ml

ddH_2O……2,8 ml

bromophenol blue to final concentration 0,02% (w/v)

10% separation SDS-PAGE gel:

H_2O…….……4 ml

1,5M Tris pH 8,8……..……2,5 ml

30% acryl-bisacryl mix…….3,3 ml

10% SDS……...100 µl

10% ammonium persulfate…..100 µl

TEMED……4 µl

5% stacking SDS-PAGE gel:

H_2O……...2,7 ml

1M Tris pH 6,8……..……...0,5 ml

30% acryl-bisacryl mix……0,67 ml

10% SDS…….....40 µl

10% ammonium persulfate.…40 µl

TEMED……4 µl

Primary and secondary antibodies used:

mouse-monoclonal anti-GFP IgG1 (ROCHE)

mouse-monoclonal anti-tubulin IgG1 (Sigma Aldrich)

goat anti-mouse HRP-conjugated (Jackson Immuno-Research Laboratories)

To visualize HRP-conjugated antibodies, WesternBright ECL HRP substrate system from Advansta was used.

3.7.2 Worm protein isolation by sonication

For the purpose of this diploma thesis, we isolated MIG-14 (*C. elegans* Wls) tagged with GFP from *C. elegans* strains carrying *huSi2* transgene. For quantification of our results, all animals needed to be synchronised, as the level of *mig-14* expression is different in different developmental stages. For *C. elegans* synchronisation, we used bleaching protocol (see chapter 3.2.3) to isolate embryos. Embryos were then cultivated o/n at RT in M9 buffer. This allowed worms to hatch and reach L1 developmental stage where they were arrested because of the lack of food. After synchronization, protein isolation was carried as follows:

1) L1 larvae were centrifuged at low speed and washed twice with M9 buffer. Then, they were washed twice again, this time in ddH_2O.
2) worm pellet was resuspended in 500 µl of HB
3) samples were then subjected to nine 1s sonication pulses with 30s breaks between pulses (Cole-Palmer Ultrasonic Homogenizer-4710 was used with highest possible output)
4) lysate was cleared by centrifugation (14000xg, 10 min, 4°C) and transferred to fresh tube
5) Laemmli sample buffer was added and sample was warmed to 95°C for 5 min
6) sample was loaded to SDS-PAGE and run for 1,5 hrs at constant current 45 mA
7) then gel was sandwiched between filter papers and membrane and proteins were transfer to membrane (Whatman Protran nitrocellulose) via Western blotting (run in blotting buffer for 1,5 hrs at constant current 500 mA)
8) nitrocellulose membrane was removed, washed 3x5min in TBST and blocked in 3% BSA in TBST for 1 hour
9) membrane was incubated o/n at 4°C in primary antibody (anti-GFP 1:10000 in 3% BSA in TBST)
10) membrane was washed 3x5min in TBST and incubated at RT in secondary antibody for 1 hour (GAM-HRP 1:10000 in 3% skim milk in TBST)
11) membrane was washed 3x5min in TBST and incubated for 2 min with excitation solution (Advansta)
12) images were taken with ImageQuant LAS 4000
13) membrane was washed 3x5min in TBST and incubated with primary antibody (anti-tubulin 1:10000 in 3% BSA in TBST) and then with secondary antibody (steps 9-12 repeated)

3.8 *C. elegans* phasmid neuron staining with DiI

To visualise *C. elegans* amphid and phasmid neurons under a fluorescent microscope, lipophilic DiI dye was used. These neurons are in direct contact with worm surroundings via specialized channels in the *C. elegans* body. Through these channels, DiI is incorporated to amphid/phasmid plasma membrane and these neurons can thus be visualized.

1) stock solution of DiI (Molecular Probes, 2 mg/ml in dimethyl formamide) was diluted 1:200 in M9 buffer
2) washed and pelleted animals were mixed with 200 µl of diluted DiI and left at RT for 2 hrs (animals can not be starved, otherwise DiI is incorporated to worm gut and interferes with phasmid visibility)
3) stained worms were transferred to a fresh NGM plate and let crawl for about 1 hour to destain excessive DiI
4) animals were observed under the fluorescent microscope (same as in chapter 3.4.1)

3.9 Other material and solutions used for *C. elegans* cultivation

M9 buffer for washing or o/n synchronization of worms

Per 500 ml of M9 buffer following ingredients were mixed:

$Na_2HPO_4*12H_2O$……..3,94 g
KH_2PO_4……..……1,5 g
NaCl……..2,5 g
1M $MgSO_4$……0,5 ml

Water added to 500 ml and then autoclaved.

Worm lysis buffer for isolation of genomic DNA

Following ingredients were mixed to final concentrations indicated:

10mM Tris pH 8,3
2.5mM $MgCl_2$
50mM KCl
0,45% NP-40 and 0,45% Tween 20

For worm lysis and DNA isolation, 100µg/ml of proteinase K was added just before use.

Worm freezing solution for long-term strain storing in -80°C

For 1 litre of freezing solution following ingredients were mixed:

NaCl……..……..5g

1M KH_2PO_4 (pH 6,0)……..50 ml

glycerol…….…..240 ml

H_2O…….710 ml

Autoclaved and 0,3 ml of 1M $MgSO_4$ added.

Tris/Borate/EDTA buffer (TBE)

To prepare 5x concentrated TBE, following ingredients were mixed to indicated final concentrations:

89mM Tris pH 7,6

89mM H_3BO_3

2mM EDTA

4. Results

4.1 RNAi screen for new regulators of Wnt signalling in *C. elegans*

On the basis of *C. elegans* RNAi feeding screen for new regulators of Wnt signalling pathway (Macůrková et al., unpublished data), candidate genes were selected, whose protein products were predicted to participate in endocytosis regulation. Selection was based on known roles in endocytosis of these proteins or their interacting partners, or their homologs in other organisms.

To identify even minor prospective Wnt pathway regulators, RNAi feeding screen was conducted in sensitized background and migration of the QL neuroblast descendants was used as a read-out of Wnt pathway activity. The nature of the mutation was a deletion in *vps-29* coding region. Worms with mutated *vps-29* allele have attenuated function of the retromer complex. This causes improper recycling of a *C. elegans* Wls protein, MIG-14, and lower EGL-20 (*C. elegans* Wnt ligand) secretion (Coudreuse et al., 2006). As a result, a proportion of *vps-29* mutated animals show a QL phenotype (Fig. 10). After RNAi feeding, penetrance of the QL phenotype is either unchanged, which means that a particular gene has no role in Wnt signalling, or increased/lowered, which indicates gene with a potential role in Wnt signalling pathway regulation. Based on pilot experiments (M. Macůrková and M. Horázná, unpublished), *sel-5* was selected for a detailed analysis (Fig. 11).

4.2 Protein kinase SEL-5 and its role in endocytosis

C. elegans SEL-5, a 1077 amino acid long protein, belongs to a Numb-associated protein kinase family (NAK) whose members were identified in a wide spectrum of organisms from *Saccharomyces cerevisiae* to humans (Sorrell et al., 2016). SEL-5 was previously described as a regulator of *C. elegans* Notch signalling important for worm development (Fares and Greenwald, 1999). In its N-terminal region, it carries a serine/threonine kinase domain which is homologous with human AP2-associated protein kinase-1 (AAK1). Its C-terminal half consists of amino acid sequence with no homology with AAK1 or other member of NAK family. This is indeed in agreement with properties of NAK family whose members are largely homologous through their kinase domains and show very little similarity outside of this domain (reviewed in Smythe and Ayscough, 2003).

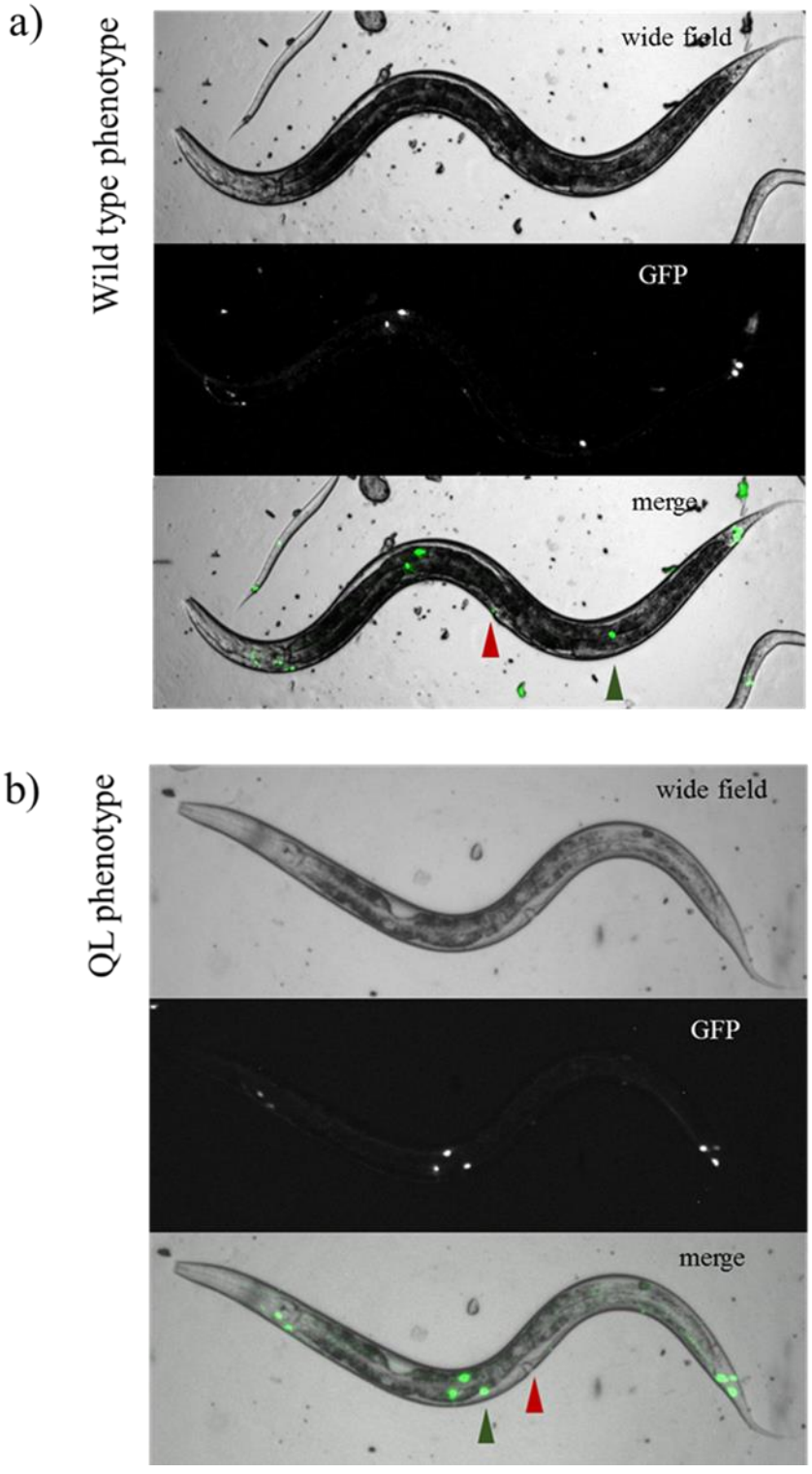


Figure 10: QL and WT position of touch receptor neurons (TRN). Worm strain with mutation in *vps-29* gene is shown here. Worms also carried *muIs32* transgene with GFP protein expressed in touch receptor neurons (TRN). This enabled TRN to be visualized under a fluorescent microscope. In **a)** animal with wild type localization of QL neuroblast descendant cells is shown here. PVM neuron (green arrowhead) is localized posteriorly to the vulva (red arrowhead). ALM, AVM and PLM neurons are also visible. In **b)** QL phenotype localization of TRN neurons is shown. Here PVM neuron (green arrowhead) is localized anteriorly to the vulva (red arrowhead). In all pictures anterior site is to the left and dorsal is up.

Human homolog of SEL-5, AAK1 was found to bind AP2 adaptor complex and phosphorylate its subunit μ2, which is important for selection, binding and internalization of cargo proteins via CME (Ricotta et al., 2002). Very often, proteins interacting with an AP2 complex do so via the α-subunit appendage domain. For this interaction to happen a short DPF/W motif needs to be present in AP2 α-subunit binding partners (Owen et al., 1999). This motif is indeed present in AAK1 and its worm homolog SEL-5, however its role in AP2 binding in *C. elegans* has not been studied so far. AAK1 mediated phosphorylation of AP2 μ-subunit causes its higher affinity towards cargo proteins (Ricotta et al., 2002). Tight regulation of AAK1 activity is necessary for a proper cycling of AP2 and clathrin between their cytosolic and CCP pools (Semerdjieva et al., 2008). We hypothesized that the endocytic role of SEL-5 kinase in nematode *C. elegans* is comparable or very similar to its human homolog AAK1. Its role in Wnt signalling could thus be either in the Wnt producing cells regulating Wls recycling, or in the Wnt receiving cells regulating endocytosis at the level of Wnt-Frizzled complex.

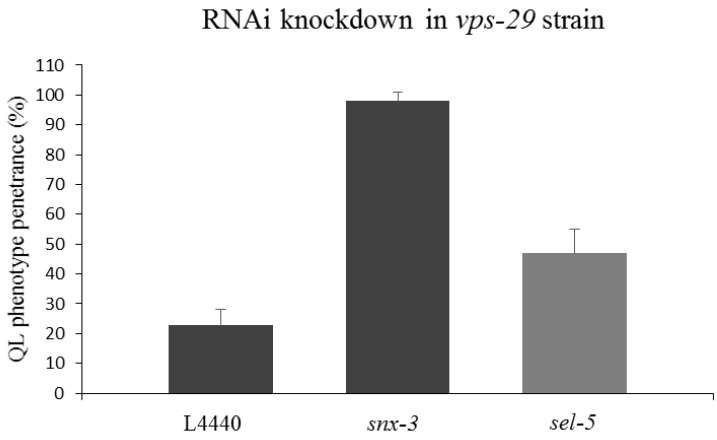


Figure 11: QL phenotype penetrance after RNAi feeding. The change of QL phenotype penetrance in *vps-29* mutated animals after they are fed with HT115 bacteria carrying plasmid with RNAi construct against indicated gene. L4440 is plasmid with no RNAi construct inserted and serves as a negative control. As a positive control, RNAi knock-down of *snx-3* gene is shown. Protein SNX-3 is known to play a role in Wls recycling (Harterink et al., 2011) and its knock-down in *vps-29* mutant animals thus have synergistic effect on QL phenotype penetrance. Several genes were identified that changed QL phenotype penetrance. However, after identification of these genes via databases such as Wormbase, most of them were rejected for being involved in processes other than endocytosis. One promising gene, *sel-5*, was identified. Knockdown of this gene caused twofold increase in QL phenotype penetrance in *vps-29* mutant worms. Its homolog in human was suggested to be involved in endocytosis regulation (see text for more details). (Adapted from Macůrková et al., unpublished data).

4.3 Confirmation of *sel-5* role in Wnt signalling

To confirm the role of SEL-5 in Wnt signalling, the results obtained with *sel-5* RNAi were first verified using *sel-5* deletion allele. No QL phenotype was observed in *sel-5;muIs32* animals, however, penetrance of the QL phenotype was strongly enhanced in *vps-29 sel-5* double mutants compared to *vps-29* alone (Fig. 12). In order to show that such effect of *sel-5* on Wnt signalling is not restricted to *vps-29* background, we utilized another member of the retromer complex, *vps-35*. As *vps-35* mutants show a fully penetrant QL phenotype, we conducted RNAi knock-down of *vps-35*. RNAi of *vps-35* produces animals with only mildly affected Wnt signalling pathway. RNAi was performed in *sel-5;muIs32* and *muIs32* animals. Very low penetrance of the QL phenotype was observed when *muIs32* animals were subjected to *vps-35* knock-down (Fig. 13). Contrary to that, RNAi knock-down of *vps-35* in *sel-5* mutated animals resulted in significant enhancement of QL phenotype penetrance (Fig. 13). These results support the role of SEL-5 in Wnt signalling.

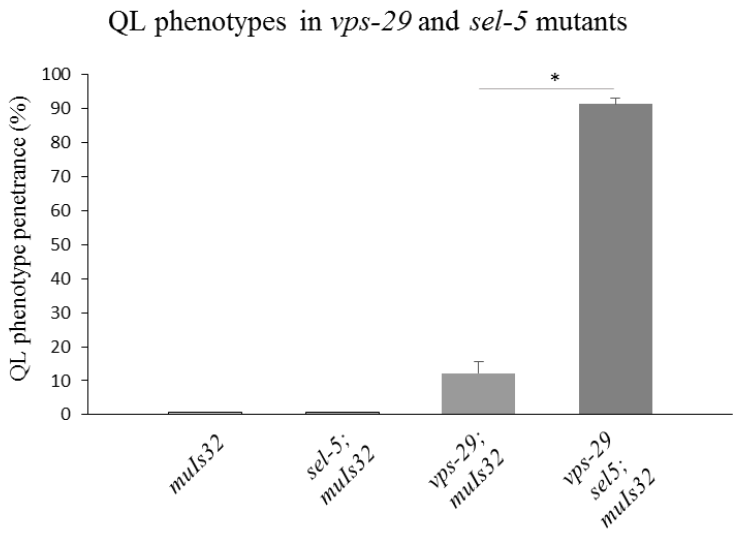


Figure 12: QL phenotypes in *vps-29* and *sel-5* single and double mutants. In this graph, a percentage of animals with a QL phenotype is shown in indicated strains. *muIs32* and *sel-5* mutated animals did not show any QL phenotype. Compared to that, *vps-29* single mutant worms showed QL phenotype penetrance slightly over 10 %. In *vps-29 sel-5* double mutants this number was significantly higher, approaching almost 100% penetrance. Results are shown as a mean of three independent experiments with error bars as SD. Statistical significance determined by unpaired *t-test*.

* $p < 0{,}0001$

Recently an article was published (Sorrell et al., 2016) where a product of a worm gene F46G11.3 was identified as a homolog of human cyclin G-associated kinase (GAK). In human, GAK and AAK1 show a partially redundant activity. In hepatitis C virus (HCV) entry via CME, both kinases are required. When their inhibitors (erlotinib and sunitinib)

inactivate these kinases, the entry of HCV is impaired. This impairment can be overcome by overexpression of either GAK or AAK1 (Neveu et al., 2015). We wondered whether worm gene F46G11.3 encoding for GAK homolog could similarly work redundantly with SEL-5 kinase in *C. elegans*. F46G11.3 gene codes for a protein product TAG-257. This protein also has a kinase domain in its N-terminal region and contains DPF motif that would enable its binding to AP2 complex. To test the hypothesis of SEL-5 and TAG-257 functional redundancy we conducted *tag-257* gene knock-down via RNAi feeding. We used strains *muIs32*, *sel-5;muIs32* and *vps-29;muIs32*. However, we did not observe any difference in QL phenotype penetrance between animals fed with empty L4440 plasmid or *tag-257* RNAi constructs.

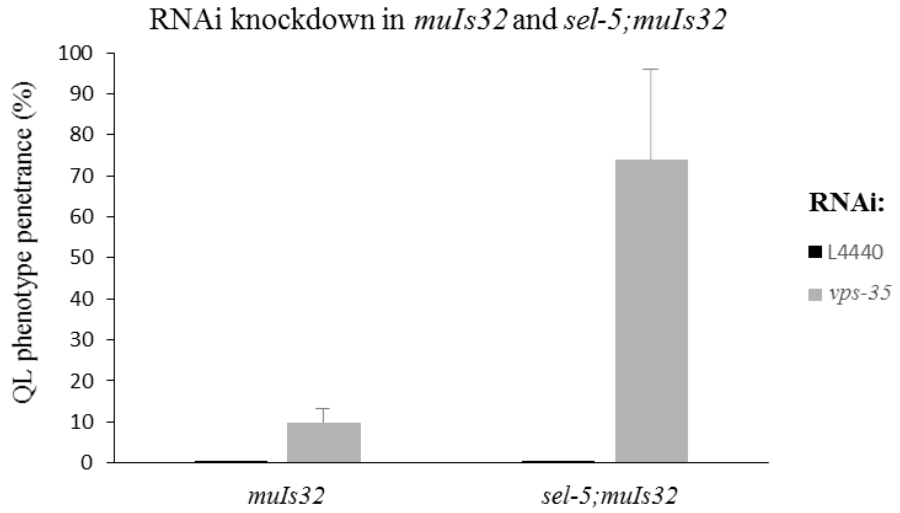


Figure 13: RNAi knock-down of *vps-35* gene in *muIs32* and *sel-5;muIs32* background. In control *muIs32* strain , knock-down of *vps-35* gene leads to a low penetrance of QL phenotype**.** In the *sel-5;muIs32* strain, same RNAi knock-down has much stronger effect on QL phenotype penetrance. Empty L4440 plasmid construct was used as a negative control. This plasmid construct does not cause QL phenotype in either of used strains. Mutation in *sel-5* gene alone does not have any effect on QL phenotype or other observable phenotypes. Results are mean of three independent experiments with error bars indicating standard deviations (SD). In each experiment at least hundred animals were scored for the QL phenotype.

4.4 Tissue specific role of *sel-5* in Wnt signalling

As mentioned above, SEL-5 could potentially regulate Wnt signalling either in Wnt producing cells at the level of Wls recycling, or in Wnt receiving cells at the level of Wnt receptor complex internalization. To discriminate between these two possibilities, tissue-

specific RNAi experiments were performed. Two transgenic lines expressing *sel-5* RNAi construct under the control of *egl-20* (Wnt producing) or *egl-17* (Wnt receiving) promoters were previously established. Pilot experiments with *vps-29* mutants revealed a significant enhancement of *vps-29* QL phenotype when using the *egl-17p::sel-5(RNAi)* transgene, while no effect was visible when using the *egl-20p::sel-5(RNAi)* transgene (M. Macůrková, unpublished). The disadvantage of the tissue-specific RNAi approach is that in case no phenotype is observed, it is difficult to verify whether the RNAi transgene is actually functional. We therefore decided to test the effect of the two RNAi transgenes also in different genetic background. For that we chose *mtm-6* mutants. *mtm-6* codes for enzyme myotubularin lipid-phosphatase and was shown to be important for recycling process of MIG-14/Wls and thus for functional Wnt signalling (Silhankova et al., 2010). Worms mutated in *mtm-6* show roughly 40% penetrance of QL phenotype, while in *mtm-6 sel-5* double mutants approximately 20% of animals display the QL migration defect (M. Macůrková and M. Horázná, unpublished). We tried to determine whether this change in QL phenotype could be reproduced by knockdown of *sel-5* in Wnt producing or receiving cells. The extrachromosomal arrays carrying the tissue specific *sel*-5 RNAi constructs were crossed into *mtm-6* mutants. The QL phenotype penetrance was then compared between *mtm-6* mutants carrying the tissue specific RNAi transgene and their non-transgenic siblings (Fig. 14). To distinguish between animals with or without extrachromosomal array, a red fluorescence of the *myo-2p::tdTomato* co-injection marker expressed in worm pharynx was used (explained in chapter 3.3).

QL phenotype penetrance in animals carrying the extrachromosomal array coding for a tissue specific *sel-5* RNAi in Wnt receiving cells and their siblings without this array shows no significant increase (Fig. 14a). Same results were observed for a tissue-specific knockdown of *sel-5* in Wnt producing cells (Fig. 14b). While the preliminary experiments in *vps-29* mutants pointed to the role of SEL-5 in Wnt receiving cells, through this follow-up experiment, we were unable to unambiguously determine the tissue where SEL-5 kinase regulation of endocytosis affects Wnt signalling.

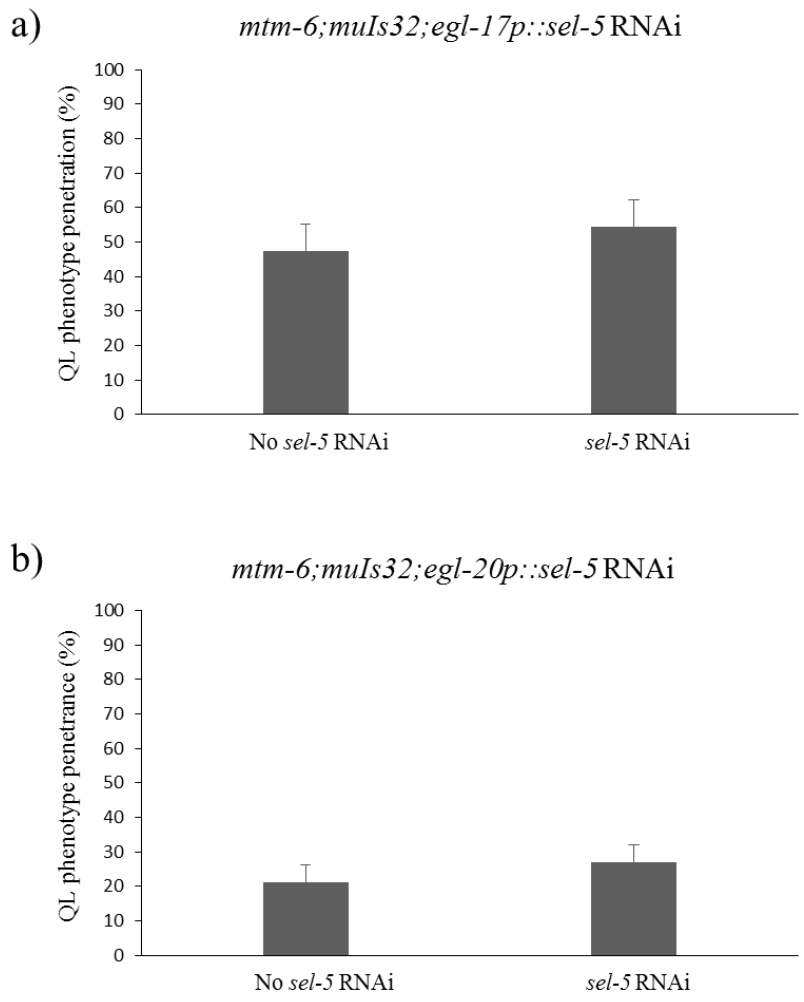


Figure 14: Tissue-specific RNAi knockdown of *sel-5* in *mtm-6* strain. a) In this picture RNAi knock-down of *sel-5* in *mtm-6* mutant animals is tissue specific for Wnt receiving cells. Sibling worms without (No *sel-5* RNAi) or with (*sel-5* RNAi) extrachromosomal array were compared for QL phenotype penetrance. **b)** Same experiment was conducted for *sel-5* RNAi in Wnt producing cells. Again, sibling worms with or without extrachromosomal array were compared. Graphs show mean results of nine independent experiments. At least 50 worms were counted in each experiment. Error bars shown are SD. Statistical significance was determined by unpaired *t-test.*

4.5 Ectopic expression of *sel-5* in Wnt producing or receiving cells

As previously shown (Macůrková, unpublished data and this work Fig. 12), double mutant worms *vps-29 sel-5* show significantly higher penetrance of QL phenotype compared to *vps-29* single mutant. Although not fully conclusive, the outcomes of experiments described in the previous section hint on the function of SEL-5 in Wnt receiving cells. To further confirm this hypothesis, we conducted injection of *vps-29 sel-5* double mutant worms with *sel-5* rescue constructs whose expression was regulated by promotors specific for either Wnt receiving (*egl-17p*) or Wnt producing cells (*egl-20p*). *sel-5* was also tagged with GFP, product of tissue specific expression of *sel-5* could thus be readily visualized under a fluorescent microscope.

Injection was successful for both rescue constructs as proportion of injected worms progeny showed red fluorescence in pharynx coming from the *myo-2::tdTomato* co-injection marker. Moreover, worms carrying the array with *egl-20p* regulated *sel-5* showed green fluorescence in Wnt producing cells around the rectum where EGL-20 is produced (Fig. 15). Unfortunately we were not able to locate GFP tagged SEL-5 in worms injected with the array containing *egl-17p* regulated *sel-5*. Both plasmid constructs were checked by DNA sequencing before injection experiments were carried out. Nevertheless, *sel-5::gfp* expression from *egl-17p* was not successful and further experiments were executed only with worms expressing *sel-5::gfp* from *egl-20p*.

QL phenotype penetrance in *vps-29 sel-5* double mutant is roughly 90% (Fig. 12). This is a significant increase, compared to *vps-29* single mutation, where QL phenotype penetrance is around 10% (Fig. 12). As there has not been any QL phenotype observed in *sel-5* single mutant worms (Fig. 12), we wanted to see whether ectopic expression of *sel-5* from *egl-20* promotor could rescue QL phenotype in *vps-29 sel-5* double mutants. Extrachromosomal arrays are inherited in a mosaic fashion, therefore the progeny of a single transgenic animal always include transgenic individuals as well as animals that have lost the array. These non-transgenic siblings serve as the control group. *vps-29 sel-5* double mutant worms without the extrachromosomal array show QL phenotype penetrance around 90% (Fig. 16) which is similar to what was observed previously (Fig. 12).

Siblings of these animals carrying extrachromosomal array containing *egl-20p::sel-5* showed mild rescue of QL phenotype (Fig. 16) which is something we did not expect. It would mean that *sel-5* ectopic expression in Wnt producing cells could prevent high QL phenotype penetrance in *vps-29 sel-5* double mutant. Possible explanation for this observation is provided in the discussion chapter.

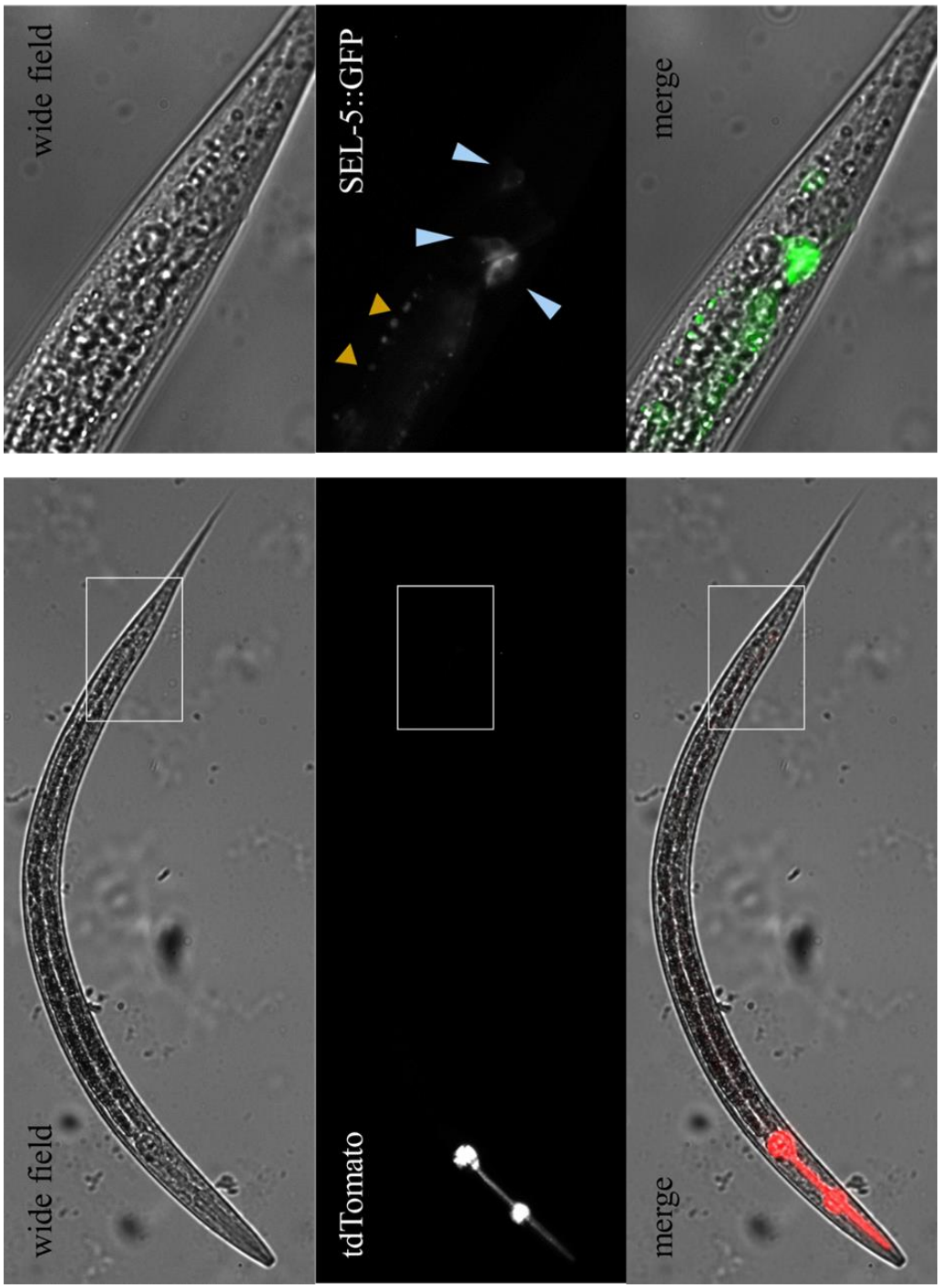


Figure 15: Worms injected with plasmid constructs. Worm strain *vps-29 sel-5* was co-injected with plasmid carrying *egl-17p::sel-5*::*gfp* and marker plasmid carrying *myo-2p::tdTomato.* Progeny of such worms was checked for individuals which expressed tdTomato in their pharynx region (anterior tip in whole animal pictures). Those animals were then checked for expression of SEL-5::GFP in EGL-20/Wnt producing cells in the posterior region of worms (light blue arrowheads in enlarged views of boxed region). In the green channel, additional fluorescence signal was detected (yellow arrowheads). This signal came from the lysosome-like granules located in a worm gut cells. In all images anterior is to the left.

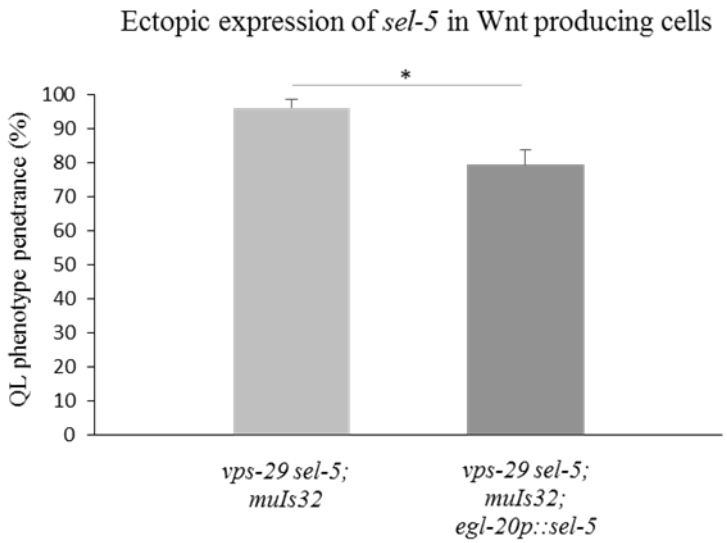


Figure 16: Tissue-specific expression of *sel-5* in Wnt producing cells. Changes in the QL phenotype penetrance after an ectopic expression of *sel-5* in Wnt receiving cells are shown here. In the left column, QL phenotype penetrance of worms without extrachromosomal array carrying *egl-20p::sel-5* plasmid construct is shown. The QL phenotype penetrance is similar to that observed previously for *vps-29 sel-5* double mutant animals (Fig. 12). In sibling worms which carried extrachromosomal array with *egl-20p::sel-5*, a partial rescue of QL phenotype penetrance was observed (right column). Results are shown as a mean of three independent experiments and error bars as SD. Statistical significance determined by unpaired *t-test*.

* $p < 0{,}005$

4.6 Level of MIG-14/Wls is independent of SEL-5 function

In Wnt producing cells, proper endocytosis is important for recovery of Wls protein from the PM and its recycling to the GA and ER for multiple rounds of Wnt ligand secretion to extracellular space. We wanted to test whether *sel-5* could play a role in MIG-14 trafficking. Such experiments could further help to discriminate between the role of SEL-5 in Wnt producing or receiving cells. We designed an experiment in which the level of *C. elegans* Wls homolog MIG-14 was measured in various strains with mutations in *sel-5* and retromer complex subunit *vps-29* or their combination. Mutation in *vps-29* leads to impaired MIG-14 recycling and its missorting to lysosomal compartment and subsequent degradation (Yang et al., 2008). As a result, level of MIG-14 is lower and Wnt signalling is partially abrogated. If SEL-5 played a role in endocytosis in Wnt producing cells its mutation would cause improper MIG-14 internalization and its accumulation at the PM. In *vps-29 sel-5* double mutant, MIG-14 would be trapped at the PM instead of degradation in lysosome and its levels would be higher compared to *vps-29* single mutant.

For this purpose we used *huSi2* transgenic strains where MIG-14 is expressed from its own promoter and is tagged with GFP at its C-terminus. Strains *huSi2*, *sel-5;huSi2*, *vps-29;huSi2* and *vps-29 sel-5;huSi2* were used in the experiment. Protein lysates were prepared from

synchronized L1 larvae and used for Western blot analysis and subsequent quantification of MIG-14 levels in individual worm strains. (Fig. 17a, b).

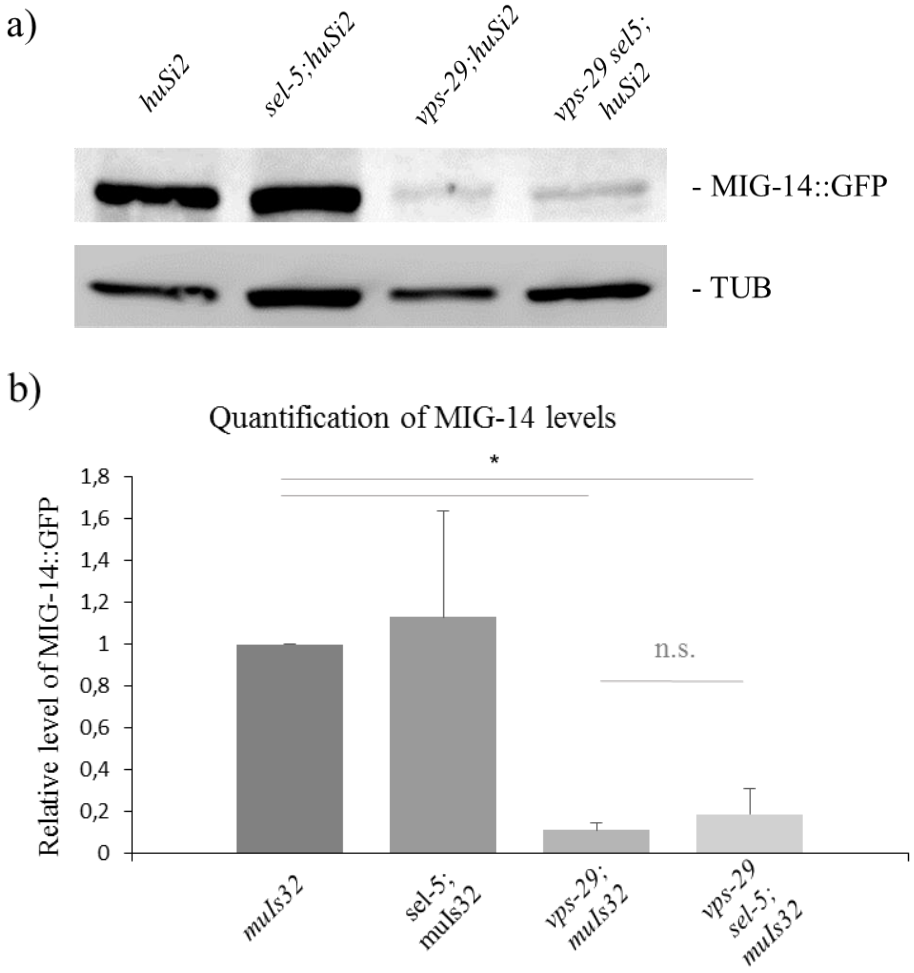


Figure 17: Levels of MIG-14/Wls protein in *vps-29* and *sel-5* mutant animals. a) Worm protein lysates of four indicated strains (*huSi2, sel-5;huSi2, vps-29;huSi2* and *vps-29 sel-5;huSi2*) were subjected to Western blot. MIG-14 is tagged with GFP in strains with *huSi2* transgene. To selectively stain MIG-14::GFP protein, primary antibody against GFP was used. As a loading control, all samples were stained with antibody against tubulin. **b)** The level of MIG-14 in strain *sel-5;huSi2* does not show significant difference compared to reference *huSi2* strain. Contrary to that, in *vps-29;huSi2* strain, MIG-14 levels were lower as expected based on previous observations (Yang et al., 2008). Similar results were obtained for double mutant worms *vps-29 sel-5;huSi2*. Picture in **a)** obtained via ImageQuant LAS4000 (GE Healthcare Life Sciences). Quantification in **b)** shows mean results of five independent experiments. Amount of MIG-14 in every loaded sample was determined by calculating the ratio between MIG-14 and loading control (α-tubulin), this ratio was then compared to the ratio calculated for protein lysates from WT animals. Error bars shown are SD. Statistical significance was determined by unpaired *t-test*.

* $p < 5.10^{-6}$; n.s., non significant

Amount of MIG-14 in *huSi2* strain was used as a reference for comparisons with the mutant strains (Fig. 17b). As shown, level of MIG-14 in *sel-5;huSi2* did not seem to differ from the *huSi2* reference strain. In *vps-29;huSi2*, MIG-14 levels are significantly lower as expected and described previously (Yang et al., 2008). Double mutant *vps-29 sel-5;huSi2* did not show any significant increase in MIG-14 levels (Fig. 17b). The lack of change in MIG-14 level in double mutant animals suggests that MIG-14 is missorted to lysosomes similarly to what is happening in *vps-29* single mutant. This would suggest that SEL-5 does not regulate MIG-14 recycling.

4.7 Role of SEL-5 in TRN polarity

So far, we have been using QL phenotype as a marker of impaired Wnt signalling pathway. A proper position of QL descendant cells in worm is affected by single Wnt ligand, EGL-20. Compared to that, polarity of ALM and PLM neurons is each directed by multiple Wnt ligands acting partially redundantly (Hilliard and Bargmann, 2006; Prasad and Clark, 2006). In the following experiments, we wanted to investigate, whether there is any role for SEL-5 kinase in Wnt signalling that helps to establish anterio-posterior polarity of ALM and PLM neurons. For this purpose, we created worm strains *mig-14;muIs35*, *sel-5;muIs35* and *mig-14;sel-5;muIs35*. Animals with mutated allele *mig-14(mu71)* show reduced activity of MIG-14, which leads to almost full penetrance of QL as well as to partial penetrance of ALM and PLM polarity defects (Pan et al., 2008). We compared QL, ALM and PLM phenotype penetrance changes in *mig-14* and *sel-5* single or double mutants.

We did not see any changes in proper position or orientation of ALM, PLM and QL descendant neurons in control worms carrying *muIs35* transgene alone (Fig. 18). Same results were obtained for *sel-5* single mutants (Fig. 18). Compared to that, we observed almost fully penetrant QL migration defect and partial penetrance of ALM phenotype in *mig-14* mutant animals (Fig. 18) as described previously (Pan et al., 2008). Surprisingly, in *mig-14;sel-5* double mutants we observed small albeit significant rescue of the QL migration defect (Fig. 18a). There was also similar trend in the level of ALM phenotype penetrance. However, this reduction was not statistically significant (Fig. 18b). Increase in PLM polarity defect was observed in *mig-14* mutant animals compared to the control, however this level was not further changed in *mig-14;sel-5* double mutants (not shown). We have shown before that after addition of *sel-5* mutation to sensitized background an elevation of QL phenotype penetrance occurs. The rescue that we observed here in QL and ALM phenotypes was thus

rather unexpected and will be discussed later in this work.

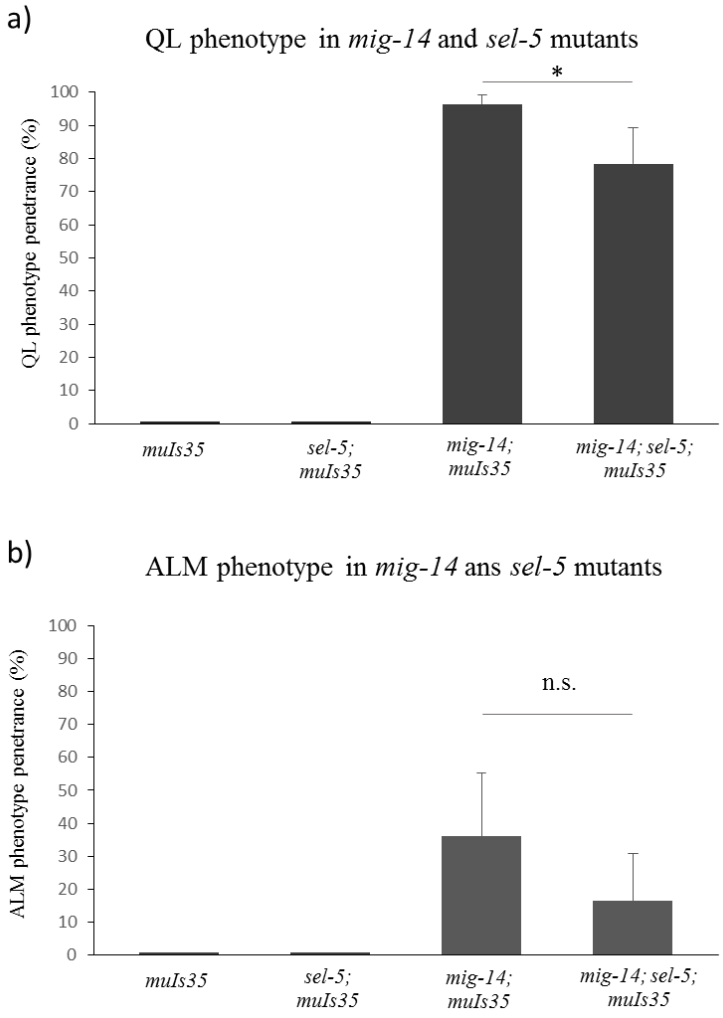
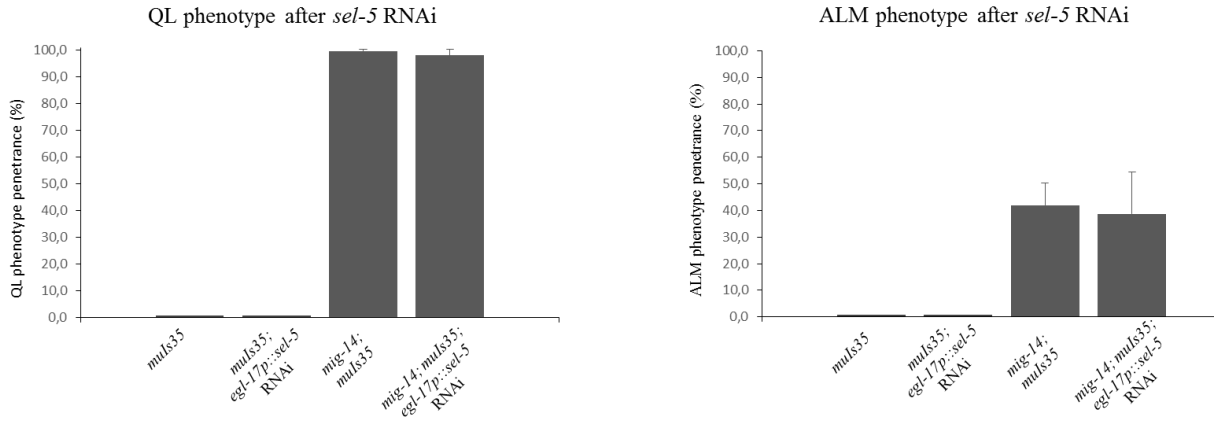


Figure 18: QL and ALM phenotype in *mig-14* and *sel-5* mutants. a) QL phenotype penetrance in worms *mig-14* and *sel*-5 and their combination. There in no QL phenotype observed in control animals with transgene *muIs35* or in single *sel-5* mutant worms. In single mutant *mig-14* worms, this phenotype reached almost full penetrance. Unexpectedly, double mutant animals *mig-14;sel-5* showed lower QL phenotype penetrance compared to single mutant worms. **b)** Similarly to observation in a), ALM phenotype penetrance was not observed in transgenic worm *muIs35* or single *sel-5* mutant. While single mutant *mig-14* showed partial penetrance of ALM phenotype, there was reduction of this phenotype in double mutant worms. However, unlike in case of QL phenotype, this reduction was not statistically significant. Mean values of five independent experiments are shown with error bars as SD. Statistical significance determined by unpaired *t-test*.
* $p < 0,01$; n.s., non significant

Similarly to previous experiments, we wanted to determine whether the partial rescue of QL and ALM phenotypes is caused by SEL-5 kinase activity in Wnt producing or Wnt receiving cells. Again, we used tissue-specific RNAi knockdown of *sel-5*, this time in *mig-14* mutant background. Contrary to what we observed in *mig-14;sel-5* double mutant, we did not detect any change in QL or ALM phenotype penetrance in *mig-14* mutants when tissue specific RNAi knockdown of *sel-5* was conducted in either Wnt producing or Wnt receiving cells (Fig. 19).

a) Wnt receiving cells



b) Wnt producing cells

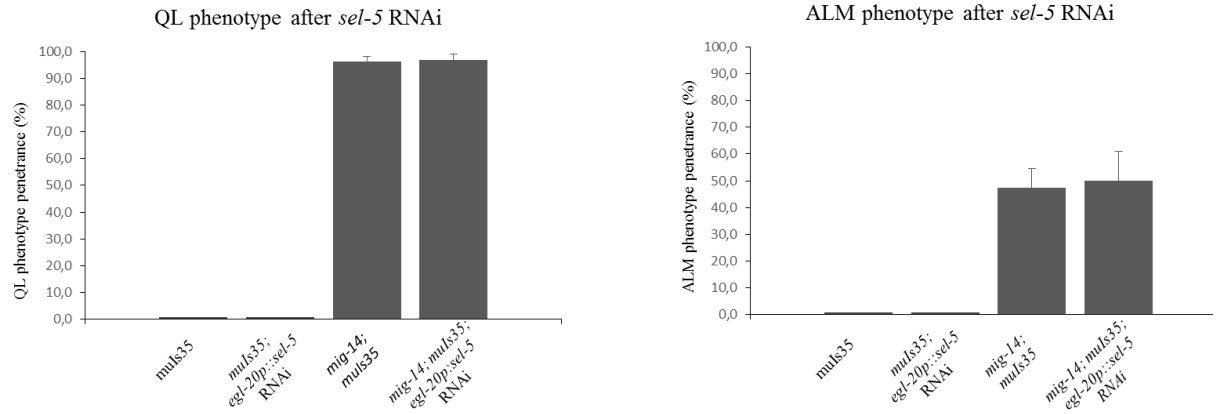


Figure 19: Tissue specific RNAi knockdown in *mig-14* and *sel-5* mutants. In these graphs results of tissue specific knockdowns of *sel-5* gene in *mig-14* and *sel-5* single or double mutant worms is shown. In **a)** *sel-5* RNAi knockdown was carried out in Wnt receiving cells. Knockdown had no effect on WT animals with *muIs35* transgene only. Strain with *mig-14* single mutant showed almost 100% and 40% penetrance of QL phenotype and ALM phenotype, respectively, as described before. We did not observe any rescue of these phenotypes after Wnt receiving cells specific *sel-5* RNAi knockdown. In **b)** results of similar experiment are shown, this time with Wnt producing cells specific RNAi knocdown of *sel-5* gene. Again, we did not observe any changes in QL or ALM phenotypes after *sel-5* RNAi knockdown. Mean results of five independent experiments are shown with error bars as SD. Statistical significance determined by unpaired *t-test.*

4.8 Role of SEL-5 in asymmetrical division of *C. elegans* T cell

During *C. elegans* ontogenesis, Wnt signalling pathway directs proper formation of socket cells in the tail region of worms (Herman et al., 2002). These socket cells form channels that enable chemosensory phasmid neurons to be in direct contact with worm surroundings. Wnt ligand LIN-44 is responsible for asymmetrical division of T cell, the socket cell precursor (Herman et al., 1995). Phasmid as well as some other chemosensory neurons can be stained with DiI dye given that socket cells developed correctly. When LIN-44 signal is not transduced properly, both T cell daughters differentiate into epidermal cells and socket cell is not formed (Herman et al., 1995). As a consequence, DiI is unable to reach phasmid neurons and stain them. Development of phasmid neurons is not affected by this; however, due to the missing socket cells they are not in direct contact with the environment and cannot sense its chemical content. We used DiI staining to detect whether *sel-5* mutation has any effect on the LIN-44-dependent T cell division. We used *dyf-3* mutant animals, whose phasmid and other chemosensory neurons cannot be stained with DiI (Starich et al., 1995), as a negative control and N2 wild type as a positive control (Fig. 20). We conducted DiI staining in control animals and in *sel-5* and *vps-29 sel-5* mutants. We did not observe any changes in phasmid neurons staining in single or double mutant worms. Both strains showed proper filling with DiI comparable to that in wild type N2 worms (Fig. 20). We therefore concluded that *sel-5* is not required for LIN-44/Wnt signalling pathway regulating asymmetric T cell division.

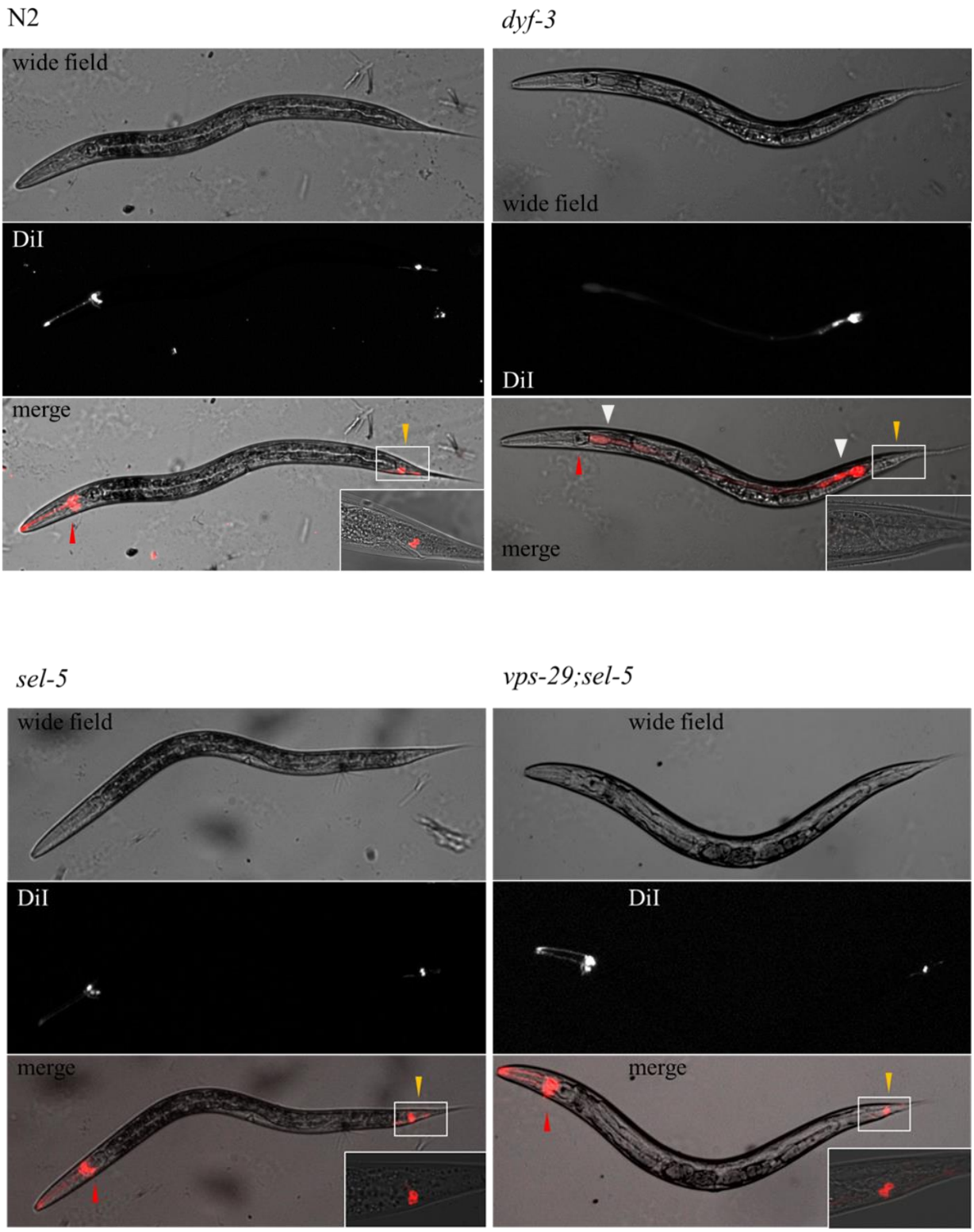


Figure 20: DiI staining of selected chemosensory neurons. (See the next page)

Figure 20: DiI staining of selected chemosensory neurons. DiI staining of indicated strains was conducted by incubating worms in DiI solution for two hours. Chemosensory neurons that are in direct contact with worms surroundings incorporate DiI dye and can be visualized under a fluorescent microscope. As a positive control, wild type worm strain N2 was used. The group of amphid neurons in the head region was stained (red arrowhead) together with two pairs of phasmid neurons in the tail region (yellow arrowhead). Only one pair of phasmid neurons is in focus on the pictures. Insets are enlarged views of the boxed region. As a negative control, *dyf-3* strain was used. In this strain phasmid and amphid chemosensory neurons are not developed properly and are therefore not stained with DiI. Observed red fluorescence was coming from the gut of the stained animals and was a consequence of DiI ingestion during staining (white arrowheads). We did not observe any change in DiI staining in *sel-5* or *vps-29 sel-5* mutant animals.

6. Discussion

6.1 Identification of new endocytosis regulators

In this work, we used nematode *C. elegans* as a model organism to identify previously unknown regulators of Wnt signalling pathway at the level of endocytosis. A whole genome screen was performed via RNAi feeding of *C. elegans* and several candidates were discovered whose knockdown led to an enhancement of a weak Wnt signalling defect, the erroneous migration of the QL neuroblast descendants. Genes, which were previously described in Wnt signalling and those that were not likely to be involved in regulation at the level of endocytosis, were removed from the list of candidates. From the candidates left on the list, we further selected those with 1) known or predicted function in endocytosis and 2) clear ortholog in higher organisms including human and mouse. Gene coding for SEL-5 kinase seemed to be promising (Fig. 11). SEL-5 is homologous to mammalian AP2-associated protein kinase-1 (AAK1). Human AAK1 was found to bind AP2 adaptor complex and phosphorylate its subunit μ2, which is important for selection, binding and internalization of cargo proteins via CME (Ricotta et al., 2002). SEL-5 thus fulfilled the above mentioned criteria and we therefore decided to investigate a possible role of this protein in Wnt signalling regulation. Interestingly, AAK1 was also identified as a potential regulator of Wnt/β-catenin signalling in an siRNA screen in mouse embryonic stem cells (Groenendyk and Michalak, 2011), further justifying our choice.

The RNAi screen was done in *vps-29* mutant background. We would not be able to identify *sel-5* gene as a candidate regulator of Wnt signalling otherwise, as *sel-5* mutation itself does not cause QL phenotype or any other Wnt-related phenotypes that we were able to observe (Fig. 12, 13, 17, 18 and 20).

First, we confirmed findings of the RNAi screen in *vps-29 sel-5* double mutants. Compared to RNAi *sel-5* knockdown in *vps-29* mutant background, we expected stronger effect in *sel-5* gene knockout in the same background. Indeed, while QL phenotype penetrance in RNAi experiment reached about 50 % (Fig. 11), in the experiment with double mutant *vps-29 sel-5* the QL penetrance was over 90 % (Fig. 12). To confirm the synergistic effect of *sel-5* mutation when retromer complex is impaired we tried knockdown of *vps-35*, another subunit of retromer. QL phenotype penetrance was significantly higher after *vps-35* RNAi in *sel-5* mutants compared to the same knockdown in WT worms (Fig. 13). These results suggest that while *sel-5* mutation itself is not sufficient for causing any observable phenotype in *C.*

elegans, it could severely aggravate penetrance of the QL phenotype when activity of the retromer complex is partially compromised.

The absence of any observable phenotypes in *sel-5* single mutants suggests that SEL-5 is only a minor regulator whose mutation could be compensated for with other regulators or that the function of SEL-5 is redundant with another protein kinase. We tested the second possibility. AAK1 and GAK1 are human homologs of worm SEL-5 and TAG-257, respectively, and they were shown to work redundantly (Neveu et al., 2015). However, we were not able to detect any QL phenotype after *tag-257* knockdown in *sel-5* mutant background. Moreover, we did not observe any increase in QL phenotype penetrance after *tag-257* knockdown in *vps-29* mutants. Either the functional interaction of those two protein kinases is different in *C. elegans* or there is yet another protein kinase that takes up the role of SEL-5 after its RNAi knockdown. According to the Wormbase database, there is one additional protein kinase in *C. elegans* that is paralogous to SEL-5 and TAG-257 kinases. This kinase, D2045.7, does not contain DPF/W motif, which is necessary for binding the AP2 complex, something that is present in both SEL-5 and TAG-257 kinases. Nevertheless, it would be interesting to test whether D2045.7 could be the kinase that functions redundantly with SEL-5.

Another possibility for the lack of QL phenotype changes after *tag-257* RNAi could be low effectivity of RNAi feeding. While generally, this method works efficiently for most genes, there are some cases described when other methods of RNAi knockdown introduction into worms had to be used to gain observable knockdown (Kamath et al., 2000).

6.2 Determining the tissue specificity of SEL-5 function in Wnt signalling

There are several possible ways how exactly SEL-5 kinase involvement in endocytosis regulation could affect Wnt signalling. The two most obvious ones are that its role is in either Wnt producing or Wnt receiving cells. In both cell types, endocytosis is necessary for proper Wnt signalling. In Wnt producing cells, recycling of Wls from the PM via CME is important for appropriate Wnt ligand secretion, especially when Wnt ligand is supposed to signal over a long distance (Coudreuse et al., 2006). It was shown that Wls recycling might not be absolutely necessary for secretion of all types of Wnt ligands (Yamamoto et al., 2013). MIG-14/Wls is, however, indispensable for proper QL neuroblast descendants localization along anterio-posterior axis of *C. elegans* (Yang et al., 2008). In Wnt receiving cells, it was shown that endocytosis is participating in the Wnt signal transduction as well as in regulation of

Wnt receptor levels at the PM (Blitzer and Nusse, 2006; reviewed in Kikuchi et al., 2007). We wanted to discriminate between these possibilities and determine whether SEL-5 kinase regulates endocytosis in Wnt producing or Wnt receiving cells. In a pilot experiment with tissue-specific *sel-5* RNAi in *vps-29* background (M. Macůrková, unpublished), Wnt signalling was found out to be dependent on a role of SEL-5 kinase in Wnt receiving cells and independent of a role in Wnt producing cells. We tried to confirm these results in different sensitized background. Previous research uncovered genetic interaction between *sel-5* and *mtm-6*, gene coding for myotubularin-related lipid phosphatase. *mtm-6 sel-5* double mutants show reduction in QL phenotype compared to *mtm-6* alone (M. Macůrková and M. Horázná, unpublished). We conducted tissue-specific RNAi knockdown of *sel-5* in *mtm-6* mutants. We did not observed any change in QL phenotype penetrance after specific *sel-5* RNAi knockdown in Wnt receiving or producing cells (Fig. 14). As we were unable to unambiguously determine the tissue specificity of SEL-5 role through tissue-specific RNAi knockdown, we tried an opposite approach and conducted a rescue experiment in *vps-29 sel-5* double mutants in which *sel-5* was ectopically expressed in either Wnt receiving or producing cells. QL phenotype penetrance in *vps-29 sel-5* double mutants reaches roughly 90% (Fig. 12) and we expected this penetrance to be markedly reduced after *sel-5* expression in Wnt receiving cells and unchanged after *sel-5* expression in Wnt producing cells. Unfortunately, we were only successful in expressing *sel-5* in Wnt producing cells. The results, however, did not confirm our hypothesis. We observed small but significant rescue after expression of *sel-5* in Wnt producing cells (Fig. 16).

This partial rescue could be possibly explained by overexpression of *sel-5* in Wnt producing cells. While in normal conditions, endogenous level of SEL-5 kinase may not be involved in MIG-14/Wls internalization, in the case of ectopic overexpression, SEL-5 surplus could interfere with endocytosis regulation. Human SEL-5 homolog was shown to increase the binding affinity of AP2 complex to its cargo proteins (such as MIG-14/Wls) and subsequently its internalization (Ricotta et al., 2002). Higher MIG-14/Wls recycling could lead to a higher Wnt ligand secretion and this could cause the partial rescue that we observed. Another possibility would be a role of SEL-5 in endocytosis generally in both types of cells, Wnt producing as well as Wnt receiving. As we have no straightforward way to verify that the *egl-20p::sel-5(RNAi)* construct is actually functional, it is still possible that our results with the tissue-specific RNAi in Wnt producing cells are falsely negative.

However, another independent line of evidence favours the role of SEL-5 in Wnt receiving cells. If SEL-5 regulates endocytosis in Wnt receiving cells and there is no regulation of

endocytosis in Wnt producing cells, MIG-14/Wls level should be independent of *sel-5* mutation. Level of MIG-14/Wls was measured in *vps-29* and *sel-5* single and double mutants and it was, indeed, confirmed that its level did not change in *vps-29 sel-5* mutants compared to *vps-29* mutants alone (Fig. 17).

It is also conceivable that SEL-5 regulates endocytosis of Wnt receptors at the PM of cells located between Wnt producing and receiving cells. Wnt ligand receptors could work as a sink for secreted Wnt ligand and thus regulate the amount of ligand that reaches Wnt receiving cells. However, we did not conduct any experiments that would address this hypothesis.

Apart from causing higher penetrance of QL phenotype in retromer complex mutants, *sel-5* mutation was also observed to cause changes in other phenotypes associated with Wnt and TRN. Namely, ALM phenotype, where ALM neurons polarity is changed. In *mig-14* mutant animals the QL phenotype reaches almost 100% penetrance and ALM are erroneously bipolar or have reversed polarity in roughly 30% of animals (Fig. 18). Unexpectedly, when *mig-14;sel-5* double mutant strain was created it was observed that the QL as well as ALM phenotypes were partially rescued (Fig. 18). Through the tissue-specific RNAi knockdown experiments, we were not able to decide whether this was caused by *sel-5* knockdown in Wnt producing or receiving cells (Fig. 19). In animals carrying *mig-14(mu71)* allele, stable MIG-14 protein is created, however its functionality is severely diminished (Yang et al., 2008). As a result, *C. elegans* EGL-20/Wnt ligand secretion is decreased leading to almost fully penetrant QL phenotype. To explain how an additional *sel-5* mutation could cause rescue of this phenotype, we suggest a role of SEL-5 in a regulation of Wnt ligand receptors at the PM of Wnt receiving cells. An amount of Wnt ligand receptors at the PM of receiving cells needs to be tightly regulated so that Wnt signalling is not excessive. It was shown in zebrafish embryo that endocytosis of LRP5/6 via CME is important for downregulation of Wnt signalling (Jiang et al., 2012). Whether similar process takes part in *C. elegans* is not known so far. However, mutation of *sel-5*, whose role is to stimulate CME, could lead to impaired CME of Wnt ligand receptors or co-receptors and their accumulation at the PM, and thus possibly to upregulation of Wnt signalling. This could cause the partial rescue in QL and ALM phenotypes penetrance that we observed in *mig-14;sel-5* double mutants (Fig. 18). Moreover, it is in agreement with our hypothesis that SEL-5 regulates endocytosis specifically in Wnt receiving cells.

To map the spectrum of Wnt-controlled processes affected by *sel-5* we also tested the role of SEL-5 in proper formation of the phasmid chemosensory neurons in the tail region of *C.*

elegans. Asymmetrical division of T cell gives rise to a structure connecting chemosensory phasmid neurons with the worm surroundings. LIN-44/Wnt ligand produced in the tail region directs this asymmetrical division (Herman et al., 1995). However, we did not observe any changes in phasmid neurons staining with DiI dye in *sel-5* mutants compared to WT animals (Fig. 20). We did not observe any changes in phasmid neuron staining in *vps-29 sel-5* mutants either. This could be explained by a close proximity of Wnt producing and receiving cells in this case. It was described previously that in some cases of Wnt producing and receiving cells close localization, retromer complex mediated Wls recycling might be dispensable for Wnt signalling (Groot et al., 2013).

Pictures of DiI stained phasmid neurons were taken one hour after staining (according to protocol in chapter 3.8). DiI stained phasmids were clearly observable even several days after staining in WT animals. However, in case of *vps-29 sel-5* mutant animals, DiI stained neurons appeared to be much weaker 24 hours post-staining compared to WT (not shown). This was observed in *vps-29* single mutant animals as well. Contrary to that, *sel-5* single mutant animals DiI stained neurons were comparable to WT animals. As the DiI dye incorporates into membranes, it is possible that this observation could reflect the overall change in membrane dynamics in *vps-29* mutants.

6.3 Possible role of SEL-5 kinase in Wnt receiving cells

Based on the results of conducted experiments, we suggest following hypothesis about SEL-5 kinase function in *C. elegans* Wnt signalling. SEL-5 phosphorylates AP2 complex μ-subunit (as does its homolog AAK1 in human), which leads to an increased binding affinity towards AP2 complex cargo proteins. In this case, the cargo proteins would be Wnt ligand receptors or co-receptors. No LRP5/6 homolog has been identified in *C. elegans* so far, nevertheless, other co-receptors such as RYK or ROR have known homologs in *C. elegans*, LIN-18 and CAM-1, respectively (Forrester et al., 2004; Inoue et al., 2004). Both, co-receptors and Fz receptor levels at the PM were shown to be required for proper Wnt signalling in *C. elegans* (Moffat et al., 2014). AP2 complex mediated internalization of these receptors/co-receptors could be involved in this regulation. Mutation of *sel-5* could lead to changes in AP2 phosphorylation pattern and deregulation of Wnt signalling at the level of Wnt receiving cells. It is likely that similarly to its human AAK1 homolog, SEL-5 works redundantly with other kinases. This is supported by the lack of any observable phenotypes when *sel-5* alone was mutated. After internalization of Wnt ligand receptors/co-receptors

they are either recycled back to the PM or they can be retained in endosomal pathway and transported to the lysosomal compartments for degradation. It is not likely that SEL-5 plays any role in these steps. However, human SEL-5 kinase homolog AAK1 was shown to have a longer splice variant that is active in regulation of multiple steps of receptor internalization and recycling (Henderson and Conner, 2007). According to the Wormbase database, there is a splice variant of SEL-5 as well, however, it is not known whether this variant plays any regulatory roles in endocytosis or recycling processes.

7. Conclusion

In this work, we were able to identify *sel-5* gene as a new regulator of Wnt signalling pathway in *C. elegans*. Through methods of molecular biology and genetics, we tried to establish the site of SEL-5 action in either the Wnt producing or Wnt receiving cells. Even though the results of our experiments were not sufficient to pinpoint SEL-5 kinase activity conclusively to the Wnt receiving cells, we gained several clues that suggest a role of SEL-5 in this location. We uncovered genetic interaction of *sel-5* with components of the retromer complex and established that *sel-5* is not required for maintenance of MIG-14/Wls levels. In this work we mainly used *C. elegans* neuronal network as a model system. We planned on conducting further experiments and testing some of our findings and hypothesis in mammal cells. It is likely that these experiments would help with more detailed description of SEL-5 role in endocytosis regulation, however, we did not manage to perform them in given time.

Further work will be necessary to understand the role of SEL-5 and its homolog AAK1 in Wnt signalling in *C. elegans* and human, respectively. Implementation of some recently developed methods could be advantageous. CRISPR mediated tissue-specific knockout of *sel-5* may provide more decisive results compared to RNAi knockdown. Watching for changes in level and localization of Wnt receptors after *sel-5* knockout/knockdown could result in more specific description of SEL-5 kinase role within Wnt receiving cells. Results gained in this diploma thesis could be used as a springboard for this further work.

8. Literature

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