Univerzita Karlova v Praze
Přírodovědecká fakulta

Studijní program: Biologie
Studijní obor: Buněčná a vývojová biologie

Bc. Monika Horázná

Mechanisms regulating MTM-6 protein activity on endosomes

Mechanismy regulace aktivity proteinu MTM-6 na endosomech

Diplomová práce

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

Praha, 2013

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

V Praze, 6.5.2013

Monika Horázná

Ráda bych poděkovala mé školitelce Marii Macůrkové, Ph.D. za vedení mého diplomového projektu a za její čas a rady, které mi věnovala během psaní této práce. Můj velký dík patří doc. Františku Půtovi za užitečné rady a pomoc při experimentech s kvasinkami. Děkuji také Liboru Macůrkovi, Ph.D. za pomoc s přípravou plasmidových konstruktů a paní laborantce Mgr. Jitce Velčevové za poskytnutí návodů a pomoc s přípravou RNAi experimentů. Za velkou pomoc děkuji členům Laboratoře regulace genové exprese, jmenovitě zejména Mgr. Martině Hálové, Mgr. Anně Valentové, Ondřeji Gahurovi, Ph.D. i všem ostatním. V neposlední řadě bych ráda poděkovala Lence Libusové, Ph.D. a dalším členům naší laboratoře za občasné konzultace a výpomoc. Na závěr velmi děkuji mým rodičům, kteří mě podporovali nejen během psaní této práce, ale i v průběhu celých pěti let mého dosavadního studia biologie.

Content

Abstrakt

Signální dráha proteinu Wnt je jednou z konzervovaných signálních drah a jejím prostřednictvím lze řídit rozhodování o osudu buňky, vývoj, regeneraci a homeostázi v tkáních dospělého organismu. Narušení či nesprávná regulace této dráhy vede často ke vzniku onemocnění. Proteiny rodiny Wnt jsou hydrofobní glykoproteiny, které potřebují speciální receptor pro transport z Golgiho aparátu na povrch buňky. U *Caenorhabditis elegans* se tento receptor nazývá MIG-14, u savců Wntless (Wls). V této práci se soustředím na studium mechanismů, které ovlivňují aktivitu proteinu MTM-6. MTM-6 je lipidová fosfatáza, u které bylo nedávno objeveno, že reguluje transport MIG-14/Wls v *Caenorhabditis elegans*. Umlčení genu *mtm-6* vede ke špatné regulaci procesů řízených Wnt signalizací, jako je například migrace potomků Q neuroblastů. V této práci jsou identifikovány nové geny, které vykazují genetickou interakci s *mtm-6* a skrz Wnt signální dráhu ovlivňují migraci potomků Q neuroblastů. Nové poznatky o genetických interakcích *mtm-6* nás přibližují k pochopení regulace signální dráhy proteinu Wnt.

Klíčová slova:

Caenorhabditis elegans, MTM-6, SEL-5, Wntless, Wnt, endosomy, fosfoinositidy, retromer

Abstract

Wnt signalling belongs to conserved pathways and mediates cell fate decision, development, regeneration and adult tissue homeostasis. Disruption or misregulation of Wnt signalling pathway often leads to disease. Wnt proteins are hydrophobic glycoproteins which need a special receptor for transport from Golgi Apparatus to cell surface, which is called MIG-14 in *Caenorhabditis elegans* and Wntless (Wls) in mammals. In this study, I focus on understanding mechanisms that regulate MTM-6 protein activity. MTM-6, a lipid phosphatase associated with endosomal membrane, has been recently identified as a regulator of MIG-14/Wls trafficking in *Caenorhabditis elegans*. Silencing of *mtm-6* leads to misregulation of some Wnt-directed processes, such as migration of Q neuroblasts progeny. This study reports identification of novel *mtm-6* genetic interactors that have been found to influence migration of Q neuroblasts progeny through Wnt signalling. New knowledge about *mtm-6* genetic interactions bring us near to understanding of Wnt signalling regulation.

Keywords:

Caenorhabditis elegans, MTM-6, SEL-5, Wntless, Wnt, endosomes, phosphoinositides, retromer

List of abbreviations and genes

AAK1	**A**P2-**a**ssociated protein **k**inase **1**
AbA	**A**ureo**b**asidin **A**
AD	**a**ctivating **d**omain
ALM	**a**nterior **l**ateral **M**T cell
AP-1, AP-2	**a**daptor **p**rotein **1** and **2**
APC	**a**denomatous **p**olyposis **c**oli
ASE	**a**mphid neurons, **s**ingle ciliated **e**ndings
BAR	**B**in/**a**mphiphysin/**R**vs
bar-1; BAR-1	**β**-catenin/**a**rmadillo **r**elated
BD	**b**inding **d**omain
BFB	**b**romo**ph**enol **b**lue
cap-1; CAP-1	**CAP**-z protein
CBD	**c**lathrin-**b**inding **d**omain
CCVs	**c**lathrin-**c**oated **v**esicle**s**
CKIα	**c**asein **k**inase **Iα**
csnk-1; CSNK-1	**c**a**s**ei**n** **k**inase **Iγ**
cup-4; CUP-4	**c**oelomocyte **up**take defective
DEPC	**di****e**thyl**p**yro**c**arbonate
dH_2O	**d**istilled water
dpy-23	**d**um**py** (μ subunit of AP2)
DTT	**di****t**hio**t**hreitol
EDTA	**e**thylene**d**iamine **t**etraacetic **a**cid
egl-20; EGL-20	**egg** **l**aying defective
ER	**E**ndoplasmic **r**eticulum
ESCs	**e**mbryonic **s**tem **c**ell**s**
farl-11; FARL-11	**f**actor **ar**rest **l**ike
FYVE	**F**ab1/**Y**OTB/**V**ac1/**E**EA1
GA	**G**olgi **ap**paratus
GAK	cyclin **G**-**a**ssociated **k**inase
GAM-HRP	**g**oat **a**nti-**m**ouse **h**orse**r**adish **p**eroxidase
GRAM	**g**lucosyltransferases, **Ra**b-like GTPase activators and **m**yotubularins

GSCs	**g**erm **s**tem **c**ell**s**
GSK3β	**g**lycogen **s**ynthase **k**inase **3β**
hmp-2; HMP-2	**h**u**mp**back
HSPGs	**h**eparan **s**ulphate **p**roteo**g**lycan**s**
IPTG	**i**so**p**ropyle-β-D-**t**hio**g**alactoside
LRP	**l**ow density lipoprotein **r**eceptor related-**p**rotein
mig-14; MIG-14	abnormal cell **mig**ration
MTM, MTMR	**m**yo**t**ubularin-**r**elated
MVBs	**m**ulti**v**esicular **b**odie**s**
PDZ	**p**ost synaptic density protein, **D**rosophila disc large tumor suppressor, **z**onula occludens-1 protein
PI	**p**hosphatidyl**i**nositol
PI(3)P	**p**hosphatidyl**i**nositol-**3**-**p**hosphate
PI(3,5)P_2	**p**hosphatidyl**i**nositol-**3,5**-bis**p**hosphate
PI(4,5)P_2	**p**hosphatidyl**i**nositol-**4,5**-bis**p**hosphate
PLM	**p**osterior **l**ateral **M**T cell
ppk-1; PPK-1	**PI**P **K**inase
PTEN	**p**hosphatase and **ten**sin homolog
PX	**p**ho**x** homology
QL, QL.d	**l**eft **Q** neuroblast, **d**aughter cell
QR, QR.d	**r**ight **Q** neuroblast, **d**aughter cell
rbbp-5; RBBP-5	**r**etino**b**lastoma protein **b**inding **p**rotein homolog
RID	**R**ho GTPase **i**nactivation **d**omain
rpn-12; RPN-12	proteasome **r**egulatory **p**article, **n**on-ATPase-like
RT	**r**everse **t**ranscriptase; **r**oom **t**emperature
SDS	**s**odium **d**odecyl **s**ulfate
sel-5; SEL-5	**s**uppressor/**e**nhancer of **l**in-12
SET	**s**u(var), **e**nhancer of zeste, **t**rithorax
SID	**S**ET-**i**nteraction **d**omain
snx-3; SNX-3	**s**orting **n**e**x**in **3**
sys-1; SYS-1	**sy**mmetrical **s**ister cell hermaphrodite gonad defect
TBE	**t**ris-**b**orate **E**DTA
TCA	**t**ri**c**hloroacetic **a**cid

TCF/LEF	**T**-**c**ell **f**actor/**l**ymphoid **e**nhancer-binding **f**actor
tes-1; TES-1	**tes**tin homolog
UAS	**u**pstream **a**ctivation **s**equence
usp-5; USP-5	**u**biquitin **s**pecific **p**rotease
VPC	**v**ulval **p**recursor **c**ells
VPS	**v**acuolar **p**rotein **s**orting
Wls	**W**nt**le**s**s**
Wnt	**w**ingless, i**nt**1
wrm-1	**w**orm a**rm**adillo
X-α-Gal	5-Bromo-4-Chloro-3-indolyl **α**-D-**gal**actopyranoside
Y2H	**y**east **2**-**h**ybrid; yeast two-hybrid

1. Theoretical background

1.1. Wnt signalling

A Wnt signalling pathway is an evolutionarily conserved pathway that is important for many processes during development and adult tissue homeostasis (Clevers, 2006; Logan and Nusse, 2004). Disruption of this pathway or its misregulation often leads to pathologies and severe diseases, such as cancer. Wnt proteins can be spread through the extracellular space to the Wnt receiving cells. There, they act as morphogens which can provide cells and tissues with positional information. This signal can guide cells to proper regulation of many processes, such as differentiation, migration, growth etc. Wnts are also important embryonic stem cell factors essential for self-renewing of ESCs (Berge et al., 2011; Kühl and Kühl, 2012).

The best characterized kind of Wnt signallig pathway is the so called canonical Wnt pathway, also often referred to as the Wnt/β-catenin pathway. The key event of the canonical pathway is the regulation of cytoplasmic β-catenin protein stability. In the absence of a Wnt signal, β-catenin is degraded as a result of the interaction with APC/Axin/CKIα/GSK3β destruction complex. β-catenin has many roles but the most important one is that it regulates the activation of transcription of the Wnt signalling pathway target genes.

Wnt/β-catenin pathway starts when a Wnt ligand binds the receptor Frizzled and the coreceptor LRP5/6, which are both found on the cytoplasmic membrane of Wnt receiving cells. In the cytoplasm, β-catenin is bound in the complex with APC, axin, CKIα and GSK3β proteins. This complex provides β-catenin phosphorylation by GSK3β and CKIα followed by polyubiquitinylation and subsequent degradation in the proteasome. After the binding of Wnt to its receptor, the destruction complex is deactivated and proteins dissociate from each other. The free β-catenin can then enter the cell nucleus and regulate the expression of target genes together with transcription factors TCF/LEF. In the absence of Wnt signal, transcription of Wnt-responsive genes is repressed by interaction of TCF/LEF factors with other factors, such as histon deacetylase. Binding of β-catenin to TCF/LEF factors provides an activating domain so the expression of target genes is activated (Clevers, 2006; Kühl and Kühl, 2012; Logan and Nusse, 2004; MacDonald et al., 2009; van Amerongen and Nusse, 2009).

1.2. Wnt signalling in *C. elegans*

The components of Wnt signalling in *C. elegans* are very similar to those in vertebrates. There are only two exceptions: first, unlike other species, *C. elegans* has four genes that encode different β-catenin-like proteins (*bar-1*, *hmp-2*, *sys-1* and *wrm-1*; Costa et al., 1998; Eisenmann et al., 1998; Kidd et al., 2005; Rocheleau et al., 1997); second, to date no LRP5/6 coreceptor homolog has been found in *C. elegans*. Canonical and noncanonical Wnt signalling pathways differ in utilization of different β-catenins; for example, WRM-1 is utilized by noncanonical Wnt signalling pathways whereas BAR-1 by canonical Wnt signalling pathways.

Canonical Wnt signalling in *C. elegans* utilizes BAR-1/β-catenin. The canonical Wnt signalling is important for VPC (vulval precursor cells) fate specification and therefore vulval induction (Eisenmann et al., 1998), formation of rays in the male tail (Hunter et al., 1999; Maloof et al., 1999) or migration of the Q neuroblasts progeny (Whangbo and Kenyon, 1999). Migration of Q neuroblasts descendants is widely used in experimental approaches as a readout of proper functioning or disruption of Wnt signalling and therefore is described in more detail.

There are two Q neuroblasts, QL and QR, born at similar positions on the left and right lateral sides of the *C. elegans* embryo. They differ in their migration directions: while QL and its descendants migrate to the posterior, QR and its descendants migrate to the anterior part of the animal`s body. The reason of this differential migration is a differential response to Wnt signalling, mediated by EGL-20 (one of the five Wnt proteins found in *C. elegans*). Both Q neuroblasts are exposed to the same level of EGL-20/Wnt, but only the QL neuroblast is sensitive to the Wnt signal and as a result its descendants migrate posteriorly (Silhankova and Korswagen, 2007).

1.3. Production of the Wnt signal

1.3.1. Wnt proteins and their posttranslational modifications

Wnt proteins function as signalling molecules with the ability to regulate various processes such as proliferation, migration, differentiation, polarization or asymmetrical cell division. They can mediate short-range as well as long-range signalling. Wnt proteins belong to the evolutionarily conserved family of glycoproteins responsible for proper development of many animal tissues. They

undergo two posttranslational lipid modifications: by palmitic acid at a conserved cystein (Willert et al., 2003) and palmitoleic acid at a conserved serin (Takada et al., 2006). Acylation of Wnt proteins is provided by Porcupine (Hofmann, 2000; Kadowaki et al., 1996). Wnts contain a relatively high number of conserved cystein residues capable of creating intra- and intermolecular disulfide bonds which might contribute to correct folding of Wnt proteins and their multimerization (Miller, 2002). Another important posttranslational modification of Wnt proteins is glycosylation. The protein responsible for glycosylation of Wnts might be Porcupine, although it is very probable that Porcupine itself would not directly glycosylate Wnts. Porcupine, as a membrane-associated acyltransferase, was suggested to bring Wnt proteins close to the ER membrane for subsequent modification (Tanaka et al., 2002). To conclude, both glycosylation and acylation are thought to be necessary for secretion of active Wnt proteins (Komekado et al., 2007; Kurayoshi et al., 2007).

Despite the predictions from amino acid sequences, Wnts are hydrophobic and insoluble. Their hydrophobicity is probably due to lipid modifications taking place in the ER. In the course of evolution, organisms developed a mechanism of vesicular transport that enables trafficking of insoluble proteins to their place of function (reviewed in Bonifacino and Glick, 2004). Proteins that are transported in vesicles usually have a receptor that binds them and enables concentration of transported proteins in a specific area of the donor membrane. This leads to creation of vesicles that are enriched in these proteins (reviewed in Pfeffer, 2007; Port and Basler, 2010). The receptor for Wnt proteins is Wls (Wntless) (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Wls is a transmembrane receptor that binds Wnt in the Golgi apparatus (GA) and brings Wnt from GA to the surface of the signalling cell. The *C. elegans* ortholog of Wls is MIG-14 (Bänziger et al., 2006; Harris et al., 1996; Thorpe et al., 1997).

1.3.2. Secretion of Wnt proteins

The precise mechanism of Wnt secretion has not been completely clarified yet, nevertheless, some of the key factors essential for proper Wnt secretion were recently discovered. Besides the Wls receptor, another component of the Wnt secretory pathway is retromer (summarized in Seaman, 2005) - the protein complex responsible for Wls recycling (Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan

et al., 2008; Port et al., 2008; Yang et al., 2008). Both Wls and retromer have important functions in the Wnt producing cells and therefore facilitate the production of sufficient amount of the signal.

Retromer is a conserved protein complex responsible for retrograde transport of cargo proteins from endosomes to the GA (summarized in Bonifacino and Hurley, 2008; Collins, 2008). The structure of retromer is conserved from yeasts to mammals and contains two discrete complexes: the core assembly and an associated dimer of PI(3)P-binding proteins. The core assembly, also often reffered to as the cargo-loading assembly, is responsible for binding cargo proteins – usually transmembrane receptors that are intended for recycling from endosomes. Subunits of the retromer core complex are VPS26, VPS29 and VPS35. The VPS26/VPS29/VPS35 complex is recruited to the endosomal membrane through interaction with a dimer of SNX (sorting nexin) proteins (in yeast Vps5/Vps17 dimer) which have the ability to bend the endosomal membrane with their BAR domains and bind PI(3)P with their PX domains (Burda et al., 2002; Carlton et al., 2004; Cozier et al., 2002; Ponting, 1996; Zhong et al., 2002). Regulation of the PI phosphorylation and PI(3)P dephosphorylation is very important process for retromer-dependent recycling and is described in greater detail in chapter 1.4.2. Regulation of the endosomal membrane lipid composition by myotubularin phosphatases.

The spreading of Wnt proteins through the extracellular space has not been clarified yet. Nevertheless, several theories were postulated about how Wnt proteins reach their target cells. First possibility is creating micelle-like particles of Wnt multimers where fatty acid chains would face the inner space. This model is supported by the discovery of protein Hedgehog nanooligomers - Hedgehog is also a lipid modified signalling protein (Chen et al., 2004). Second possibility is association of Wnt proteins with lipoprotein particles, that enables especially the long-range travelling of Wnt (and Hedgehog) proteins (Panáková et al., 2005). Next, Wnt proteins could be secreted on vesicular carriers like argosomes or exosomes (Greco et al., 2001; Gross et al., 2012) and travel bound to these. Wnt has also been shown to bind to heparan sulphate proteoglycans (HSPGs) which can facilitate its movement through the tissue (reviewed in Lin, 2004).

1.3.3. Overview of events in a Wnt producing cell

The Wnt signalling pathway begins with the synthesis of a Wnt protein in the Wnt producing cell. In the GA, Wnt binds its receptor Wls (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006) and together they are transported to the cell surface. After releasing of Wnt, Wls is endocytosed and from endosomes recycled in a retromer-dependent manner back to the GA (Belenkaya et al., 2008; Franch-Marro et al., 2008; Pan et al., 2008; Port et al., 2008; Yang et al., 2008). There it can bind another Wnt protein and the whole process is repeated (Fig. 1A). In case the recycling pathway of Wls is disrupted, receptors accumulate in MVBs and are subsequently degraded in lysosomes. Therefore the production of Wnt signal decreases and the whole pathway is weakened (Fig. 1B) (reviewed in Willert and Nusse, 2012).

1.3.4. An important role of endocytosis in Wnt producing cell

Endocytosis is an essential cell ability that enables uptake of extracellular material and internalization of transmembrane molecules from plasma membrane. Clathrin-mediated endocytosis is a first step in the recycling pathway of Wls. The importance of endocytosis in *C. elegans* MIG-14/Wls recycling has been suggested on the basis of Wnt signalling defects in *dpy-23* mutants. *dpy-23* encodes the μ subunit of AP-2 clathrin adaptor complex and its disruption leads to accumulation of MIG-14/Wls at or near plasma membrane (Pan et al., 2008; Yang et al., 2008). Dependance of Wls internalization on clathrin mediated endocytosis was further confirmed by discovery of a conserved endocytosis motif in the third intracellular loop of Wls. Mutation in this motif led to accumulation of Wls protein on the plasma membrane (Gasnereau et al., 2011).

1.4. Myotubularins

Myotubularins are a family of proteins with dual protein phosphatase specificity (they are able to dephosphorylate both phosphotyrosine and phosphoserine) (Laporte et al., 1998) and lipid phosphatase activity (they are able to dephosphorylate PI(3)P and PI(3,5)P_2 at the 3` position) (Schaletzky et al., 2003; Taylor et al., 2000). Although some myotublarins are catalytically inactive, they still

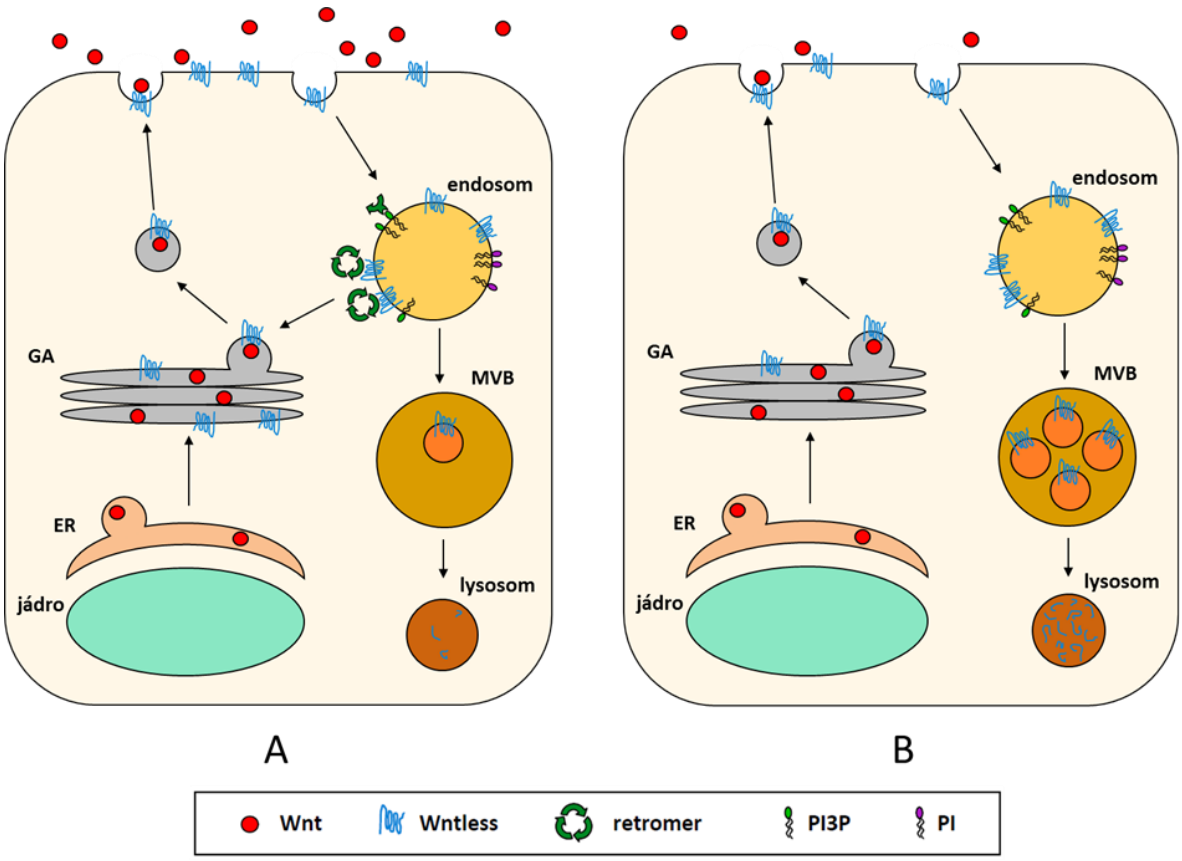


Fig. 1: **Model of the retromer and Wls roles in the secretion of Wnt by the Wnt producing cell.**
A) recycling of Wls in a cell with functional retromer complex, B) nonfunctional recycling of Wls in the absence of retromer (after Franch-Marro et al., 2008)

can act as important regulators; for example, catalytically inactive myotubularins can form complexes with active myotubularins and therefore regulate their activity or intracellular localization (Dang et al., 2004; Kim et al., 2003). For example, MTMR9, an inactive phosphatase, has recently been shown to regulate the enzymatic activity and substrate specificity of an active phosphatase MTMR8 (Zou et al., 2012). List of all to date known *C. elegans* myotubularins and their mammalian orthologs is shown in Tab. 1.

Table 1. **List of *C. elegans* myotubularins and their mammalian orthologs.**

mammalian myotubularins	*C. elegans* myotubularins
MTM-1 MTMR-1 MTMR-2	MTM-1
MTMR-3 MTMR-4	MTM-3
MTMR-5 MTMR-13	MTM-5
MTMR-6 MTMR-7 MTMR-8	MTM-6A, MTM-6B
MTMR-9	MTM-9
MTMR-10, 11, 12, 14	?

(after www.wormbase.org, www.omim.org, Laporte et al., 2003)

The domain composition is common for all myotubularins – they contain several conserved domains (Fig. 2):

- GRAM domain probably regulates the membrane binding and protein interactions (Doerks et al., 2000)
- catalytic domain contains a conserved Cx_5R motif in the catalytic centre – this motif is related to the active sites of tyrosine-phosphatases, dual protein phosphatases, and a lipid phosphatase PTEN
- SID (SET-interaction domain) domain could regulate interaction with SET domain-containing proteins
- PDZ-binding motiv probably binds proteins with PDZ domain.

Some myotubularins can contain different additional domains, for example FYVE or PH domains (Laporte et al., 2001) that mediate interaction with specific phosphoinositids.

In the *C. elegans* genome, there are five genes coding different myotubularins (*mtm-1*, *mtm-3*, *mtm-5*, *mtm-6* and *mtm-9*). MTM-1, MTM-3 and MTM-6 are catalytically active, while MTM-5 and MTM-9 are catalytically inactive phosphatases.

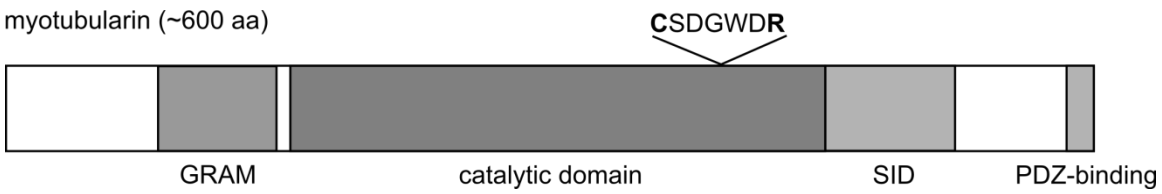


Fig. 2: **Domain composition of myotubularins.**

Scheme represents a general domain composition common to myotubularins. Position of the Cx_5R motif within the calatytic domain is indicated as CSDGWDR amino acid sequence. (after Wishart et al., 2001)

1.4.1. MTM-6 and MTM-9 as regulators of Wnt signalling

MTM-6 is a *C. elegans* lipid phosphatase that is found in two isoforms: MTM-6A and MTM-6B. Since the MTM-6B isoform is presumably unstable and does not rescue the *mtm-6* knock down phenotype (Dang et al., 2004), the following text will be only about the MTM-6A isoform (which will be, to simplify, termed MTM-6). The functional structure of MTM-6 corresponds to the general domain composition of myotubularins and is schematically represented in Fig. 3 along with domain structure of MTM-9.

MTM-6 is an active lipid phosphatase creating a functional complex with MTM-9. These two myotubularins were described as proteins important for endocytosis by *C. elegans* coelomocytes (Dang et al., 2004) and as regulators of PI(3)P level on the endosomal membrane (Dang et al., 2004; Xue et al., 2003), which is a central regulator of endosomal trafficking. In coelomocytes, both MTM-6 and MTM-9 are mostly cytosolic with some membrane localization (Dang et al., 2004). The regulatory role of MTM-6 and MTM-9 in the Wnt signalling pathway was indentified recently by Silhankova et al., 2010. They show that mutations in *mtm-6* or *mtm-9* genes cause defects in several Wnt-dependent processes, such as the posterior migration of QL.d neurons. The MTM-6/MTM-9 complex has the ability to dephosphorylate PI(3)P on the endosomal membranes. It was therefore suggested that the MTM-6/MTM-9 complex activity regulates binding of SNX proteins on endosomal membranes containing Wls and thus influence the efficiency of retromer-dependent recycling of MIG-14/Wls (Silhankova et al., 2010). Interestingly, while mutations in other *C. elegans* myotubularins do not cause a perturbation of the Wnt signalling pathway suggesting that individual myotubularins have different functions, in *mtm-6;mtm-9* double mutant strain other myotubularins appear to partially compensate for MTM-

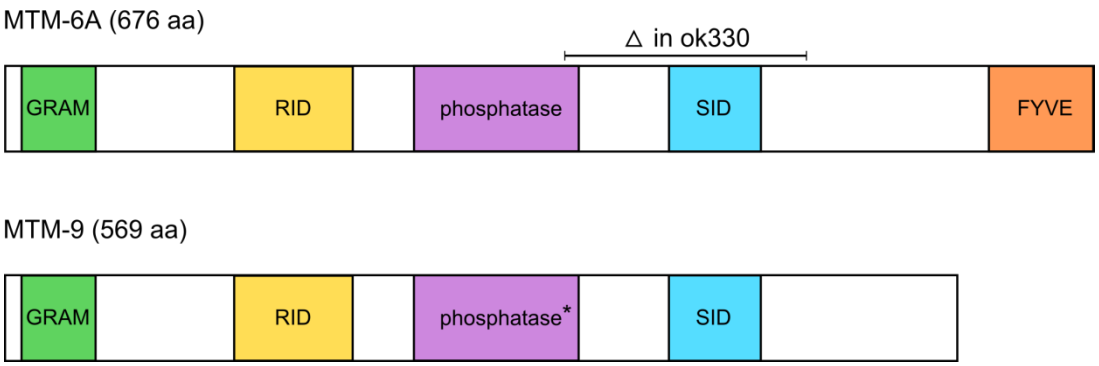


Fig. 3: **Domain composition of MTM-6A and MTM-9 proteins.** Protein domains are predicted from cDNA sequences. The ok330 deletion is present in the *mtm-6*(ok330) allele in *C. elegans* strains mutated in *mtm-6* gene. Asterisk indicates that phosphatase domain lacks amino acid residues in the Cx_5R motif that are required for its activity. (after Dang et al., 2004)

6/MTM-9 absence. On the basis of these observations, the MTM-6/MTM-9 myotubularin complex is thought to be required for Wnt secretion and retromer-dependent recycling of MIG-14/Wls in *C. elegans* Wnt producing cells.

1.4.2. Regulation of the endosomal membrane lipid composition by myotubularin phosphatases

A lipid composition of the endosomal membrane is influenced by many proteins. Key players are lipid kinases and phosphatases – their enzymatic activity determines the level of diversely phosphorylated phosphoinositides. These phosphoinositides create binding epitopes for other proteins and thereby allow them to bind the endosomal membrane or, conversely, prevent them from binding it.

As already mentioned, myotubularins are lipid phosphatases important for the regulation of the lipid composition on the endosomal membranes. It was previously shown in mammalian cells that myotubularins (namely MTM1 and MTMR2) are involved in local regulation of PI(3)P together with Rab5 and Rab7 GTPases and Vps15/Vps34 kinase complex. MTM1 plays a primary role in sorting transmembrane receptors from early endosomes, MTMR2 regulates the following transit out of late endosomes (Cao et al., 2008, 2007).

The proces of PI phosphorylation is initiated with the exchange of GTP for GDP resulting in Rab5 and Rab7 activation and association with the early and late endosomal membrane, respectively. An important effector of Rab5 and Rab7 is the

Vps15/Vps34 kinase complex that creates a PI(3)P microdomains within the endosomal membrane (Murray et al., 2002; Stein et al., 2003). When the PI(3)P microdomains are created, PI(3)P-binding proteins, such as retromer, are recruited to the endosomal membrane. Myotubularins are also recruited to domains with a local burst of PI(3)P and their recruitment initiates the PI(3)P degradation. When the myotubularin binds the Vps15/Vps34 kinase complex, the Rab5 or Rab7 GTPase is displaced and the Vps15/Vps34 complex is therefore deactivated. Nevertheless, this interaction also leads to deactivation of the myotubularin because its catalytic domain becomes sterically occluded by the Vps15 subunit (Fig. 4A) (Cao et al., 2008, 2007). Due to the cycling between activation and inactivation of the Vps15/Vps34 complex, endosomes may go through waves of PI phosphorylation and dephosphorylation (Fig. 4B) (Cao et al., 2008, 2007). Such a mechanism enables the precise recruitment and release of trafficking proteins and thus a spatio-temporal regulation of membrane transport.

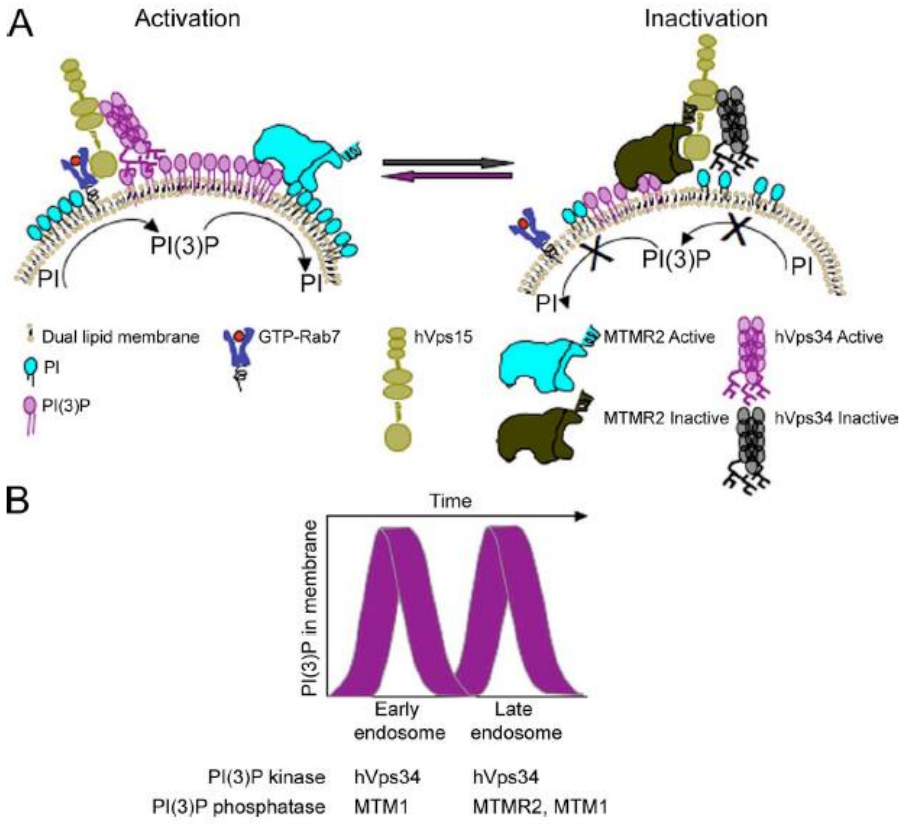


Fig. 4: **Model of PI and PI(3)P homeostasis through the cooperation between the Vps15/Vps34 kinase complex and the myotubularin phosphatases.**

A) After being activated by Rab~GTP, the Vps15/Vps34 kinase complex creates a burst of PI(3)P. Myotubularin is recruited on the endosomal membrane enriched in PI(3)P molecules and degrades them. When myotubularin interacts with the Vps15/Vps34 complex, both are deactivated. B) A schematic graph of PI(3)P synthesis and degradation on early and late endosomal membrane. (Cao et al., 2008, 2007)

2. Objectives

It was suggested that MTM-6/MTM-9 complex regulates binding of SNX proteins on endosomal membranes through modulation of PI(3)P levels and thus affects the efficiency of Wls recycling (Silhankova et al., 2010). However, there are still many unresolved questions regarding the exact mechanism of myotubularin involvement in Wnt signalling. For example, it is not clear whether the activity of the MTM-6/MTM-9 complex is constitutive or whether it is regulated in some way. An intriguing possibility would be that a similar interplay between myotubularins, Vps34 kinase and Rab GTPases that was described above, also exists in *C. elegans*.

Interestingly, *C. elegans vps-34* was also shown to affect Wnt signalling (Silhankova et al., 2010), while mammalian Rab GTPases Rab5 and Rab7 are required for retromer-dependent traffic (Rojas et al., 2008). Moreover, MTM-6 and MTM-9 are not the only myotubularins that could be involved in regulation of Wnt signalling. Silhankova et al. discovered that *mtm-5* and *mtm-1* also significantly influence WNT signalling, although their influence is revealed only in *mtm-6* mutant background. Genetic interactions among *C. elegans* myotubularins have been thoroughly studied (Silhankova et al., 2010) but not much is known about interactions among their protein products.

The main objective of this diploma thesis project is to describe mechanisms regulating MTM-6 protein activity on endosomes. To achieve this objective, these subtasks were postulated:

1. Test interactions between these candidate proteins: MTM-1, MTM-3, MTM-5, MTM-6, MTM-9, RAB-5, RAB-7, VPS-34 and ZK930.1.
2. Search for new binding partners of MTM-6.
3. Search for genetic enhancers or supressors of *mtm-6* that are involved in regulation of Wnt signalling.

3. Materials

3.1. Organisms

3.1.1. *E. coli*

Strains of *Escherichia coli* that were used:

TOP10 competent cells used for molecular cloning

DH5α competent cells used for molecular cloning

OP50 strain used for common *C. elegans* feeding

HT115 strain used for *C. elegans* feeding during RNAi experiments

3.1.2. *S. cerevisiae*

Strains of *Saccharomyces cerevisiae* that were used:

GOLD strain used for transformation and crossing

Y187 strain used for transformation and crossing

Information about yeast strains genotypes are described in Matchmaker™ Gold Yeast Two-Hybrid System User Manual (Protocol No. PT4084-1; Version No. PR033493).

3.1.3. *C. elegans*

Following tables (Table 2 and 3) show *Caenorhabditis elegans* strains, mutations and transgenes that were used.

Table 2. ***C. elegans* strains.**

strain	genotype	use
N2	wild type	RNA isolation, crossing
CF700	*muIs32*	RNAi
KN1040	*mtm-6*(ok330);*muIs32*	RNAi, crossing
KN1151	*cup-4*(ok837);*muIs32*	RNAi
RB638	*sel-5*(ok363)	crossing

Table 3. **Mutant alleles and transgenes.**

mutant allele/transgene	reference
cup-4 (ok837)	Fares and Greenwald, 2001
mtm-6 (ok330)	Silhankova et al., 2010
sel-5 (ok363)	Caenorhabditis Genetics Center, St. Paul, MN
muIs32[Pmec-7::gfp; lin-15(+)]	Ch'ng et al., 2003

3.2. Cultivation media

3.2.1. Culturing of *E. coli*

Bacteria are cultured on plates with LB (Luria-Bertani) medium with appropriate antibiotics for selection.

For one liter of LB (agar) medium following ingredients were used:

tryptone	10 g
yeast extract	5 g
NaCl	10 g
(agar	15 g)
H_2O	1 l

After autoclaving in 120°C, it was optional to add these antibiotics:

ampiciline (50 mg/ml)	1 ml
kanamycine (25 mg/ml)	1 ml
tetracycline (5 mg/ml)	2.5 ml

3.2.2. Culturing of *S. cerevisiae*

Yeasts are cultured either on plates with YPAD medium, which is a complex medium for rutine growth, or SD (synthetic dextrose minimal medium) medium.

For one liter of YPAD (agar) medium following ingredients were used:

peptone	20 g
yeast extract	10 g
adenine hemisulphate	100 mg
(agar	20 g)
H_2O	900 ml

After autoclaving in 120°C, following solutions were added:

20% glucose	100 ml
ampiciline (100 mg/ml)	1 ml

For one liter of SD (agar) medium following ingredients were used:

yeast nitrogen base	6.7 g
drop-out supplements	1.394 g
(agar	20 g)
H_2O	900 ml

After autoclaving in 120°C, following solutions were added:

20% glucose	100 ml
ampiciline (100 mg/ml)	1 ml
100× concentrated solution of	
Leu, Trp, His, Ura	10 ml

Media for different selection were prepared by adding different combination of leucine, tryptophan, histidine and uracil.

3.2.3. Culturing of *C. elegans*

Worms are cultured on NGM (Nematode Growth Medium) agar plates with a grown layer of *E. coli* OP50 strain, which serves as a source of nutrients (Brenner, 1974). Worms can be washed out of plates with M9 buffer.

For one liter of NGM agar medium following ingredients were used:

NaCl	3 g
tryptone	2.5 g
agar	17 g
H_2O	972 ml

After autoclaving in 120°C, following solutions were added:

1 M $CaCl_2$	1 ml
cholesterol (5 mg/ml in EtOH)	1 ml
1 M $MgSO_4$	1 ml
1 M K-phosphate buffer (pH = 6.0)	25 ml

When preparing plates for RNAi experiments, following supplements were added:

ampiciline (50 mg/ml)	1 ml
tetracycline (5 mg/ml)	2.5 ml
1 M IPTG	1 ml

For one liter of M9 buffer following ingredients were used:

$Na_2HPO_4 \cdot 7H_2O$	6 g
KH_2PO_4	3 g
NaCl	5 g
1 M $MgSO_4$	1 ml

Before autoclaving, it is optional to add a small amount of gelatin into the mixture. The reason of adding the gelatin is to prevent worms from sticking to plastic during pipetting and manipulation in Eeppendorf tubes.

Plates with the agar medium were left at room temperature overnight, and simultaneously *E. coli* OP50 were cultured in liquid LB medium at 37°C. The next day, liquid culture of *E. coli* OP50 was pipetted onto plates and spreaded with a glass stick. Plates with bacterias were left in the room temperature overnight.

When preparing RNAi experiments, 6-well plates were prepared in the same way as described in the previous paragraph. The *E. coli* HT115 strain containing the appropriate RNAi construct was pipetted onto the wells agar and during the overnight incubation at room temperature, the dsRNA production was induced by IPTG.

Lysis buffer

30 mM Tris pH 8, 8 mM EDTA, 100 mM NaCl, 0.7% NP40, 0.7% Tween 20
Proteinase K (200 μg/ml)

3.3. Material for cloning

3.3.1. Vectors

List of vectors (plasmid maps are shown in Fig. 5, 6 and 7):

pJET1.2	cloning vector, Fermentas
pPC86	Y2H vector, Clontech
pPC97	Y2H vector, Clontech
pGBKT7	Y2H vector, Clontech
pGADT7	Y2H vector, Clontech

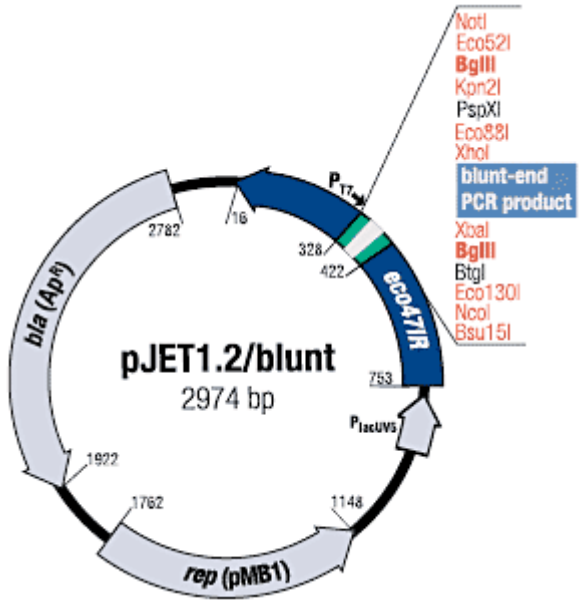


Fig. 5: **pJET1.2 plasmid map.**

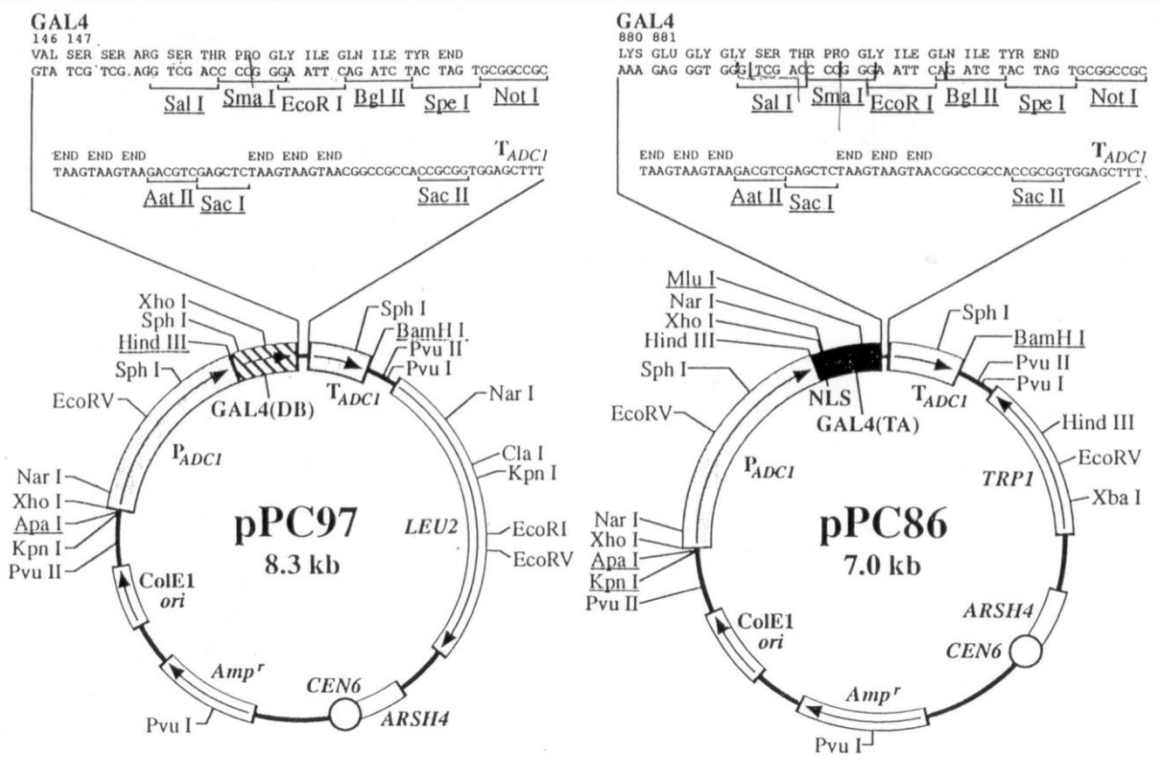


Fig. 6: **pPC86 and pPC97 plasmid maps.**

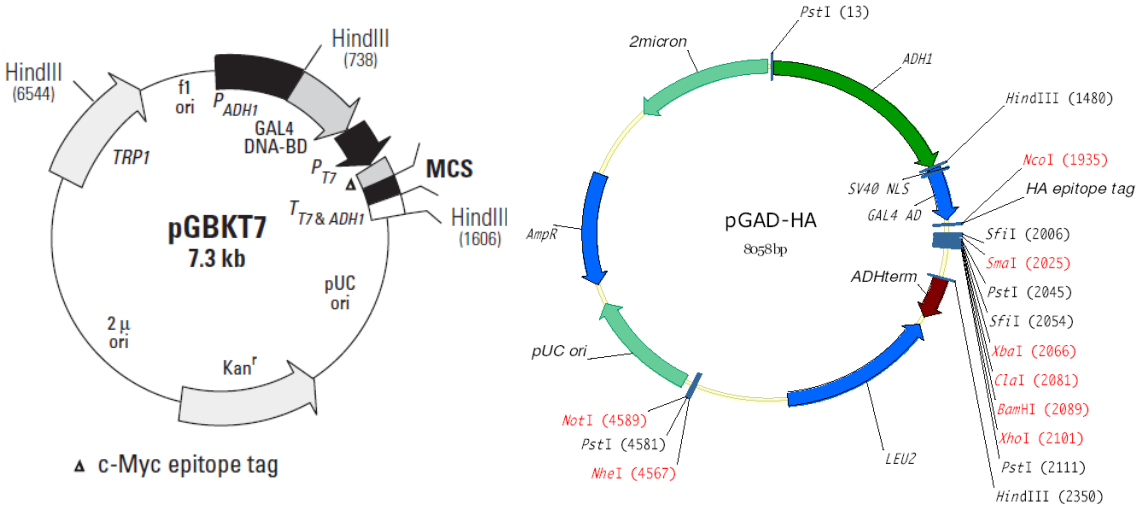


Fig. 7: **pGBKT7 and pGAD-HA plasmid maps.**

3.3.2. DNA electrophoresis

TBE (Tris/Borate/EDTA) buffer (5× concentrated)

89 mM Tris pH 7.6, 89 mM boric acid, 2 mM EDTA

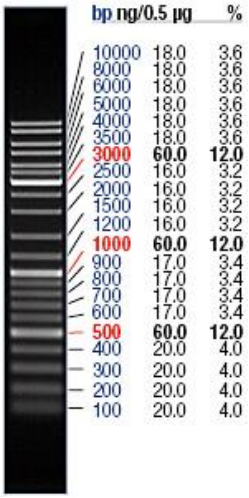
Glycerol/bromophenol blue loading buffer (6× concentrated):

glycerol	3 ml
bromophenol blue	25 mg
dH_2O	to 10 ml

EtBr

1 mg/ml stock solution, final concentration in gel 0.5 µg/ml

DNA size marker (GeneRuler™ DNA Ladder Mix (Thermo Scientific))



3.4. Material for manipulation with proteins

3.4.1. Protein extract preparation

For preparing protein extracts from yeasts following chemicals and solutions were used:

2 M NaOH

β-mercaptoethanol

50% TCA (trichloroacetic acid)

1 M TRIS base

2× Laemmli buffer

0.112 M TRIS-Cl pH 6.8, 3.42% SDS, 12% (v/v) glycerol, 0.002% BFB

1 M DTT

3.4.2. SDS-PAGE and Western blotting

Running buffer

25 mM TRIS base, 192 mM glycin, 0.1% SDS

For 5 liters of Running buffer following ingredients were used:

H_2O	5 l
TRIS	15 g
glycin	72 g
SDS	5 g

After dissolving of all ingredients on magnetic stirrer, the solution was filtered.

Blotting buffer (Towbin)

25 mM TRIS base, 192 mM glycin, 20% methanol

For 5 liters of Blotting buffer following ingredients were used:

H_2O	4 l
TRIS	15 g
glycin	72 g
methanol	1 l

After dissolving of all ingredients on magnetic stirrer, the solution was filtered. The required final pH value (8.1 – 8.5) was achieved without any adjustment.

TBST, pH 7.4

10 mM TRIS-Cl, 150 mM NaCl, 0.05% Tween 20

For 5 liters of TBST following ingredients were used:

H_2O	5 l
TRIS-Cl	6.05 g
NaCl	43.8 g
Tween 20	2.5 g

pH value 7.4 was adjusted by HCl, then the solution was filtered.

Amido Black Stain

For 100 ml of Amido Black Stain following ingredients were used:

methanol	40 ml
acetic acid	10 ml
Amido Black 10B	0.1 g

Coomassie Briliant Blue Stain

0.2% CBB R-250, 40% ethanol, 10% acetic acid

For 1l of Coomassie Briliant Blue Stain following ingredients were used:

95% ethanol	420 ml
acetic acid	100 ml
CBB R-250	2.0 g
H_2O	to 1 l

The solution was filtered before use.

Destain

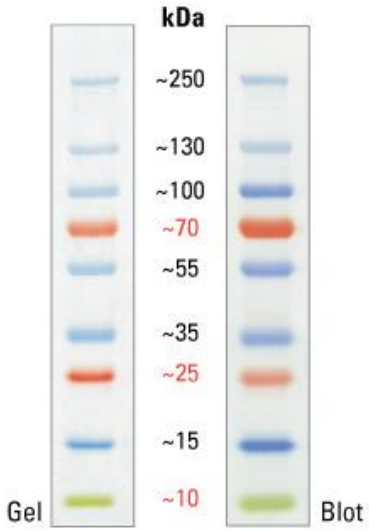
10% ethanol, 7.5% acetic acid

For 1l of Destain solution following ingredients were used:

ethanol	105 ml
acetic acid	75 ml
H_2O	to 1 l

Protein size marker

PageRuler Plus Prestained Protein Ladder (Thermo Scientific)



Antibodies

primary antibodies:

mouse monoclonal anti-c-myc (9E10) (Exbio; 11-433-C100)

mouse monoclonal anti-PSTAIR (Sigma; P 7962)

secondary antibodies:

GAM-HRP (Jackson ImmunoResearch; 115-035-146)

Primary antibodies were diluted in 3% BSA (prepared as 0.18 g BSA in 6 ml of TBST buffer), secondary antibodies were diluted in 3% milk (prepared as 0.18 g milk in 6 ml of TBST buffer).

Horseradish peroxidase detection system

SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used to visualize the secondary antibody GAM-HRP for subsequent exposion to photographic film.

3.5. Other materials

Primers used for cloning - sequences (5'-3'):

mtm1ex1F-XmaI	CCCGGGAATGGATGACAGAGGGAACAATAG
mtm1ex11R-NotI	GCGGCCGCCTAGGCGGTCAATTTTTGAG
mtm3ex1-FSalI	GTCGACGATGACGGTTACTTCTTCTGCG
mtm3ex9-RBglII	AGATCTTCAGAGAGGTTGCTCATTCACATC
mtm5ex1-FSalI	GTCGACGATGCGAGATCCGGATAAGGTG
mtm5ex21-RNotI	GCGGCCGCCTAATCACGAATAACTTGTTCAATGC
mtm6ex1F-NdeI	CATATGCGATTCGAGGACATTGGC
mtm6ex8R-SalI	GTCGACTCAATCAGTTTTGCACTTGTCAC

Primers used for control PCR reactions - sequences (5'-3'):

pGAD-HAF	TACGACGTACCAGATTACGC
mtm9ex3F	TCTGATTGCTTCATCGTTGGC
mtm9ex5R	CTACATTGGCGACAAAACGC

Primers used for *sel-5* and *mtm-6* genotyping - sequences (5'-3'):

mtm6ex4-F1	ATGTGCACAGCCTCTGACG
mtm6ex4-F2	TCTCATCGAGAAGGATTGGC
mtm6int-R	CACGAAGAGGTTGCCATTT
sel5ok363ins-F	GAAACCAGCAGCTGAAGCTG
sel5ok363out-F	CCACAACCTATCATTCATCGC
sel5ex11-R	GTAGAGATACTGGTTGTGCAG

Primers used for amplification of *mtm-3* and *mtm-5* cDNA - sequences (5'-3'):

mtm3ex1-FSalI	GTCGACGATGACGGTTACTTCTTCTGCG
mtm3ex9-RBglII	AGATCTTCAGAGAGGTTGCTCATTCACATC
mtm5ex1-FSalI	GTCGACGATGCGAGATCCGGATAAGGTG
mtm5ex21-RNotI	GCGGCCGCCTAATCACGAATAACTTGTTCAATGC

Photographic films (AGFA)

4. Methods

4.1. Working with DNA

4.1.1. Izolation and purification of plasmid DNA

There are two ways how plasmid DNA was isolated:

a) Preparation of plasmid DNA with NucleoSpin® Plasmid kit (Macherey-Nagel), using the protocol supplied by the manufacturer (protocol *5.1 Isolation of high-copy plasmid DNA from E. coli)*. This approach was used for isolating small amounts of plasmid DNA from 1 to 5 ml of bacterial culture.

b) Preparation of plasmid DNA by alkaline lysis. For greater yield of plasmid DNA the following protocol was used.

- A bacterial clone carrying plasmid DNA was inoculated on a plate with LB agar medium and appropriate antibiotic and cultivated overnight at 37°C.
- One colony of bacteria was inoculated into 10 – 15 ml of LB medium with antibiotic and cultivated overnight with vigorous shaking (37°C, 180 rpm).
- The culture was cooled on ice for 5 – 10 minutes, transferred into polypropylen centrifuge tube, and centrifuged (2800 g, 20 min, 4°C). The pellet was resuspended in x[1] ml of ice-cold solution I. The tube was taken out from the ice, *2x* ml of solution II[2] were added, and the tube was gently shaken. After 5 minutes, the tube was put back on ice and *1.5x* ml of ice-cold solution III were added. Next incubation took about 15 – 30 minutes and led up to formation of a white precipitate, which was centifuged (17000 g, 20 min, 4°C). The supernatant was transferred into a new centrifuge tube, mixed with 0.6 volume of ice-cold isopropanole, and immediately centrifuged (2800 g, 5 min). The supernatant was discarded and the pellet allowed to dry. After drying, the pellet was dissolved in 300 µl of mpH_2O (mili-pore H_2O) and transferred into a microtube. Then the same volume of 10 M LiCl was added and the mixture was incubated for 10 minutes at -80°C. At this step it was possible to leave the mixture at -80°C for longer storing.
- The mixture from the previous step was centrifuged (16000 g, 5 min) and the supernatant was transferred into a new microtube. The plasmid DNA was precipiteated by one volume of 96% EtOH (10 min, -80°C) and centrifuged (16000 g, 5 min). The supernatant was discarded, the pellet

was washed with 1 ml of 70% EtOH and centrifuged again (16000 g, 5 min). The supernatant was discarded once more and the pellet was allowed to dry. After drying, the pellet was dissolved in 30 – 200 µl of mpH_2O.

Solutions: solution I: 25 mM Tris-HCl (pH 8.0), 10 mM EDTA - NaOH, 1% glucose
solution II: 1% SDS, 0.2 M NaOH
solution III: 3 M potassium acetate (CH_3COOK), 2 M acetic acid, pH 5.4

[1] The amount of solution I is given by the starting volume of the bacterial culture; it is usually 0.5 - 1 ml for 10 ml of culture, 2 - 4 ml for 50 ml of culture.
[2] Before using the solution II, it was warmed to approximately 20°C to allow the SDS dissolve.

4.1.2. Electrophoresis in agarose gel

The DNA electrophoresis was performed in an apparatus for a horizontal gel electrophoresis. The gel was prepared from 1× TBE buffer and agarose at 1% concentration. Ethidium bromid (EtBr) was used as a DNA labeling dye and added to the gel before its solidification. The electrophoresis was run in 1× TBE buffer.

4.1.3. PCR

Polymerase chain reaction was used in the course of preparing plasmid constructs or genotyping *C. elegans*. The standard PCR mixture for one reaction was prepared as follows:

deionized H_2O	15.25 µl
dNTPs (10 mM)	0.5 µl
forward primer (10 µM)	0.5 µl
reverse primer (10 µM)	0.5 µl
DreamTaq buffer (Fermentas)	2 µl
Taq polymerase	0.25 µl
template DNA	1 µl

Typical setting of PCR machine was:

95°C	5 min	initial denaturation	
95°C	20 s	DNA denaturation	
55°C	40 s	primer annealing	30-35×
72°C	1 min	second DNA strand synthesis	
72°C	5 min	final synthesis	
12°C	0-59 min	hold	

Any deflections from this setting are described in specific cases.

4.1.4. Preparing of plasmid constructs

For the Y2H analysis, two sets of recombinant plasmids were prepared – one for fusion of the candidate genes with the GAL4 DNA binding domain (plasmid pPC97) and one for fusion with the GAL4 activation domain (plasmid pPC86) (Chevray and Nathans, 1992). Most cDNA sequences that were required had already been cloned in the pJET1.2 vector (Fermentas) except for *mtm-3* and *mtm-5*. The latter two were amplified from *C. elegans* total RNA and were cloned into the pJET1.2 vector (the process of RNA isolation and cDNA synthesis is described in following chapters).

The proces of plasmid constructs preparation could be divided into five main steps:

1. digestion
2. electrophoresis
3. isolation of DNA fragments
4. ligation
5. selection of positive clones

1. digestion:

cDNA sequences were excised from the pJET1.2 vector and cloned into the opened pPC plasmids. In some cases it was necessary to generate blunt ends, which was done by T4 DNA polymerase after the first digest. Restriction enzymes used for this cloning were purchased from Fermentas and NEB, T4 DNA polymerase from Fermentas.

Typical composition of a digest mixture:

DNA sample[1]	x µl
restrictase 1	0.5 µl
restrictase 2	0.5 µl
10× buffer	2 µl
H_2O	20–(2+2×0.5+x)

[1] The final amount of DNA differed depending on the further use of the digested DNA. In a control digestion, the final amount was 200 – 500 ng. In a digestion for further cloning, the final amount was usually 500 – 1000 ng. In case it was needed to change the buffer for second restrictase or when the inzert was extremely small, the final amount of DNA was approximately 2 – 4.5 µg).

The digest temperature was usually 37°C, for some restrictases 30°C. The optimal digest conditions were designed on the basis of information provided on Fermentas web site: http://www.thermoscientificbio.com/webtools/doubledigest/

2. electrophoresis:

After the digest termination, products were separated by gel electrophoresis (see chapter 4.1.2. Electrophoresis in agarose gel) and bands containing open pPC plasmids and cDNA sequences excised from pJET1.2 vector were cut from the gel.

3. isolation of DNA fragments:

DNA fragments were isolated from the agarose gel using *Gel/PCR DNA Fragments Extraction Kit* (Geneaid) as recommended by the manufacturer. The only step that was done differently was the DNA elution – DNA was eluted from the column by ddH_2O instead of the Elution Buffer provided with the Kit.
(http://www.geneaid.com/sites/default/files/100DF.pdf)

4. ligation

Ligation mixture was prepared in 20 µl volume. The vector:insert molar ratio was usually 1:3; only when problems with ligation appeared, different vector:insert ratio was tried.

Typical composition of a ligation mixture:

vector DNA	x µl
insert DNA	20–(x+2+1) µl
10× buffer	2 µl
T4 DNA ligase	1 µl

The ligation mixture was incubated for 1 hour at 37°C followed by an overnight incubation at 4°C. The tube with the mixture was placed into polystyrene box with water heated on 37°C so during the time of incubation, the mixture went through the temperature gradient from 37°C to 4°C which provided optimal conditions for ligation.

5. selection of positive clones

The product of ligation was transformed into TOP10 or DH-5α competent cell. using heat shock method. Bacteria were spread on Petri dishes with selective LB agar medium and icubated overnight at 37°C. Grown colonies were tested for presence of the recombinant plasmid using PCR (amplification by forward primer located in the insert and reverse primer located in the vector) and gel electrophoresis. The connection at the 5` end of the insert was always sequenced to make sure that there was no shift in the reading frame.

Altogether, cDNA of nine genes was cloned into the multiple-cloning site of pPC86 and pPC97 plasmids; *mtm-6* cDNA was also cloned into pGBKT7 plasmid (Clontech). The list of cloned inserts, lenghts of cDNA inserts and cloning sites are listed in Tab. 4 (for plasmid maps see chapter 3.3.1. Vectors).

Table 4 Plasmid constructs

name	characteristics
pPC86/mtm-1	full length cDNA *mtm-1* ; insert 1767 bp; cloned XmaI-NotI into XmaI-NotI, in frame with GAL4(TA)
pPC86/mtm-3	full length cDNA *mtm-3* ; insert 3021 bp; cloned SalI-BglII into SalI-BglII, in frame with GAL4(TA)
pPC86/mtm-5	full length cDNA *mtm-5* ; insert 5235 bp; cloned SalI-NotI into SalI-NotI, in frame with GAL4(TA)
pPC86/mtm-6	full length cDNA *mtm-6* ; insert 2031 bp; cloned SalI-MscI into BglII*-SalI, in frame with GAL4(TA)
pPC86/mtm-9	full length cDNA *mtm-9* ; insert 1710 bp; cloned BamHI*-BglII into SalI*-BglII, in frame with GAL4(TA)
pPC86/rab-5	full length cDNA *rab-5* ; insert 627 bp; cloned XhoI-BglII into SalI-BglII, in frame with GAL4(TA)
pPC86/rab-7	full length cDNA *rab-7* ; insert 630 bp; cloned SalI-BamHI into SalI-BglII, in frame with GAL4(TA)
pPC86/vps-34	full length cDNA v*ps-34* ; insert 2706 bp; cloned SalI-NotI into SalI-NotI, in frame with GAL4(TA)
pPC86/ZK930.1	full length cDNA *ZK930.1* ; insert 4065 bp; cloned EcoRI-NotI into EcoRI-NotI, in frame with GAL4(TA)
pPC97/mtm-1	full length cDNA *mtm-1* ; insert 1767 bp; cloned XmaI-NotI into XmaI-NotI, in frame with GAL4(BD)
pPC97/mtm-3	full length cDNA *mtm-3* ; insert 3021 bp; cloned SalI-BglII into SalI-BglII, in frame with GAL4(BD)
pPC97/mtm-5	full length cDNA *mtm-5* ; insert 5235 bp; cloned SalI-NotI into SalI-NotI, in frame with GAL4(BD)
pPC97/mtm-6	full length cDNA *mtm-6* ; insert 2031 bp; cloned SalI-MscI into BglII*-SalI, in frame with GAL4(BD)
pPC97/mtm-9	full length cDNA *mtm-9* ; insert 1710 bp; cloned BamHI*-BglII into SalI*-BglII, in frame with GAL4(BD)
pPC97/rab-5	full length cDNA *rab-5* ; insert 627 bp; cloned XhoI-BglII into SalI-BglII, in frame with GAL4(BD)
pPC97/rab-7	full length cDNA *rab-7* ; insert 630 bp; cloned SalI-BamHI into SalI-BglII, in frame with GAL4(BD)
pPC97/vps-34	full length cDNA v*ps-34* ; insert 2706 bp; cloned SalI-NotI into SalI-NotI, in frame with GAL4(BD)
pPC97/ZK930.1	full length cDNA ZK930.1; insert 4065 bp; cloned SmaI-NotI into SmaI-NotI, in frame with GAL4(BD)
pGBKT7/mtm-6	full length cDNA *mtm-6* ; insert 2031 bp; cloned NdeI-SalI into NdeI-SalI, in frame with GAL4(BD)

* = blunt end was created in this restriction site

4.1.4.1. Total RNA isolation

N2 (wild type) worms were collected from plates with M9 buffer and washed for three times. The pellet was left in 30 µl of M9 buffer and 300 µl of Trizol reagent (Invitrogen) were added. Sample was frozen in liquid nitrogen and after that incubated at 65°C for 15 minutes. In the next step, 60 µl of chloroform were added, sample was vortexed hard and left at room temperature for 3 minutes. Then the sample was centrifuged for 15 minutes at 21382×/4°C; the upper fraction was collected, 312 µl of isopropanol (0.8 volume) were added, the sample was vortexed and left at room temperature for 10 minutes. Then the sample was centrifuged for 10 minutes at 21382×/4°C; the supernatant was discarded, the pellet washed with 500 µl of 70% ethanol and centrifuged. The supernatant was discarded once more, the pellet air-dried and dissolved in DEPC H_2O. The isolated RNA was used for cDNA synthesis and the rest was stored at –80°C.

4.1.4.2. cDNA synthesis

For synthesis of cDNA, the *SuperScript III First-Strand Synthesis System for RT-PCR* (Invitrogen) was used. The reaction was mixed as follows:

RNA (~120 ng/µl)	8 µl
primer	1 µl
10 mM dNTP mix	1 µl

Two separate reactions were prepared: one with 50 µM oligo(dT) primers, the second one with random hexamers (50 ng/µl). This mixture was incubated for 5 minutes at 65°C and than placed on ice for one minute. Following components were added after the incubation on ice (pre-mixed in the indicated order):

10× RT buffer	2 µl
25 mM $MgCl_2$	4 µl
0.1 M DTT	2 µl
RNAseOUT	1 µl
SuperScript III RT	1 µl

The reaction mixture was gently mixed by pipetting, collected by brief centrifugation and incubated for 50 minutes at 50°C (for random hexamer primed reaction, this

incubation was preceded by ten-minute incubation at 25°C). The reaction was terminated at 85°C for 5 minutes and stored at –80°C.

4.1.4.3. Amplification of *mtm-3* and *mtm-5* cDNA

PCR reactions were prepared as described in chapter 4.1.3. PCR. The the total reaction volume was 50 µl, therefore the amount of all ingredients was multiplied 2.5× - except for the cDNA sample (2 µl) and Phusion polymerase (0.5 µl). Four separate reactions were prepared: first doublet with the cDNA sample prepared using oligo(dT) primers, second doublet with the cDNA sample prepared using random hexamers. From each dublet, one reaction was run with primers for *mtm-3* amplification and second reaction was run with primers for *mtm-5* amplification (for primer sequences see 3.5 Other materials).

PCR program (35 cycles for denaturation – annealing – synthesis):

98°C	30 s
98°C	10 s
55.5°C	30 s
72°C	3 min
72°C	10 min
12°C	-

PCR products were analyzed by gel electrophoresis (see chapter 4.1.2. Electrophoresis in agarose gel); only reactions with the cDNA sample prepared using oligo(dT) primers gave products corresponding to *mtm-3* and *mtm-5* sequences. *mtm-3* and *mtm-5* DNA fragments were excised from gel, isolated and cloned into pJET1.2 vector. Sequencing of randomly chosen clones revealed deletion of six nucleotides in the *mtm-3* sequence when compared to sequence from database (www.wormbase.com). These mutations were appearing repeatedly so I decided to edit the sequence by PCR mutagenesis.

4.1.4.4. PCR mutagenesis

Two reactions were prepared for PCR mutagenesis: one with all ingredients, one without the polymerase (that served as a control). The reaction was mixed as follows:

10× Pfu buffer	5 μl
10 mM dNTP mix	1 μl
10 μM primer F	1.25 μl
10 μM primer R	1.25 μl
template DNA (250 ng/μl)	2 μl
Pfu Turbo polymerase	1 μl
H_2O	8.5 μl

Primer sequences (5'-3'):

mtm3del639-640-F	GATGAAGAAGAACATTATACGCAAATCTTCAGTCCAAAGGAG
mtm3del639-640-R	CTCCTTTGGACTGAAGATTTGCGTATAATGTTCTTCTTCATC

PCR program (18 cycles for denaturation – annealing – synthesis):

95°C	30 s	
95°C	30 s	
55°C	1 min	
68°C	6 min	(1 minute/kb of plasmid length)
12°C	-	

In the next step, the template DNA was digested by adding 1 μl of DpnI directly to the PCR reaction (DpnI has the ability to digest only methylated DNA – the substrate). The reaction was then incubated at 37°C for 1 hour. After incubation, 1.5 and 15 μl of the reaction were used for transformation of competent cells. Further sequencing confirmed succes of the mutagenesis.

4.2. Working with proteins

4.2.1. Preparation of yeast protein extracts

Protein extrats from yeasts were prepared as follows:

- One fresh colony of a yeast strain was inoculated into 5 ml of medium (either appropriate selection medium or YPAD medium for strains that do not carry any plasmids for selection) and cultivated overnight with shaking at 30°C.
- The culture was inoculated into 10 ml of a fresh medium to a final OD_{600}~0.1. After reaching OD_{600}~0.3, the culture was centrifuged (1625×g, 3 min, 23°C); sediment was resuspended in 1 ml of ddH_2O, transferred into

1.5 ml plastic tube and centrifuged again (26829×g, 30 s, RT). The supernatant was discarded and the pellet was incubated at –20°C for 15 minutes, for longer storing at –80°C.

- The pellet was resuspended in 100 µl of NaOH/β-mercaptoethanol mixture (93 µl of 2 M NaOH and 7 µl of β-mercaptoethanol), vortexed (4×30 s vortex and 4×30 s incubation on ice) and incubated for 10 minutes on ice. From this moment it was necessary to keep the lysate on ice.
- 100 µl of 50% TCA were added to the lysate, then the lysate was incubated for 5 minutes on ice and centrifuged (22860×g, 10 min, 4°C). The supernatant was discarded, the sediment was gently resuspended in 500 µl of 1 M Tris and centrifuged. The supernatant was discarded and the sediment was resuspended in 100 µl of 2× Laemmli buffer (preheated at 95°C for better solubilization of proteins). The lysate was incubated at 95°C for 5 min and after that kept at –80°C.

4.2.2. Western blotting

Western blotting was used to test whether the MTM-6 protein is being expressed in yeasts (in pGBKT7 plasmid, MTM-6 is fused with c-Myc epitope tag and therefore can be detected on WB by anti-Myc antibody). Protein lysates were prepared as described in previous chapter. Shortly before loading, samples were mixed with 1 M DTT in the amount 1/20 of sample volume and incubated at 95°C for 5 min.

- Proteins were separated by SDS-PAGE (BioRad), 5 ml of separation gel + 2 ml of stacking gel. Ingredients for gels were mixed in this order:

  For 10% separating gel (10 ml/2 gels):

dH_2O	4 ml
1.5 M Tris (pH 8.8)	2.5 ml
30% acryl-bisacrylamide mix	3.3 ml
10% SDS	100 µl
10% ammonium persulphate	100 µl
TEMED	4 µl

For 5% stacking gel (4 ml/2 gels):

dH_2O	2.7 ml
1 M Tris (pH 6.8)	0.5 ml
30% acryl-bisacrylamide mix	0.67 ml
10% SDS	40 µl
10% ammonium persulphate	40 µl
TEMED	4 µl

- First, 5 ml of the 10% gel were pipetted between two glasses of the electrophoretic apparatus and overlayed with 200 µl of isopropanol (to protect the gel prom oxygen which inhibits polymerization of the gel). After solidification, the isopropanol was removed, the gel surface was washed by dH_2O and the residual water was removed by filter paper. Then, 2 ml of the 5% gel were pipetted onto the surface of the 10% gel and a comb was inserted into the gel.
- The electrophoresis was run at 130 V for 10–15 min and then at 140 V, after the proteins focused at the interface between stacking and separating gels.
- After the separation was finished, proteins were transferred from the gel onto a nitrocellulose membrane using wet blot (BioRad; 200 mA) in ice bath. The membrane was then incubated in Amido Black Stain for 1 min to confirm succesfull transfer of proteins, washed in dH_2O and blocked in 3% BSA/TBST for 1 hour. After blocking, the membrane was transferred into 6 ml of 3% milk/TBST with anti-c-myc (Exbio) primary antibody (diluted 1000×) and incubated overnight at 4°C.
- The membrane was washed 3×10 min in TBST and transferred into 6 ml of 3% milk/TBST with secondary antibody GAM-HRP (Jackson ImmunoResearch) (diluted 10000×). After incubation at RT for 90 min, the membrane was washed again 3×10 min in TBST. For visualisation of the protein bands by luciferase assay, the membrane was incubated in 2 ml of *SuperSignal West Pico Chemiluminescent Substrate* (Thermo Scientific). Membrane was then covered with Saran foil and exposed to photographic film (AGFA).

- To compare the amount of proteins in visualized bands, second antibody staining was performed. The procedure was performed as described in the previous article. The primary antibody was anti-PSTAIRE (Sigma) (diluted 8000×), the secondary antibody GAM-HRP (diluted 10000×).

4.3. Manipulation with bacteria

4.3.1. Culturing and long-term maintaining

E. coli TOP10 and DH5α were cultivated and maintained on LB agar plates with appropriate antibiotics. Liquid LB medium was used for growing bacteria in liquid culture.

E. coli OP50 were cultivated and maintained on LB agar plates without antibiotics, *E. coli* HT115 were cultivated and maintained on LB agar plates with ampiciline and tetracycline. For growing bacteria in liquid culture, liquid LB medium without antibiotics or with ampiciline and tetracycline was used.

In the long term, bacterial strains were stored in 15% glycerol at -80°C.

4.3.2. Transformation

Bacteria were transformed on the basis of standard protocols using the heat shock method. Transformed cells were seeded onto plates with LB agar medium containing an antibiotic for selection of transformed clones (see 3.2.1 Culturing of *E. coli*).

4.4. Manipulation with yeasts

4.4.1. Culturing and long-term maintaining

S. cerevisiae GOLD and Y187 were cultivated and maintained on YPAD agar plates. For selection of transformants and their subsequent maintaining, SD medium without appropriate amino acids was used. Liquid YPAD and SD medium were used for growing yeasts in liquid culture.

In the long term, yeast strains were stored in 30% glycerol at -80°C.

4.4.2. Transformation

Yeasts were transformed on the basis of standard LiAc/SS-DNA/PEG method protocol using the heat shock method. When greater efficiency of transformation was

required (especially for transformation of library plasmids), the protocol was modified by adding 98% EtOH to the final concentration of 10% (as described in Lauermann, 1991): the EtOH was added to the mixture of cells, LiAc, ssDNA and H_2O and the mixture was then incubated for 5 min at RT; after that, PEG was added and the protocol continued normally. Transformed cells were transferred on plates with SD agar medium deficient for certain nutrients (most often leucine, tryptophan, histidine, uracil or combination of these; see chapter 3.2.2. Culturing of *S. cerevisiae*).

4.4.3. Mating

Yeast mating was used for Two-Hybrid library screening and preparing controls for Western blotting. For yeast mating, the protocol *Matchmaker™ Gold Yeast Two-Hybrid System user manual (*Clontech *Cat. No. 630489)* was used. One step in the protocol was changed: the mating culture was not incubated on a shaker (as described on page 18) but transferred onto 9 cm Petri dishes with YPAD agar medium (about 1.5 ml of yeast culture per dish). The incubation time was 6 hours at 3O°C and gave sufficient amount of mated clones.

4.4.4. Yeast Two-Hybrid Assay

Yeast Two-Hybrid assay is a molecular biology technique designated for studying protein-protein interactions. The premise for the testing is activation of a reporter gene by binding of a transcription factor on the upstream activation sequence (UAS) of that gene. The key to this method is that the binding and activating domains of most eucaryotic transcriptional factors are modular and can work without direct binding.

Y2H assay utilizes genetically modified yeast strains with defects in certain biosynthetic pathways, such as for synthesis of essential amino acids or nucleobasis. These strains fail to survive on media that lack these essential nutrients; nevertheless, they are able to accept special plasmids that compensate for these defects. This is a mechanism for selection of yeasts that have received during transformation plasmids coding a tested protein fused either to GAL4 binding domain or to activating domain.

The Y2H system that I used (Matchmaker™ Gold Yeast Two-Hybrid System provided by Clontech) enables selection on four reporter genes in order to detect protein interactions: *AUR1-C*, *HIS-3*, *ADE-2* and *MEL-1*. *AUR1-C* encodes an enzyme

that confers resistance to a toxic drug Aureobasidin A; *HIS-3* permits cells to grow on –His minimal medium; *ADE-2* permits cells to grow on –Ade minimal medium; *MEL-1* encodes enzyme that turns blue a chromogenic substrate X-α-Gal.

The Y2H assay may be used also for cDNA library screening. In that case, there are cDNA sequences cloned in the plasmid with the activation domain and the plasmid with the binding domain encodes one certain protein that is being tested for new interactions. For Y2H cDNA library screen, I used the *ADE-2* selection system: this system is based on red/white colour detection where red colonies congest an intermediate product od adenine synthesis (which is red) and do not synthesis adenine whereas white colonies have functional pathway of adenine synthesis and do not congest the intermediate product.

4.4.5. cDNA library screen in yeast

cDNA library screen in yeast is a widely used method when searching for new binding partners of a known protein. The library used in this work was provided by Dualsystems Biotech (*Caenorhabditis elegans* yeast two-hybrid cDNA library; P02105). The library was prepared from whole animals at the adult stage, complexity of the library is $5{,}7\times10^6$ independent clones.

First it was necessary to copy the library DNA, which was done in bacteria (see chapters 4.3.2. Transformation; 4.1.1. Izolation and purification of plasmid DNA). The transformation and subsequent plasmid DNA isolation was repeated for four times until sufficient amount of clones was collected – altogether approximately 5 265 000 independent bacterial clones. The Y187 yeast strain was transformed with this DNA (see chapter 4.4.2. Transformation) and approximately 966 800 independent yeast clones were collected. Cells from all Petri dishes were washed from the agar, frozen in glycerol and kept at –80°C for later use.

Next step was transformation of pGBKT7/mtm-6 plasmid into the GOLD yeast strain (the plasmid was prepared as described in the chapter 4.1.4. Preparing of plasmid constructs; transformation of the GOLD strain was performed as described in the chapter 4.4.2. Transformation). The mating was performed as described in the protocol *Matchmaker™ Gold Yeast Two-Hybrid System user manual (Cat. No. 630489)* with the change described in chapter 4.4.3. Mating. What was done differently from this protocol was using a different selection system for the first screen.

4.5. Manipulation with worms

4.5.1. Culturing and long-term maintaining

Worms are usually cultured on Petri dishes with a bacterial lawn grown on the NGM agar medium (see part 3.2.3). The optimal temperature of their growth is 20°C, but they can be kept alive also in lower temperatures where they slow down their growth. All strains were kept at 20°C to prevent changes in their QL.d migration phenotype (in our laboratory we have an experience that the *mtm-6*(ok330) strain exhibits significant difference in the QL phenotype when the culturing temperature changes from 20°C to 15°C and vice versa.

For long-term storing, *C. elegans* strains were frozen and kept at –80°C.

4.5.2. Microscopic preparations

When working with worms, there is often a need to evaluate their phenotype, for example when performing RNAi experiments. Animals were washed away from plates and pipetted onto a microscope slide with a drop of 2% agarose containing 10 mM NaN_3 (which paralyzed the animals and prevented them from moving on the slide). The phenotype evaluation was performed on a fluorescence microscope (Olympus BX40).

4.5.3. Synchronization

Some experiments require synchronized population of worms – it means that all or the majority of worms are at the same developmental stage. There are two methods how a *C. elegans* culture can be synchronized: hypochlorite treatment and washing.

For the hypochlorite treatment a fully grown plate with many gravide hermaphrodites is required. The plate was washed with M9 buffer and worms were transferred into 1.5 ml plastic eppendorf tube. The mixture of animals and buffer was then centrifuged in a microcentrifuge (30 sec, 1073×g). The pellet was resuspended in 300µl of M9 buffer and subsequently 150 µl of sodium hypochlorite (SAVO) and 5 M KOH mixture (in 2:1 ratio) was added. For larger volume of animals, bigger amounts of M9 buffer and SAVO/KOH mixture were used. The mixture was then incubated at room temperature for approximately 3-4 minutes. The key point to observe was when the adults started to disintegrate and the embryos were released.

At the point when more than half of worms were disintegrated, the embryos were centrifuged, supernatant discarded and the pellet was washed two times with M9 buffer. The embryos were transferred on a fresh agar plate with bacterial lawn, where they hatched and grew into the required stage. This protocol was also used to clean *C. elegans* stocks from bacterial or fungal contaminations. In order to reduce the risk of contamination recovery, several larvae were transferred to a fresh plate the day after the treatment.

For the synchronization by washing, a plate with many laid embryos was used. All worms were washed away and only the embryos stayed on the plate because they sticked to the bacterial lawn. After one hour the plate was washed once again to get rid of first hatched larvae and worms that could have resisted the first washing. After three hours the plate was washed and hatched larvae were centrifuged (30 sec, 603×g) and transferred to a fresh plate. This process was repeated after another three hours in order to get more animals.

The hypochlorite treatment poses a stress on the animals and therefore can influence the penetrance of the QL phenotype. The synchronization by washing is a more physiological method and easier to be done, so I used it preferentially. The population of worms is also better synchronized, which is another advantage of this method.

4.5.4. Crossing

The ability to create compound mutants is an important genetic skill for phenotype analysis and crossing is a way how to prepare these mutant animals. For the crossing it is necessary to know the mechanism of sex determination in worms, which is the XX/X0: hermaphrodites carry two X chromosomes whereas males carry only one.

As the natural representation of males in worms population is very low (on average just 0.05 %), the first step that needs to be done before crossing two different strains is to increas the number of males in the population. This could be achieved by crossing of N2 (wild type) male and hermaphrodite in the larval stage 4 (approximately six males and three hermaphrodites per plate). The advantage of worm reproduction is that hermaphrodites prefer to be fertilized by male sperms, if males are available. This enables to increase the percentage of males in progeny.

The scheme of crossing is indicated below on the example of preparing the *mtm-6*;*sel-5*;*muIs32* strain from these (homozygotic) strains:

N2	wt
KN1040	*mtm-6*(ok330);*muIs32*
RB638	*sel-5*(ok363)

preparing males:

$$\text{N2}\ ♂ \times \frac{mtm-6}{mtm-6};\frac{muIs32}{muIs32}\ ☿$$

P0 generation: crossing

$$\frac{mtm-6}{+};\frac{muIs32}{+}\ ♂ \times \textit{sel-5}\ ☿$$

F1 generation: selection of L4 larvae (~8)

$$\frac{mtm-6 \quad +}{+ \quad sel-5};\frac{muIs32}{+}\ ☿$$

After laying progeny, in the parent animal:

- the *muIs32* transgene carrying larvae are recognizable under fluorescence
- the *sel-5*(ok363) mutation is present in all worms
- the *mtm-6*(ok330) mutation is detected by genotyping

F2 generation: selection of L4 larvae (~30)

- from the progeny of animal positive for *mtm-6*(ok330)

After laying progeny, in the parent animal:

- the *muIs32* transgene carrying larvae are recognizable under fluorescence
  - homozygotic worms give stronger signal than heterozygotes and were selected preferentially
- the *sel-5*(ok363) mutation
  - homozygotic worms are detected by genotyping
- the *mtm-6*(ok330) mutation
  - homozygotic worms are detected by genotyping

F3 generation: selection of L4 larvae (~15)

- from the progeny of animal homozygotic in one mutant allele and heterozygotic in the other one

• the *muIs32* transgene carrying larvae are confirmed under fluorescence
• homozygotic worms in the *sel-5*(ok363) mutation are detected by genotyping
• homozygotic worms in the *mtm-6*(ok330) mutation are detected by genotyping

This step is not necessary to do if the previus step gave animals homozygotic in both mutant alleles and carrying the *muIs32* transgene.

4.5.5. Genotyping

Genotyping was used as a method determining the genotype of individual animals during crossing. One or several animals were transferred into 200 µl plastic PCR tubes with 10 µl of lysis buffer containing proteinase K and lysed in PCR machine (65°C/60 min – 95°C/15 min). Lysates were used immediately or stored at –20°C.

The presence of mutant allele was detected by using two sets of primers, where for example the reverse primer (R) is same for both sets and forward primer is different (F1 or F2). One forward primer is complement to 5` end of the gene before the place of mutation, the second forward primer is located into the deleted region. The reverse primer is placed to the 3` end of the gene so that the deletion is between forward and reverse primer (Fig. 8).

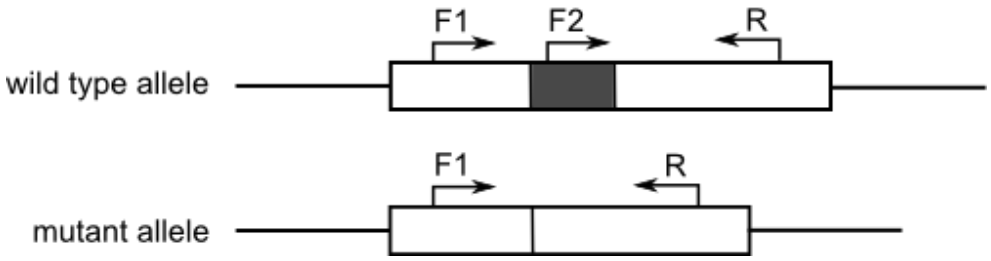


Fig. 8: **Scheme of a potential placement of primers for genotyping.**

Two separate PCR reactions were prepared (one with F1 and R primes, the second one with F2 and R primers) as described in chapter 4.1.3. PCR. The setting of PCR machine was:

94°C	2 min	
94°C	20 s	
58°C	40 s	35×
72°C	1.5 min	
12°C	-	

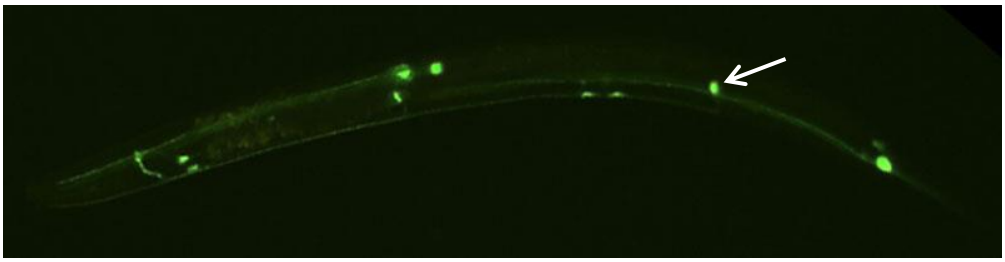
The PCR results were analyzed by gel elecrophoresis (see chapter 4.1.2. Electrophoresis in agarose gel). If a wt allele was present in the sample DNA, two bands of different size appeared on the gel. If a mutant allele was present in the sample DNA, only one band appeared on the gel.

4.5.6. Test of QL neuron`s migration

Migration of Q neuroblasts` descendants is a great tool to determine functioning of Wnt signalling. As the principle of Wnt-dependent migration is characterized in the Theoretical background (chapter 1.2. Wnt signalling in *C. elegans*), here I will describe the practical use of this phenomenon.

For observing the QL neuron`s migration, *C. elegans* strains carrying the *muIs32* transgene were used (Ch'ng et al., 2003). This transgene encodes GFP (green fluorescent protein) under a tissue specific promoter (*mec-7*), that is activated in ALM neurons, PLM neurons, and one descendant of the QL and QR neuroblasts. This enables an easy visual observation of labeled neurons which give a special pattern in wild type animals and in mutant ones (Fig. 9).

A) **wild type**



B) **Wnt loss of function**

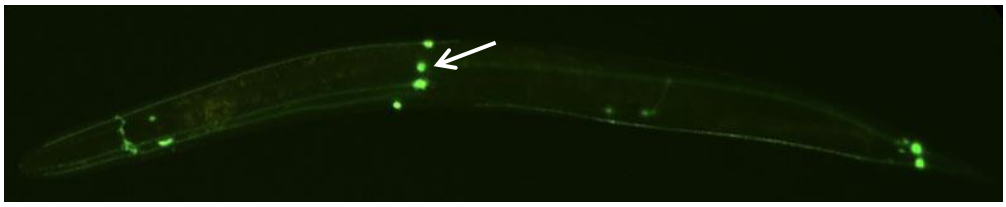


Fig. 9: **QL neuron`s migration in *C. elegans*.**

A) wild type animal: QL neuron is in the posterior third of the animal body, B) mutant animal: QL neuron migrate anteriorly. The anterior part of body is on left, posterior is on right. White arrows indicate position of the QL neuron (M. Macůrková, unpublished).

I evaluated animals with well visible QL.d and QR.d neurons. The key point in determining the QL.d neuron`s migration was their position in relation to vulva. Animals with the QL.d neuron located posteriorly and the QR.d neuron anteriorly to vulva were taken as ‘wild type‘. Animals with both neurons placed anteriorly to vulva were taken as ‘defective‘ in QL migration. In case it was not possible to determine the position of the QL.d neuron, animals were excluded from the scoring.

4.5.7. RNAi

The principle of RNAi in worms is that the animals are fed with bacteria expressing dsRNA molecules homologous to a target gene. dsRNA molecules are then released from bacteria during digestion and spread through the whole body.

RNAi experiments were performed on 6-well plates with NGM agar containing IPTG (for preparation see section 3.2.3) that induces the expression of RNA strands in bacteria. Individual bacterial clones from *C. elegans* genomic library (Kamath et al., 2003) were plated on two separate wells and grown overnight at laboratory temperature. The next day, 4-10 worms in the L4 stage (synchronized by washing) were transferred to each well. When their progeny reached at least the stage L2 (optimally L4), which took about 4 days, population of each doublet was mixed together and used for QL phenotype scoring. (A schematic representation of the RNAi method is shown in Fig. 10)

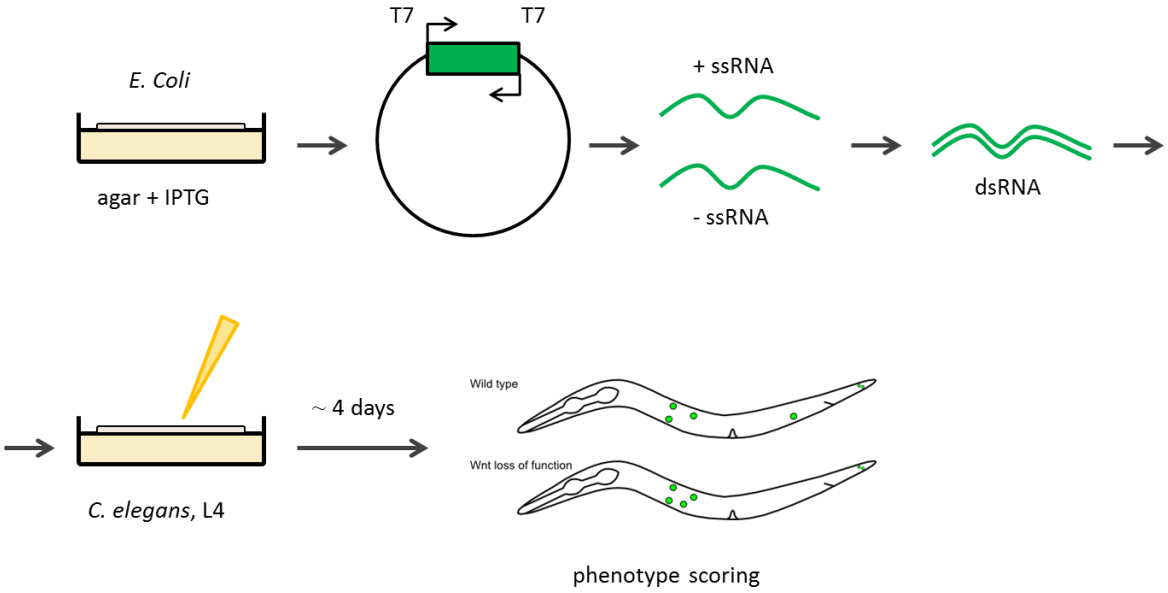


Fig. 10: **A schematic representation of the RNAi method.**

Bacteria from genomic library (Kamath et al., 2003) are plated onto agar plates with IPTG that induces the expression from T7 promoters. This results into synthesis of dsRNA strands. Simultaneously, worms in the L4 larval stage – which is an important stage just before adulthood – are placed onto plates with these bacteria. In the intestine, digested bacteria release dsRNAs which get into all body cells, incuding germ cells. From the very beginning of the embryonal development of the L4 progeny, the RNAi influences phenotype of newly born individuals.

5. Results

5.1. Interactions between lipid kinases and phosphatases

C. elegans MTM-6 regulates recycling of the Wnt cargo receptor Wls/MIG-14, presumably by regulating the levels of PI(3)P on endosomal membrane (Silhankova et al., 2010). However, the mechanism of MTM-6 functioning on endosomes and factors regulating MTM-6 activity are currently not clear. I decided to test a possible model of regulation of the endosomal membrane lipid composition. In mammalian cells, hVps34/hVps15 protein complex (activated by Rab7~GTP) phosphorylates phosphatidylinositols on the endosomal membrane and therefore enables binding of recruited proteins, such as retromer, on the endosomal membrane. MTM1 and MTMR2 are mammalian phosphatases that dephoshorylate PI(3)P and PI(3,5)P_2 and are important for establishing the homeostasis of lipid composition on the endosomal membrane. hVps15 binds Rab7 or MTMR2 to form an active or inactive complex, respectively (Cao et al., 2008, 2007) (for more details see chapter 1.4.2. Regulation of the endosomal membrane lipid composition by myotubularin phosphatases). I wanted to determine whether a similar model as published for mammalian cells might work also in *C. elegans* during myotubularin-dependent Wls trafficking. It was previously shown that knock-out of *mtm-6* leads to an increase of the QL phenotype, which reflects disruption of the Wnt signalling pathway. Moreover, when *mtm-6* mutant animals were treated with RNAi against *vps-34* or *ZK930.1* (which is a *C. elegans* homolog of hVps15), the QL phenotype was significantly rescued (Silhankova et al., 2010). Considering the published data, I chose several candidate proteins to be tested for interactions by the Yeast Two-Hybrid Assay: five myotubularins (MTM-1, MTM-3, MTM-5, MTM-6 and MTM-9), two Rab GTPases (RAB-5 and RAB-7) and two subunits of lipid kinase complex (VPS-34 and ZK930.1).

Two sets of plasmids were prepared for the Y2H assay (as described in Methods, chapter 4.1.4. Preparing of plasmid constructs). cDNA of all previously described genes was cloned into pPC86 and pPC97 plasmids carrying the GAL4 activating and DNA binding domain, respectively. Combinations of prepared pPC86 and pPC97 plasmids were transformed together into the GOLD yeast strain. Yeasts were then seeded on SD –Leu/–Trp agar medium and five clones from each combination were tested for interaction on SD –Leu/–Trp/–His agar medium (Fig. 11).

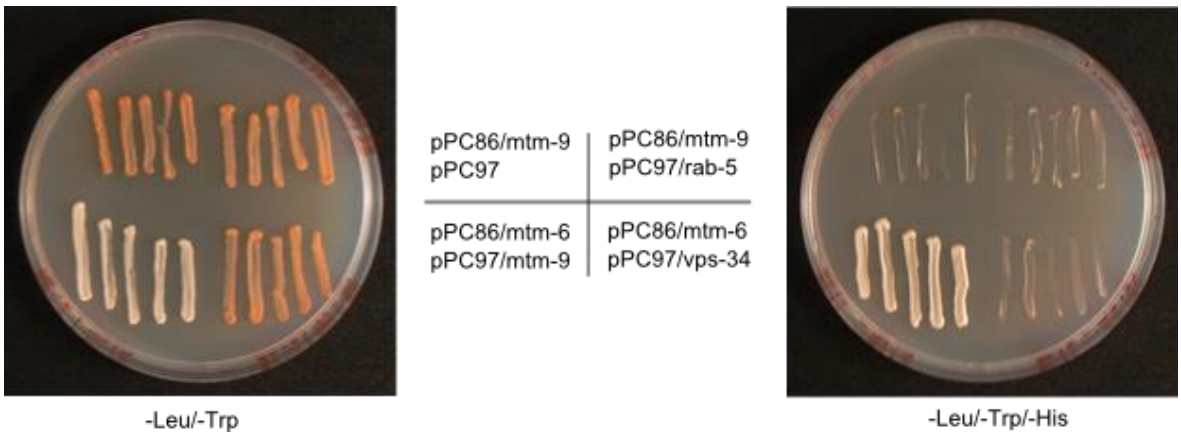


Fig. 11: **Verification of the interaction between MTM-6 and MTM-9 proteins in the *S. cerevisiae* Y2H analysis.**

This figure shows an example of how the *S. cerevisiae* GOLD strain grows on SD –Leu/–Trp or SD –Leu/–Trp/–His agar medium. There are strains with four different combinations of pPC86 and pPC97 plasmids. In the lower left corner of both dishes there is the positive control combination pPC86/mtm-6 + pPC97/mtm-9 where yeasts grow on the SD –Leu/–Trp/–His agar medium and have got a white colour. An example of yeast strain expressing proteins that seem to not interact is pPC86/mtm-9 + pPC97/rab-5 in the upper right corner (yeasts do not grow on the SD –Leu/–Trp/–His agar medium because the transcription of *his-3* gene is not activated).

The results of the Y2H assay are summarized in Fig. 12. It is clear that this experiment did not reveal any new interactions (possible reasons for these results are discussed later). The interaction between MTM-6 and MTM-9 was previously published in literature (Dang et al., 2004) and in this combination served as a positive control for the whole experiment. The MTM-3 protein seems to exhibit a certain degree of autoactivation because clones with pPC97/mtm-3 plasmid grew on SD agar medium –Leu/–Trp/–His (data not shown). Nevertheless, the colour of colonies was not white (as for MTM-6 and MTM-9 control), but red. In addition, the reverse combination of plasmids and inserts did not activate reporter gene for histidine synthesis. From these observations it can be concluded that the MTM-3 protein autoactivates the *his-3* reporter gene in this Y2H system.

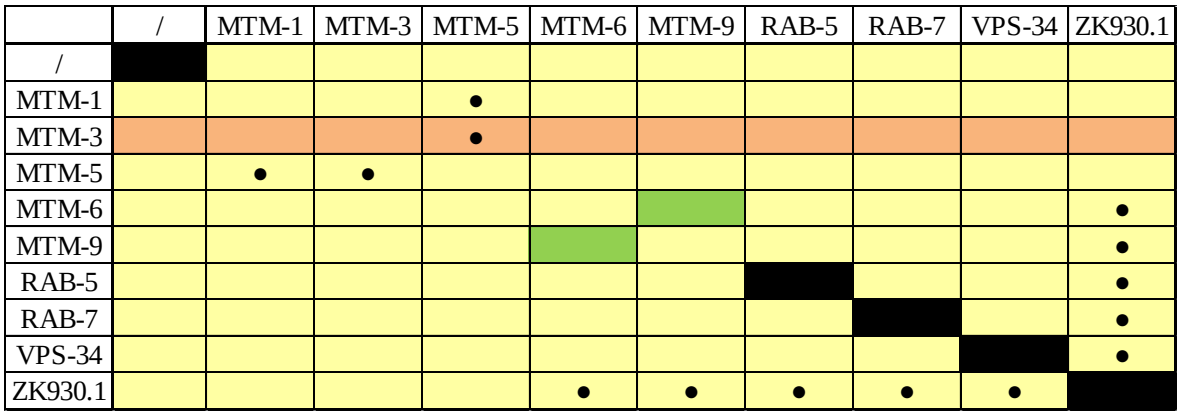


Fig. 12: **Yeast Two-Hybrid Assay results.**

In the horizontal line there are inserts cloned in pPC86 plasmid (activating domain), in the vertical line there are inserts cloned in pPC97 plasmid (DNA binding domain). Colours indicate the presence or absence of an interaction (green: interaction was confirmed; yellow: interaction was not confirmed) and presumable autoactivation (orange); black color stands for interactions that were not tested. Black dots indicate interactions that were predicted based on published literature.

5.2. Searching for new binding partners of MTM-6

As the candidate approach did not reveal any MTM-6 interacting proteins I decided to use an unbiased approach to look for new binding partners of MTM-6 which might bring more light into the mechanism of MTM-6 functioning. For this purpose I chose the Y2H cDNA library screen as a suitable method. The *mtm-6* cDNA was cloned in the pGBKT7 plasmid which expressed MTM-6::GAL4-BD protein. The cDNA library was provided in the pGAD-HA plasmid (for more details see chapter 4.4.5. cDNA library screen in yeast).

5.2.1. cDNA library complexity verification

Before starting with library screening, it is advisable to check the complexity of the library (which should be 95%). In this case, a sample of the library was transformed into bacteria, plasmid DNA from twelve random bacterial clones was digested with HindIII restrictase and the digested mixture was analyzed by gel electrophoresis (Fig. 13). Two almost same reaction mixtures were prepared for each plasmid – the difference was that only one contained the restrictase. In Fig. 13 there are twelve pairs of columns, each pair represents two reaction mixtures: one column shows the mixture without HindIII (–) and the another one shows the mixture with

HindIII (+). HindIII cuts the pGAD-HA plasmid at two sites (position 1480 and 2350) that are close to the multiple cloning site (MCS) and each is on the other side from the MCS. The result of digestion gives two or more bands on the gel where the band size 7188 bp represents the pGAD-HA plasmid and the other bands indicate presence of an insert. According to the electrophoretic analysis, the cDNA library seems to be suitable for a screen.

5.2.2. cDNA library screen

Yeast strain GOLD (carrying pGBKT7/mtm-6 plasmid) was mated with Y187 strain (carrying *C. elegans* cDNA library plasmids). When the mated yeast culture was seeded on Petri dishes with SD –Leu/–Trp/–His selection medium it was supposed that only clones carrying both plasmids (selection for Leu and Trp) whose protein products bind each other (selection for His) would survive. Nevertheless, I observed growth of red colonies (hundreds on each of eight 18 cm Petri dishes). As the red colour indicates a problem in synthesis of adenin and therefore possible false positive results, 26 randomly chosen clones were transferred on SD –Leu/–Trp/–Ade selection medium (*ADE-2* is one of target genes to test the protein-protein interaction in this Y2H system; see Methods, chapter 4.4.4. Yeast Two-Hybrid Assay). No clones survived on the SD –Leu/–Trp/–Ade medium so it is very probable that they really were false positives. Nevertheless, the positive control GOLD×Y187[pPC86/mtm-6; pPC97/mtm-9] did survive the transfer and grew as white colonies. To conclude, no

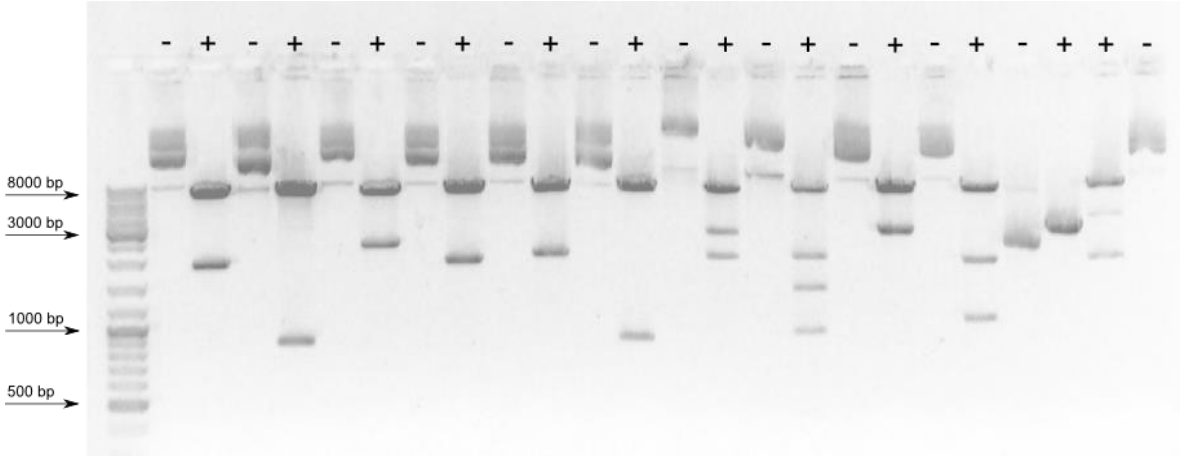


Fig. 13: **Verification of cDNA library complexity – digest of randomly chosen clones by HindIII.**

positive clones with proteins interacting with MTM-6 were found in the cDNA library screen.

5.2.3. Troubleshooting

As I had a serious difficulty with finding yeast clones carrying plasmids expressing MTM-6 and a protein that would interact with MTM-6, I decided to perform several control experiments to find out where might be a problem. MTM-6 is known to interact with the MTM-9 phosphatase (Dang et al., 2004, Silhankova et al., 2010; see above) so one would expect to uncover at least this interaction in the Y2H library screen. *mtm-9* gene is therefore a good positive control for testing whether the whole system works well (as for the Y2H analysis).

Is MTM-9 present in cDNA library?

First, in order to interact, *mtm-9* cDNA has to be present in the library. I was not sure whether the plasmid with *mtm-9* cDNA remained in the library after copying it, so the first control experiment was PCR of the original cDNA library and its copies. Two reactions were prepared for each sample DNA which differed in used primers: the first reaction contained one primer in the plasmid sequence (pGAD-HAF) and one in the last exon of *mtm-9* (mtm9ex5R), the second reaction contained one primer in exon 3 of *mtm-9* (mtm9ex3F) and one in the last exon of *mtm-9* (mtm9ex5R). The presence of *mtm-9* in both, the original library and its copies, was proved by agarose gel electrophoresis of these PCR reactions (Fig. 14). The presence of multiple bands in the first combination of primers is likely due to presence of shorter than full length variations of *mtm-9* cDNA sequence in the Y2H cDNA library. I concluded that *mtm-9* cDNA sequence is present in the original library and its copies.

How many clones are sufficient for screen?

Next, it was possible that not enough clones were screened to find an interaction. Therefore I performed an experiment to identify how many clones are sufficient for testing in order to find a certain interaction, in this case between MTM-6 and MTM-9.

A frozen sample of DH5α bacteria that were previously transformed with the cDNA library was cleaned from glycerol and seeded in several dilutions on Petri

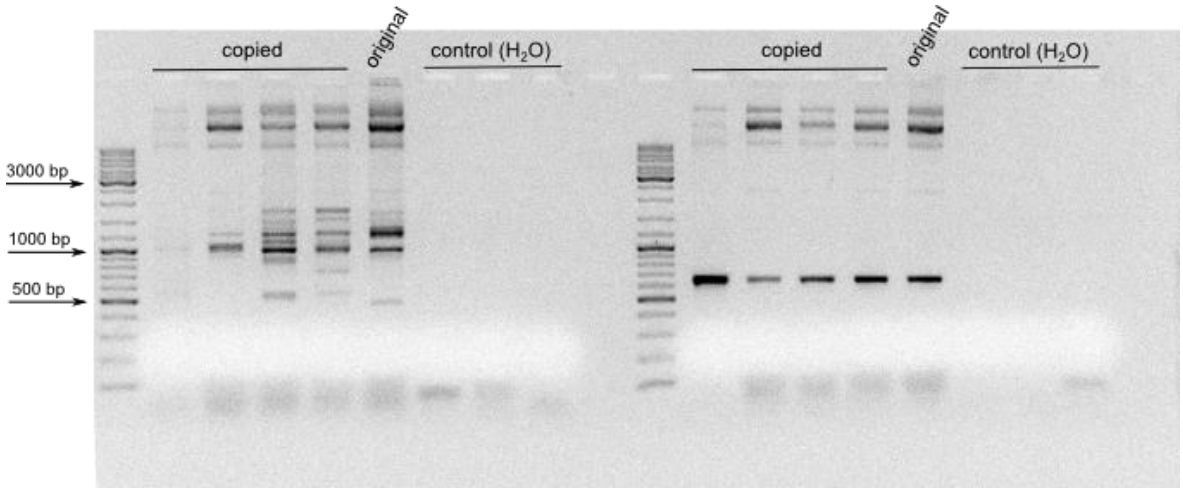


Fig. 14: **Verification of the *mtm-9* presence in the cDNA library.**
The left half of the figure shows reactions with primers pGAD-HAF and mtm9ex5R (product size ~ 1800 bp); the right half of the figure shows reactions with primers mtm9ex3F and mtm9ex5R (product size 652 bp).

dishes with LB/Amp agar medium. Different numbers of clones grew on each dish. Bacteria were washed from dishes, their plasmid DNA was isolated and tested by PCR for presence of the *mtm-9* sequence. Again, two reactions were prepared which differed in primers as described above. The agarose gel electrophoresis shows that *mtm-9* is still present in a library sample isolated from 1 675 000 and 335 000 clones (Fig. 15). As almost 1 000 000 yeast clones were screened, it is very probable that *mtm-9* would have been found among them.

Testing of MTM-6 protein expression

I also decided to test whether the MTM-6 protein is even expressed in yeast. Protein extracts were prepared from three different yeast strains: GOLD [pGBKT7/mtm-6], Y187×GOLD [pGBKT7/mtm-6;pGADT7] and Y187×GOLD [pPC86;pPC97]. MTM-6 is expressed as a fused protein with Myc tag, therefore the presence of MTM-6 protein was detected by antibody against Myc tag (mouse monoclonal anti-c-myc; Exbio). As shown in Fig. 16, MTM-6 fusion protein is expressed in both strains carrying the pGBKT7/mtm-6 construct.

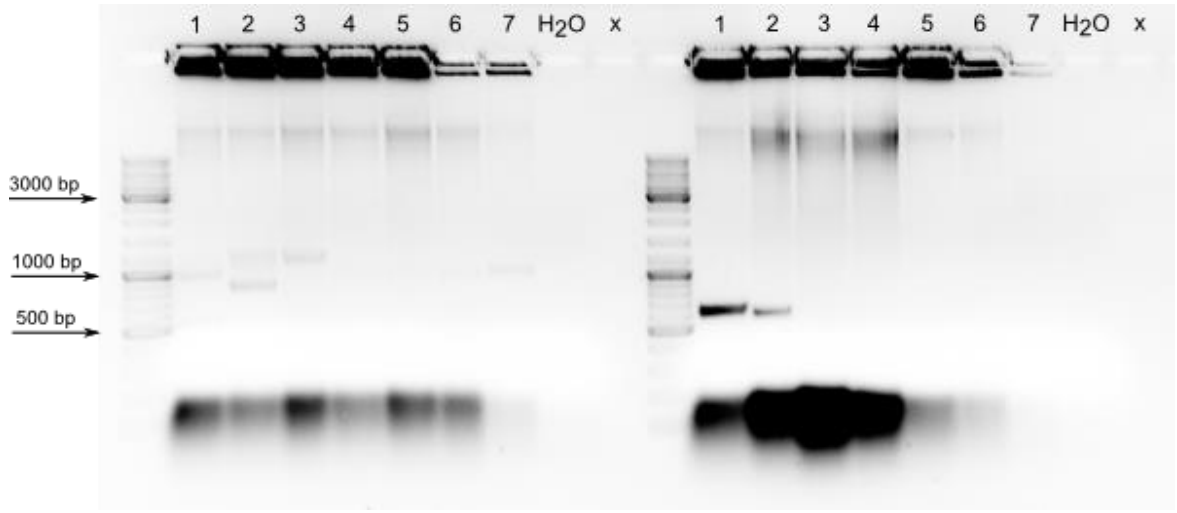


Fig. 15: **PCR analysis of diluted cDNA library.**

left half of the figure: primers pGAD-HAF and mtm9ex5R (product size ~ 1800 bp)

right half of the figure: primers mtm9ex3F and mtm9ex5R (product size 652 bp)

Number of clones that grew from diluted samples: 1) 1675000; 2) 335000; 3) 67000; 4) 13400; 5) 6700; 6) 2700; 7) 540. H_2O served as a negative control.

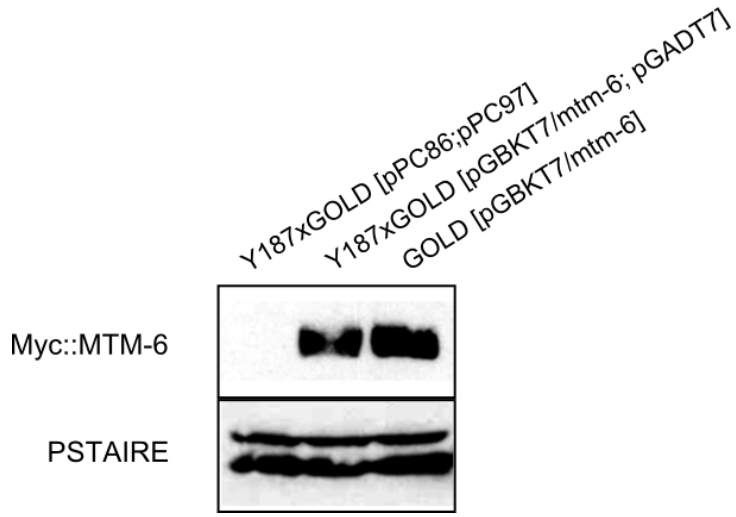


Fig. 16: **MTM-6 expression in yeast.**

The first line shows expression of Myc::MTM-6 phusion protein in GOLD strain and GOLD×Y187 crossed strain, both carrying the pGBKT7/mtm-6 plasmid. The control GOLD×Y187 strain carrying empty pPC86 and pPC97 plasmids does not exhibit expression of Myc::MTM-6.

5.3. Searching for genetic interactions of *mtm-6*

Since the Y2H experiments did not reveal any MTM-6 interacting partners, I decided to use an alternative approach to better understand the role of *mtm-6* in Wnt signalling. I switched to the *C. elegans* model system and searched for genetic interactions of *mtm-6* by RNAi. I made use of the fact that a genome-wide RNAi screen for novel Wnt pathway regulators was recently performed in the lab resulting in a list of candidate genes. The screen was performed on *C. elegans* strain mutated in the *vps-29* gene, which encodes a retromer subunit VPS-29 (Seaman et al., 1998). Both VPS-29 and MTM-6 are important regulators of MIG-14/Wls trafficking in *C. elegans* and they act at a similar level of the trafficking route. Therefore it was interesting to test whether *vps-29* and *mtm-6* genes exhibit similar genetic interactions with genes coding proteins that could be involved in Wnt signalling regulation. Although this approach may not necessarily reveal direct interacting partners of MTM-6, it could still bring important insight into the mechanisms of Wnt pathway regulations dependent on MTM-6.

5.3.1. QL.d migration defect in *mtm-6* mutants

One process that is regulated by Wnt signalling in *C. elegans* is the migration of Q neuroblasts progeny. QL and QR are two neuroblasts born at similar positions on left and right lateral sides of the *C. elegans* body. Their migration directions are influenced by EGL-20/Wnt which activates a canonical Wnt signalling pathway that leads to expression of *mab-5* gene. In QL and its progeny, *mab-5* changes the default migration direction and directs QL.d migration to the posterior. QR and its progeny are not sensitive to EGL-20/Wnt signalling and therefore QR.d migrate to the anterior (Silhankova and Korswagen, 2007).

The principle of the so called QL migration assay is that the *muIs32* transgene (Ch'ng et al., 2003) carrying animals express GFP in one QL and one QR descendant. Therefore it is possible to visualize their migration directions and score the penetrance of the QL.d migration defect (see chapter 4.5.6. Test of QL neuron`s migration) in population. In wild type animals, QR.d neuron migrates anteriorly from vulva and the QL.d neuron stays more or less at the same position. In case the QL.d neuron also migrates anteriorly to vulva, the animal exhibits the so called QL.d

migration defect. For the purpose of this thesis, the QL.d migration defect will be referred to as the QL phenotype (summarized in Silhankova and Korswagen, 2007).

It has been reported recently that MTM-6 is a regulator of MIG-14/Wls trafficking in *C. elegans* (Silhankova et al., 2010). It is therefore logical that *mtm-6* mutants have problems in Wnt signalling. This is confirmed by the fact that the penetrance of the QL phenotype is about 50% in the *mtm-6*;*muIs32* animals. This number was gained during RNAi experiments when worms were fed on HT115 bacteria expressing mock plasmid L4440 (plasmid for RNAi with no insert). The L4440 plasmid containing bacteria served as negative control and therefore it was possible to determine the influence of RNAi against other genes on the QL phenotype.

5.3.2. Genetic interactions of *mtm-6*

In order to search for genetic enhancers and supressors of *mtm-6*, RNAi experiments on *mtm-6* mutant animals were performed (strain *mtm-6*;*muIs32*). In total, 26 genes were selected to be tested for their influence on Wnt signalling based on their function, intracellular localization and genetic interaction with *vps-29* (Tab. 5). The QL phenotype was scored on the animals treated with RNAi. *mtm-6*;*muIs32* animals exposed to bacteria containing the L4440 plasmid served as a negative control (see above), RNAi against *snx-3* was used as a positive control. The *snx-3* gene encodes a member of the sorting nexin family. SNX-3 was found to mediate the MIG-14/Wls trafficking in *C. elegans* by recruiting the cargo-loading subcomplex of retromer to endosomal membranes containing PI(3)P. Together with the retromer complex, SNX-3 functions in recycling of MIG-14/Wls from endosomes and is therefore essential for Wnt secretion (Harterink et al., 2011; Silhankova et al., 2010). The results of the RNAi experiments are summarized in Fig. 17. Apart from the QL phenotype I also observed embryonic lethality with *smo-1* and *trr-1* RNAi and partial embryonic and larval lethality with RNAi against *cap-1*, *cogc-4* and *ppk-1* genes.

Table 5. **List of genes that were tested by RNAi for genetic interaction with MTM-6.**

name	protein function	reference
C42C1.4	vps8-like (in *S. cerevisiae*: prevacuoles to GA transport)	Chen and Stevens, 1996
cap-1	α subunit of actin capping protein	Waddle et al., 1993
cap-2	β subunit of actin capping protein	Waddle et al., 1993
cogc-4	subunit of COG complex (glycosylation in GA)	Kubota et al., 2006
csnk-1	casein kinase 1; negative regulator of PPK-1	Panbianco et al., 2008
dab-1	adaptor protein; endocytosis, trafficking	Holmes, 2007
dyf-3	ortholog of human CLUAP1; cilium morphogenesis	Murayama et al., 2005
F08A8.5	alpha-1,2-fucosyltransferase	Zheng et al., 2008
farl-11	FAR11-like; inhibitor of DHC-1 (dynein heavy chain)	O'Rourke et al., 2007
K04G7.1	genetic interaction with *bar-1* (β-catenin)	Byrne et al., 2007
mon-2	Arf GEF-like; endosome to GA retrograde transport	Kanamori et el., 2008
plc-3	phospholipase C gamma homolog; IP_3 signalling	Yin et al., 2004
plx-2	plexin, transmembrane receptor of semaphorin	Nakao et al., 2007
ppk-1	putative PI(4)P 5`kinase	Weinkove et al., 2008
rab-8	Ras GTPase; clathrin-dependent trafficking	Kaplan et al., 2010
rack-1	receptor of activated C kinase; cytokinesis	Ai et el., 2009
rbbp-5	histone H3 lysine 4 methylation	Li and Kelly, 2011
rpn-12	subunit of proteasom complex	Davy et al., 2001
sel-5	AP2 kinase; LIN-12/Notch-mediated signaling	Fares and Greenwald, 1999
smo-1	ortholog of SUMO	Bhalla et al., 2008
snx-3	sorting nexin; retromer subunit	Silhankova et al., 2010
T25B9.10	uncharacterized protein	
usp-5	ubiquitin specific protease	Kahn et al., 2008
tes-1	asymmetrical division, similar to human TESTIN	Coutts et al., 2003
trr-1	similar to mammalian TRRAP	Ceol and Horvitz, 2004
wht-3	ATP-binding cassette (ABC) transporter	Sundaram et al., 2006
Y18D10A.11	uncharacterized protein	

Information about individual genes were collected from www.wormbase.org and listed references.

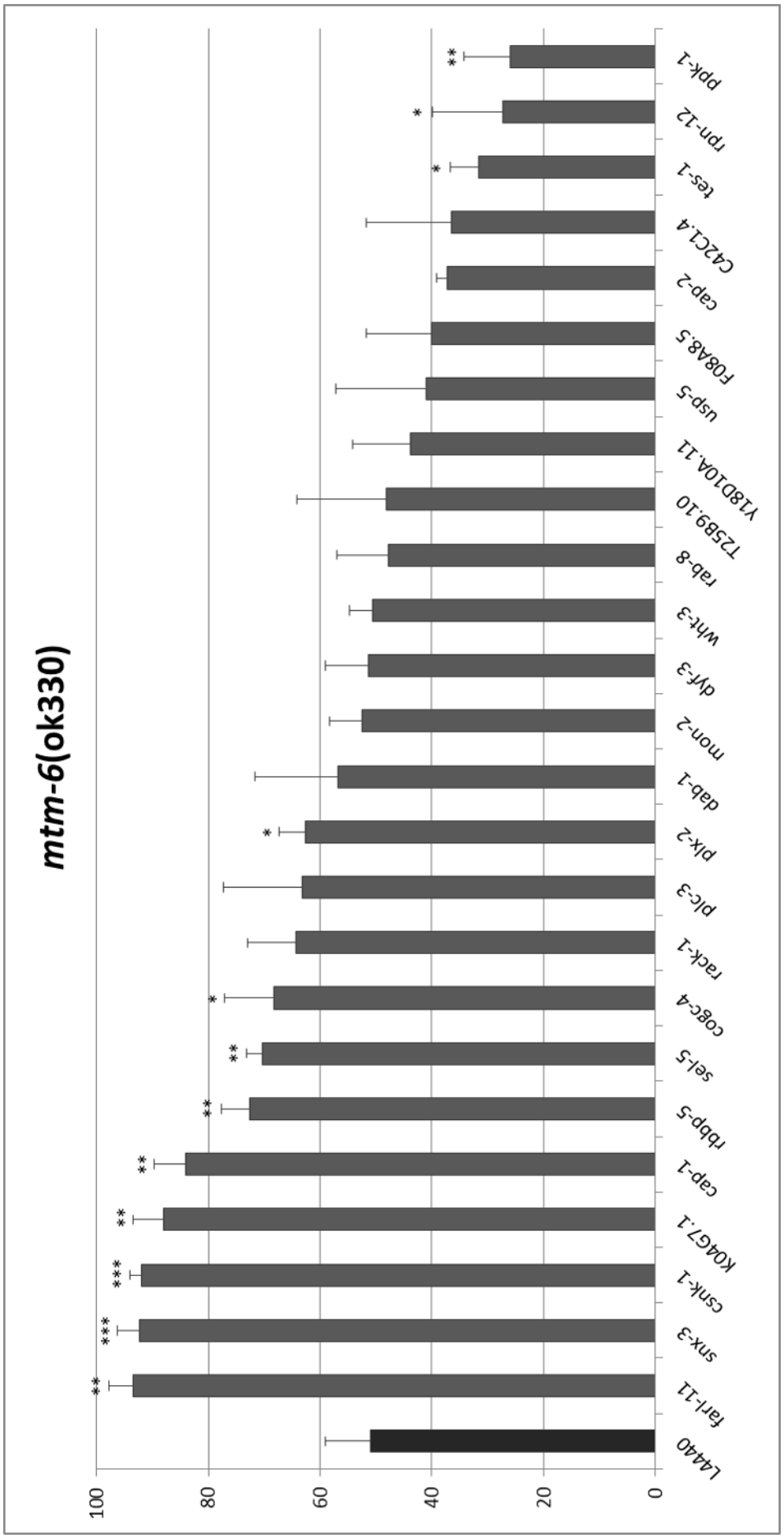

mtm-6(ok330)
L4440
farl-11
snx-3
csnk-1
K04G7.1
cap-1
rbbp-5
sel-5
cogc-4
rack-1
plc-3
pik-2
dab-1
mon-2
dyf-3
wht-3
rab-8
T25B9.10
Y18D10A.11
usp-5
F08A8.5
cap-2
C42C1.4
tes-1
rpn-12
ppk-1
0
20
40
60
80
100

Fig. 17: **List of genes tested for influencing the QL phenotype in *mtm-6;muIs32* strain.**

Bars represent mean values of three or four independent experiments (except for snx-3 which represents data from all experiments with *mtm-6*;*muIs32* strain – 9 in total); error bars represent standard deviations. Total number of tested animals was in all experiments more than 120.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; unpaired *t*-test (compared to L4440).

The RNAi experiments in the *mtm-6* mutant strain revealed that, unlike in the *vps-29* mutant strain, some of these genes did not change the QL phenotype at all or even decreased the phenotype to values lower than mock control (Fig. 17). The most surprising result was the penetrance of the QL phenotype in *mtm-6* mutant animals treated with RNAi against *tes-1*, *rpn-12* and *ppk-1*. All these three genes exhibit statistically significant decrease of the penetrance of the QL phenotype in *mtm-6* mutant animals compared to *vps-29* mutant animals, where the effect is opposite (M. Macůrková, unpublished data). On the other hand, *plx-2* significantly increases the penetrance of the QL phenotype in *mtm-6* mutant animals whereas in *vps-29* mutant animals the phenotype is decreased. Finally, there are two genes, *rab-8* and *mon-2*, that both significantly increase the penetrance of the QL phenotype in in *vps-29* mutants but have no effect on the phenotype in *mtm-6* mutants. These results suggest interesting information about function of these genes. Possible function of some of these genes is discussed later.

From these RNAi experiments in *mtm-6* mutant strain, eight most interesting genes were selected for further testing (see next chapter 5.3.3. Genetic interactions of *cup-4*); the criterium for selection was the function or intracellular localization of their protein products. All of the selected genes were either distinctly increasing or decreasing the penetrance of the QL phenotype in *mtm-6* mutant strain which indicates that their products could be involved in the Wnt signalling or its regulation.

5.3.3. Genetic interactions of *cup-4*

To further verify the significance of these genetic interactions, these genes were subsequently tested for genetic interaction with *cup-4* (Fig. 18). *cup-4* is required in *C. elegans* coelomocytes for efficient endocytosis of fluids. The *cup-4* gene encodes CUP-4 protein, a membrane protein with homology to mammalian nicotinic acetylcholine receptors. CUP-4 localizes to the plasma membrane, endosomes and GA and depletion of CUP-4 leads to blocking of endocytosis in coelomocytes (Patton et al.,

2005). There is a low defect in QL.d migration in *cup-4* mutants; however, in *mtm-6;cup-4* double mutants the penetrance of the QL phenotype is almost 100% and therefore it was suggested that CUP-4 and MTM-6 cooperate in the regulation of QL.d migration (Silhankova et al., 2010). RNAi experiments were performed on *cup-4*(ok837);*muIs32* animals. Control RNAi experiments with these genes were also performed on animals carrying only the *muIs32* transgene (that are taken as wild type). *cap-1* exhibited a very low penetrance of the QL phenotype (4% ± 0.7), none of the other genes exhibited any QL phenotype at all (data not shown).

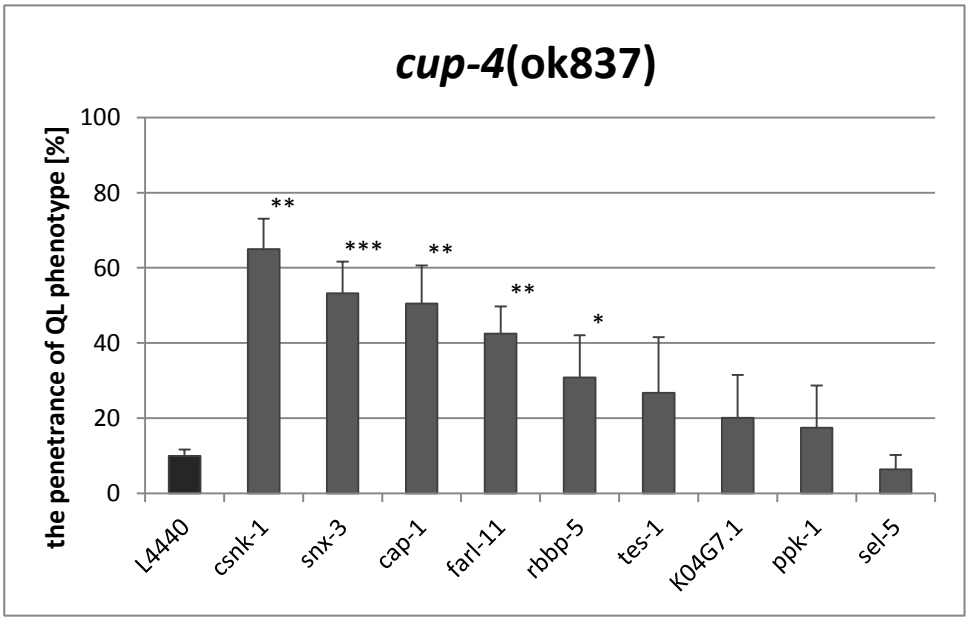


Fig. 18: **Candidate genes for Wnt signalling regulation tested in *cup-4* mutant strain.**
The graph shows RNAi in *cup-4* mutants. About half of genes exhibit similar trends in affecting the QL.d migration when compared to RNAi in *mtm-6* mutants; *K04G7.1*, *sel-5*, *tes-1* and *ppk-1* act differently. Bars represent mean values of three or four independent experiments; error bars represent standard deviations. Total number of tested animals was in all experiments more than 120.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; unpaired *t*-test (compared to L4440).

5.3.4. Verification of RNAi experiment on *mtm-6;sel-5* double mutants

It is known that organisms with gene knock-down do not necessarily exhibit exactly the same phenotype as organisms with gene knock-out. Therefore it is reasonable to verify results from RNAi experiments on real knock-out animals.

Unfortunately, many of the genes that were tested have no viable mutants available and therefore this confirmation could not be performed.

The most interesting gene from those who have available mutant strains in *C. elegans* is *sel-5*. To test whether the *sel-5* knock-out would recapitulate the phenotype seen with *sel-5* RNAi, *sel-5* mutant strain was crossed with *mtm-6* mutant strain to give *mtm-6;sel-5;muIs32* strain (see chapter 4.5.4. Crossing). The penetrance of the QL phenotype was counted for the *mtm-6;sel-5;muIs32* strain and compared to single mutants *mtm-6;muIs32* and *sel-5;muIs32* (Fig. 19). Unexpectedly, the penetrance of the QL phenotype in *mtm-6;sel-5;muIs32* strain was distinctly decreased compared to *mtm-6;muIs32* control whereas the penetrance of the QL phenotype in *mtm-6;muIs32* strain treated with *sel-5* RNAi was distinctly increased (see Discussion for possible explanation).

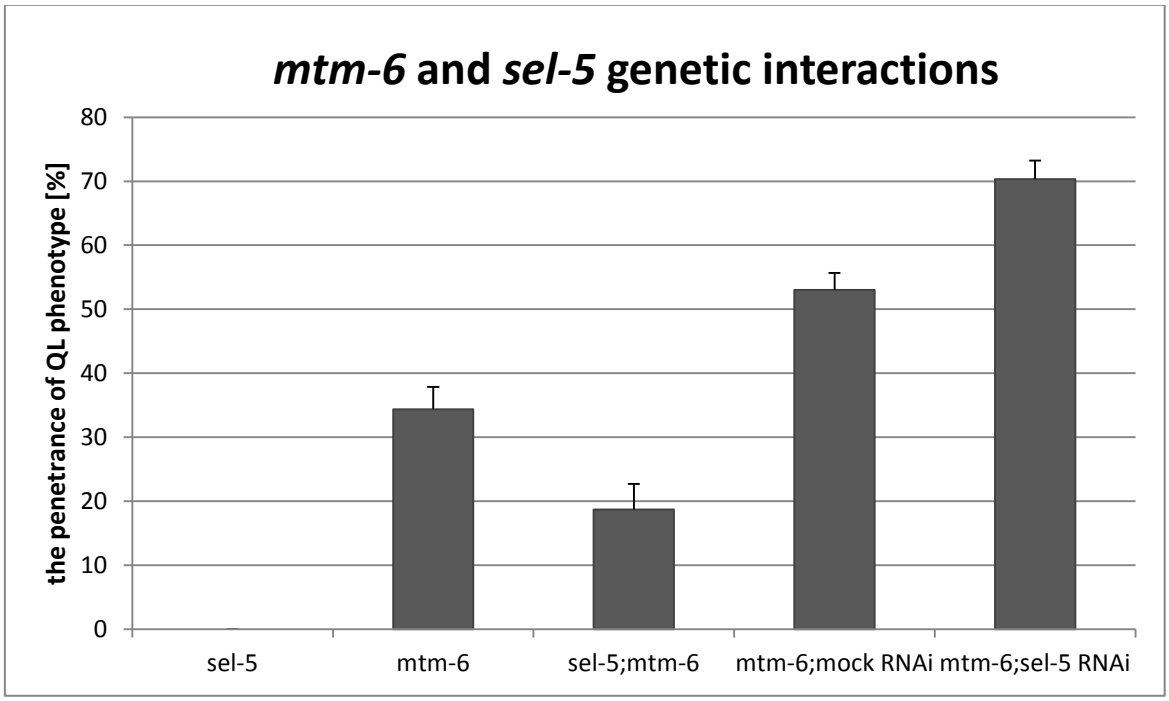


Fig. 19: **QL.d migration defect in *mtm-6;sel-5* double mutant *C. elegans* strain.**

The figure shows the penetrance of the QL phenotype in *sel-5* and *mtm-6* single mutants and *mtm-6;sel-5* double mutant. For better comparison, previous results from RNAi experiments are shown in the right two columns. The different QL phenotype in *mtm-6* strain and *mtm-6* strain treated with mock RNAi is due to using different bacteria for both experiments (OP50 for standard cultivation, HT115 for RNAi experiments) – these strains differently affect several aspects of *C. elegans* biology, e.g. metabolism or lifespan (Reinke et al., 2010) and also Wnt signalling (M. Macůrková, unpublished).

6. Discussion

In this work I have attempted to bring new insight into the regulation of the Wnt signalling pathway in *C. elegans*, more specifically to the step dependent on the myotubularin-related lipid phosphatase MTM-6. The two main experimental approaches were to search for MTM-6 protein interactions by yeast two-hybrid technique and to search for genetic interactions of *mtm-6* by RNA interference in *C. elegans*. The outcomes of my research are discussed below.

6.1. Regulation of the endosomal membrane lipid composition

I wanted to test a model that would describe the mechanism of regulation of the endosomal membrane lipid composition and the regulation of MTM-6 in this process. A hypothesis was postulated that the *C. elegans* lipid kinase complex VPS-34/ZK930.1 and the phosphatase complex MTM-6/MTM-9 could work like the mammalian hVps34/hVps15 complex and MTM1 and MTMR2 proteins (see chapter 1.4.2. Regulation of the endosomal membrane lipid composition). It means that the VPS-34/ZK930.1 kinase complex would be first activated by RAB GTPase which is then displaced by the MTM-6/MTM-9 phosphatase complex. This mutual interaction would then inhibit the enzymatic activity of both complexes. Unfortunately, I did not detect any interactions between the kinase and phosphatase complex components, nor there was any detectable interaction between the kinase complex and Rab GTPases. It is therefore not possible to declare whether this model could work in *C. elegans*.

Although many interactions among the candidate proteins were predicted as very likely, among them the interaction between the two subunits of the kinase complex VPS-34 and ZK930.1/Vps15, none of them was confirmed by the Y2H assay (see Results, 5.1. Interactions between lipid kinases and phosphatases). There are several explanations for this result. In general, Y2H assays are prone to give false negative results. The specific reasons may be as follows:

- It is possible that tested proteins actually do not interact - the mechanism on the endosomal membrane in *C. elegans* might be different from the mechanism in mammals.

- Proteins may not be able to interact because they are not properly posttranslationally modified; there could be an important difference in posttranslational modification system in *S. cerevisiae* compared to *C. elegans* which would prevent the proteins from being properly modified.
- All tested proteins are normally cytosolic and the mammalian homologs bind each other while being associated with the endosomal membrane. They may therefore have a problem to interact inside the cell nucleus, which is a very different environment from the cytosol and in addition, there is not the possibility to associate with a membrane similar to the endosomal membrane.

6.2. Searching for new binding partners of MTM-6

I also wanted to find new binding partners of the MTM-6 protein to gain insight into the mechanisms regulating MTM-6 activity. Although MTM-6 was correctly expressed in the mated yeast strain GOLD×Y187 I was not succesfull in finding any new interacting partners of MTM-6. Unfortunately, I was not able to recover even the already known partner MTM-9, although the *mtm-9* cDNA was present in the cDNA library. The impossibility of finding the control interaction may be due to presence of *mtm-9* cDNA with partially shorten N-terminus which would prevent MTM-9 from binding MTM-6.

The reason for these negative results are not clear. Some steps during the cDNA library screen could be done differently and a change in some parts of the protocol would possibly lead to more satisfactory results. For example, it might be better to perform the first selection of mated yeasts as suggested in the protocol supplied by the manufacturer (which means to use the SD –Leu/–Trp/X-α-Gal/AbA agar medium for the first selection instead of the SD –Leu/–Trp/–His agar medium). Nevertheless, the experiment was designed as it is for financial reasons and after discovering the problems it was not possible to repeat the whole screen due to lack of time.

6.3. Placement of tested genes into the Wnt producing cell

On the basis of results that came from the genome-wide screen performed on *C. elegans vps-29* mutants, I chose 26 candidate genes for further testing on genetic

interactions with *mtm-6*. From this primary set of genes, eight were subsequently selected for testing on genetic interactions with *cup-4*.

According to their function and intracellular localization, most proteins coded by genes that were tested by the RNAi method (see chapter Results, Tab. 5) could be roughly placed into individual processes that happen in Wnt producing cell or into distinct compartments within the cell. Proteins that are not discussed further are those that cause embryonal lethality during RNAi experiments performed on *mtm-6* mutants (*smo-1*, *trr-1*) and genes that did not influence the QL phenotype significantly. Possible functions of the remaining genes and their genetic interactions with *mtm-6* are discussed later.

To make a short introduction, I would like to propose a scheme for visualization of a potentional placement of tested genes that significantly influence the QL phenotype into the inner compartments of the Wnt producing cell. Considering published data, most genes could be divided into several groups that reflect possible functions of their protein products on the route plasma membrane-endosomes-GA-plasma membrane. (Fig. 20). Possible roles of some of these genes and their connection with Wnt signalling are described in the text below.

Except for those described in Fig. 20, there are three other genes for which it is difficult to estimate their involvement in Wnt signalling or place them into defined cellular compartments: *rpn-12*, *K04G7.1* and *tes-1*. RPN-12 is a proteasome subunit (Davy et al., 2001). An interesting result is that *rpn-12* exhibits increase of the penetrance of the QL phenotype in *vps-29* mutants (M. Macůrková, unpublished) but decrease of the penetrance of the QL phenotype in *mtm-6* mutants. According to its function, RPN-12 could be involved in protein turnover of some Wnt signalling regulators or even in degradation of β-catenin. *tes-1* and *K04G7.1* are discussed below because they were both tested also for genetic interactions with *cup-4* (*rpn-12* was not and therefore is not discussed further).

cogc-4 encodes a subunit of COG (conserved oligomeric Golgi) complex that is responsible for glycosylation in GA (Kubota et al., 2006). Within Wnt signalling, *cogc-4* would probably be involved in receiving vesicles transported from endosomes to GA (Miller and Ungar, 2012). *plx-2* encodes a transmembrane protein plexin, a receptor of *C. elegans* class 2 semaphorin MAB-20 (Nakao et al., 2007). It does not seem probable that *plx-2* would have a specific function in Wnt signalling, more likely

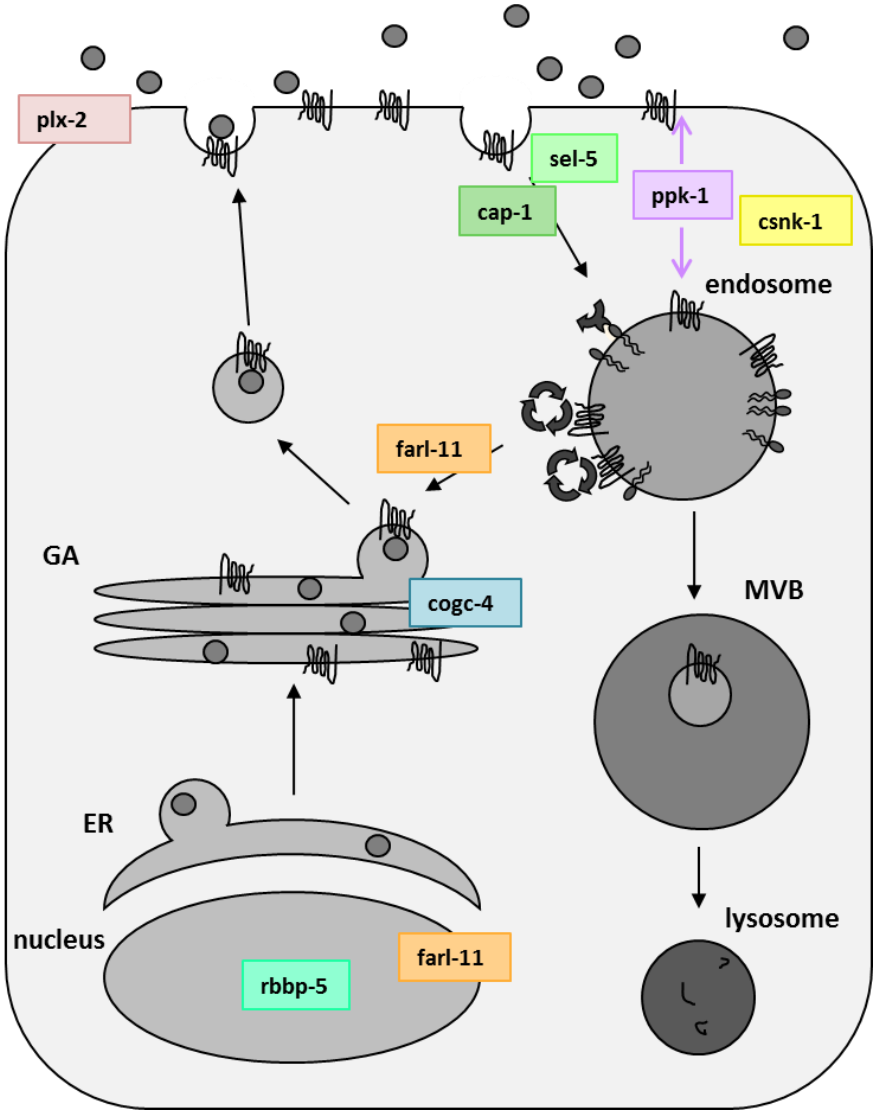


Fig. 20: **A potential placement of genes that significantly influence the QL phenotype into processes in Wnt producing cells.**

PLX-2 is a transmembrane protein (Nakao et al., 2007); CAP-1 is probably involved in endocytosis (Waddle et al., 1993); PPK-1 associates with both plasma membrane and endosomes (Weinkove et al., 2008); SEL-5 is involved in clathrin-associated trafficking and endocytosis (Fares and Greenwald, 1999); CSNK-1 is associated with plasma membrane (Panbianco et al., 2008); COGC-4 participates on glycosylation in GA (Kubota et al., 2006); FARL-11 is an inhibitor of dynein and exhibits a pronuclear localization (O'Rourke et al., 2007); rbbp-5 participates in H3K4 methylation (Li and Kelly, 2011).

plx-2 may affect neuronal migration in general. *cogc-4* and *plx-2* genes are not discussed later because their influence on the penetrance of the QL phenotype in *mtm-6* mutants is very low.

In order to prepare the ground for further discussion, I would like to introduce brief characteristics of all selected genes that were tested for genetic interactions with both *mtm-6* and *cup-4*:

• *cap-1* encodes CAP-1 protein, a homolog of mammalian CapZα subunit of actin capping protein (Waddle et al., 1993).

• *csnk-1* encodes casein kinase 1γ (CSNK-1), a Ser/Thr protein kinase (Davidson et al., 2005) which is associated with the plasma membrane during the whole cell cycle.

• *farl-11* encodes FAR11-like protein that exhibits pronuclear and nuclear localization and *in vivo* inhibits dynein heavy chain (DHC-1) (O'Rourke et al., 2007).

• The function of *K04G7.1* gene product has not been discovered yes, neither anything is known about ist homologs in other species. Nevertheless, according to a global analysis of genetic interactions, *K04G7.1* was found to interact with *bar-1*, the *C. elegans* gene coding β-catenin (Byrne et al., 2007). As the influence of K04G7.1 depletion on QL neurons migration is both high and significant, this genetic interaction could be relevant in terms of the regulation of Wnt signalling.

• *ppk-1* encodes phosphatidylinositol-4-phosphate 5' kinase (PPK-1), the only type I PIP kinase in *C. elegans* (Weinkove et al., 2008). PPK-1 was suggested to play an essential role in cytoskeleton organization, IP_3 signalling and ovulation (Xu et al., 2007).

• *rbbp-5* encodes a homolog of mammalian retinoblastoma binding protein 5 (RbBP5) which is a member of methyltransferase complexes.

• *sel-5* encodes two isoforms of SEL-5 protein. SEL-5 plays role in LIN-12/Notch signalling, probably before or during the release of the intracellular domain after binding of the ligand. (Fares and Greenwald, 1999; Tax et al., 1997).

• *tes-1* encodes an ortholog of human TESTIN and LMCD1. Human TES protein was shown to localise to actin stress fibres, cell-cell contact sites and focal adhesions (Coutts et al., 2003) which suggests that it is involved in events associated with cell adhesion and motility.

6.4. Genetic interactions of *mtm-6* and *cup-4*

From the initial set of genes I chose eight that seemed to be most relevant to the Wnt signalling pathway considering their genetic interactions with *mtm-6*. These genes were further tested for genetic interactions with *cup-4*. Here I would like to summarize results of the RNAi experiments and link them to already published experimental data from literature (*tes-1* and *K04G7.1* are not discussed because lack of information about their function). Information about gnetic interactions with *vps-29* were kindly provided by M. Macůrková (unpublished data).

In order to get more information about the genetic interaction between *mtm-6* and *sel-5*, I decided to cross *C. elegans* strains *mtm-6*(ok330);*muIs32* and *sel-5*(ok363) to create a strain with knock-out of both genes. Scoring of the QL phenotype in *mtm-6*; *sel-5* strain gave unexpected but considerably interesting results: in contrast to the experiment with *mtm-6* mutant animals treated with *sel-5* RNAi (increase of the QL phenotype), the penetrance of the QL phenotype in *mtm-6*;*sel-5* double mutant strain was considerably decreased (see chapter 5.3.4. Verification of RNAi experiment on *mtm-6;sel-5* double mutants). There are two main explanations for the different results that came from experiments with RNAi compared to experiments with double mutants. First, there could be off-target effects of the RNAi so the observed phenotype would then reflect knock-down of other genes than *sel-5*. Second, the ok363 allele might not act as a null allele and there is still a functional protein product in *sel-5* mutants. Additional search in literature has shown that the second option appears to be very probable.

The *sel-5* gene encodes two isoforms, SEL-5A and SEL-5B, which share N-terminal region but differ in C-terminus (Fares and Greenwald, 1999). At the very beginning of the N-terminus there is a kinase domain; the ok363 deletion starts downstream from the end of the kinase domain and therefore does not influence its amino acid sequence (Fig. 21). The start of the ok363 deletion is within intron 5 and the end is within exon 10; the mRNA transcript would very probably continue after exon 5 with exon 11 and even will be translated because the open reading frame is not disrupted by the ok363 deletion. What is really interesting is the possible consequence of this deletion. It is tempting to speculate that the ok363 deletion is within a regulation domain of the SEL-5 kinase. It is therefore possible (and according to our results it seems even very probable) that the ok363 deletion does not cause a

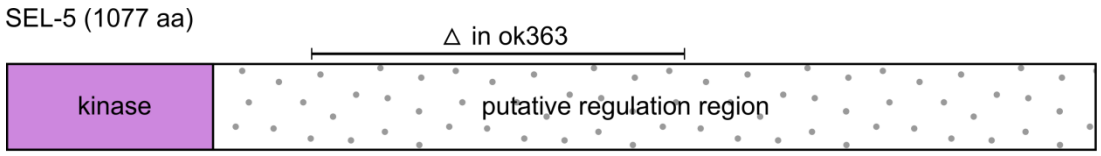


Fig. 21: **Domain composition of SEL-5.**

The scheme of SEL-5 domain composition is predicted after information available at www.wormbase.org. The ok363 deletion is present in *sel-5* mutant allele in *C. elegans* strains mutated in the *sel-5* gene.

(designed after www.wormbase.org and Fares and Greenwald, 1999)

gene knock-out but rather a disruption of the regulation domain and the result could be a permanently active SEL-5 kinase. To sum up, it seems that *sel-5* RNAi caused in *mtm-6* mutant strain a real knock-down of SEL-5 kinase whereas *sel-5*(ok363) deletion in *sel-5*;*mtm-6* double mutant strain caused a constitutively active SEL-5. It would be certainly worth to cross *mtm-6*(ok330) strain with another mutant strain that carry a null allele of the *sel-5* gene and compare the results with *sel-5* RNAi in *mtm-6* mutants.

SEL-5 is a Ser/Thr kinase homologous to mammalian AP2-associated protein kinase 1, AAK1. Not many information about SEL-5 have been explored yet, therefore I discuss its possible intracellular localization and function considering published data about its mammalian ortholog AAK1. AAK1 was found to phosphorylate AP2 µ2 subunit (Conner and Schmid, 2002; Ricotta et al., 2002) which enhances affinity of AP2 for sorting signals of cargo proteins (Ricotta et al., 2002) and is essential for clathrin-mediated endocytosis (Olusanya et al., 2001). The intracellular localization of AAK1 was shown to be mainly in association with membranes and AAK1 was also found to cofractionate with AP complexes and clathrin (Conner and Schmid, 2002). These findings strongly support the idea that AAK1 regulates AP and clathrin function.

Our results indicate that SEL-5 participates in regulation of Wnt signalling. There are several possibilities how SEL-5 could be involved in Wnt signalling regulation. Should SEL-5 phosphorylate AP-2 as does its ortholog AAK1 (Conner and Schmid, 2002; Olusanya et al., 2001; Ricotta et al., 2002), the function of SEL-5 would probably be in formation of CCVs budding from the the plasma membrane. This

possible function of SEL-5 is supported by the fact that Wls internalization from plasma membrane is dependent on AP-2 (Pan et al., 2008; Port et al., 2008).

Other two very interesting genes that should be discussed in more detail are *csnk-1* and *ppk-1*. As shown in previous text, *csnk-1* exhibits very strong and statistically significant genetic interaction in all genetic backgrounds tested – the penetrance of the QL phenotype was always strongly enhanced. On the other hand, knock-down of *ppk-1* by RNAi causes a significant decrease in the penetrance of the QL phenotype in *mtm-6* mutants, a negligible increase in *cup-4* mutants and an increase up to more than 90% in *vps-29* mutants.

CSNK-1 was suggested to be a negative regulator of PPK-1, which is supported by the discovery that loss of CSNK-1 by RNAi caused increased levels of PPK-1 and $PI(4,5)P_2$ (Panbianco et al., 2008). In vertebrates, members of casein kinase 1 family have important functions in Wnt signalling (Davidson et al., 2005; Del Valle-Pérez et al., 2011; Tanneberger et al., 2011). For example, CK1α is part of the destruction complex that routes β-catenin for degradation (Liu et al., 2002) and CK1γ phosphorylates the Wnt coreceptor LRP5/6 and thereby integrates the extracellular signal with the intracellular transducing machinery (Davidson et al., 2005; Zeng et al., 2005). Although there is no to date evidence that CK1γ homolog CSNK-1 has a role in Wnt signalling in *C. elegans*, there are several possibilities of CSNK-1 function in *C. elegans* Wnt signalling or its regulation.

First, CSNK-1 may have a very similar role as CK1γ in vertebrates, although this potentiality has not been supported yet by finding a gene encoding a homolog of LRP5/6 coreceptor in the *C. elegans* genome. Nevertheless, the possibility that a gene encoding LRP5/6-like coreceptor is present in *C. elegans* genome should not be ruled out because Wnt pathway genes of *C. elegans* could be quite diverged and it could be just a matter of time to find one.

Secondly, CSNK-1 as a Ser/Thr kinase (Davidson et al., 2005) may be involved in phosphorylation of some other components of Wnt signalling pathway. This is a very general hypothesis that would need further testing but should not be excluded considering the importance of CK1γ in vertebrates.

Third, the role of CSNK-1 could be in regulation of PPK-1 lipid kinase. The regulation of PPK-1 was described in *C. elegans* one-celled embryos where PPK-1 was

posteriorly enriched by CSNK-1 action which led through PI(4,5)P_2 signalling to posterior spindle displacement (Panbianco et al., 2008). This is probably not the case of influencing the Wnt signalling pathway but it shows a direct regulation between these two proteins. Considering the fact that in *mtm-6* mutant animals knock-down of *csnk-1* causes a dramatic increase of the QL phenotype (up to 92%) and knock-down of *ppk-1* causes a decrease of the QL phenotype (up to 26%), there appears a possibility for CSNK-1 to be a negative regulator of PPK-1. The rescue of the increased QL phenotype in *mtm-6* mutants by *ppk-1* knock-down indicates that both genes could regulate processes with opposite impact on Wnt signalling.

RNAi against *ppk-1* revealed unexpected but very interesting results. It was previously shown that *C. elegans* mutants in *cup-4* exhibit a significant reduction in PI(4,5)P_2 levels at the plasma membrane of coelomocytes (Patton et al., 2005). If PI(4,5)P_2 levels are important for proper Wnt signalling, one would expect that loss of *ppk-1* and loss of *cup-4* in combination with *mtm-6* should have similar consequences. However, this is not the case. In *mtm-6* mutants treated with *ppk-1* RNAi the penetrance of the QL phenotype is significantly decreased from 50 to 27%, so it seems that the influence of *mtm-6* knock-out is compensated by *ppk-1* knock-down. On the other hand, *cup-4*;*mtm-6* double mutants exhibit almost 100% penetrance of the QL phenotype (Silhankova et al., 2010). One possible explanation is that *ppk-1* acts in the same cells as *mtm-6* (i.e. Wnt producing cells) and the effects of these PI metabolizing enzymes cancel each other, while *cup-4* may act in different cells and the effects enhance each other. The second part is supported by the fact that *cup-4* seems to be expressed only in coelomocytes (Patton et al., 2005). Overall, these findings suggest that a controlled homeostasis of different phosphoinositides is crucial for proper Wnt signalling.

cap-1 knock-down by RNAi results in a great and statistically significant increase in the penetrance of the QL phenotype in *mtm-6*, *cup-4* and *vps-29* mutants. Except for the QL phenotype, an embryonic lethality was also observed in the RNAi experiments, which indicates either a strong genetic interaction between *cap-1* and those three genes or an importance of CAP-1 in essential cellular processes. As it is not possible to create a viable *cap-1* knock-out mutant, CAP-1 is suggested to be important for cell viability. Furthermore, animals carrying only the *muIs32* transgene also exhibited significant embryonic lethality when treated with *cap-1* RNAi. These

findings suggest that *cap-1* has an essential function within cell and the possibility that this function also influences Wnt signalling should not be ruled out.

CAP-1 is an ortholog of the human F-actin-capping protein subunit alpha (CapZα) (Waddle et al., 1993), which inhibits the addition and loss of actin subunits at the barbed ends of actin filaments (Pollard and Cooper, 2009). Interestingly, the CapZ proteins are also parts of the dynactin complex (Hodgkinson et al., 2005; Schafer et al., 1994) that is required for dynein activity (Kardon and Vale, 2009). Its function is therefore connected with both actin and microtubule cytoskeleton. Taken this into account together with the fact that *mtm-6* and *cup-4* mutant animals treated with *cap-1* RNAi had serious problems to hatch and survive early larval stages (visual observation during RNAi experiments), the role of CAP-1 may be more general in the context of the whole cell/animal rather than specific for Wnt signalling. Yet there are several possibilities of CAP-1 involvement in Wnt signalling, whether in Wnt producing or Wnt receiving cell. The steps most probable hit by loss of *cap-1* in the Wnt producing cell would be during internalization of Wls from the plasma membrane by clathrin-mediated endocytosis or during transport of vesicles carrying the Wls cargo. Nevertheless, the regulation of vesicular transport from endosomes to GA by CAP-1 would be very probably not specific for Wnt signalling. In the Wnt receiving cell, CAP-1 would probably play a role during internalization of Wnt/Frizzled receptor from the plasma membrane. All these events are essential for proper Wnt signalling.

FARL-11 protein was found to be a supressor of dynein heavy chain. FARL-11 has a pronuclear and nuclear localization in early *C. elegans* embryos (O'Rourke et al., 2007) but it is not clear what function could FARL-11 have in cell nucleus. FARL-11 is a highly conserved ortholog of *S.cerevisiae* Far11 which takes part in DNA damage response and regulation of autophagy in yeast (Lisa-Santamaría et al., 2012). *farl-11* knock-down by RNAi exhibits a very high and statistically significant increase of the QL phenotype in *mtm-6* and *cup-4* mutants. Strong genetic interactions with *mtm-6* and *cup-4* indicate involvement of FARL-11 in processes where MTM-6 and CUP-4 also take part. From the little information known about FARL-11 I would like to emphasize its negative influence on dynein heavy chain (O'Rourke et al., 2007). It has been shown that retromer-coated vesicles rely on dynein mediated transport on the way from endosomes to the GA and that this transport is mediated by interaction

between sorting nexins and the dynactin complex (Wassmer et al., 2009). FARL-11 could thus interfere with the recycling of Wls from endosomes. Importantly, unlike *cap-1*, loss of *farl-11* does not seem to cause any phenotype that would suggest a more general function in the cells which supports the idea that FARL-11 could be more specific regulator of Wnt signalling.

RBBP-5 is a *C. elegans* homolog of mammalian RbBP5, a component of Set1/MLL methyltransferase complex. In *C. elegans*, RBBP-5 activity is essential for H3K4 di- and tri-methylation in both the embryonic germline and adult GSCs (germ stem cells) (Li and Kelly, 2011). Though *rbbp-5* knock-down leads to a significant and relatively high increase of the QL phenotype in *mtm-6* and *cup-4* (and also in *vps-29*) mutants, it is very difficult to estimate its role in Wnt signalling because of limited information about this gene and its protein product function. Based on the RBBP-5 requirement in maitaining the H3K4me2 and H3K4me3 modifications, there is a possible role for RBBP-5 in *C. elegans* Wnt signalling. Considering the fact that H3K4me2 and H3K4me3 are epigenetic markers of transcriptionally active chromatin (summarized in Lachner et al., 2003) and that *rbbp-5* silencing negatively influences the Wnt signalling, RBBP-5 could be involved in transcription activation of some positive regulators of Wnt signalling.

rbbp-5 could also participate on more general mechanisms influencing left/right asymmetry of cell fate decision in *C. elegans* neurons. *rbbp-5* was identified as a regulator of left/right fate decision in ASE neurons where in *rbbp-5*(ot86) mutants ASEL neuron adopts ASER fate (Sarin et al., 2007). This situation is very similar to QL.d and QR.d neurons fate: according to my results from RNAi experiments (see chapter 5.3.2. Genetic interactions of *mtm-6*), in *mtm-6* mutant animals treated with *rbbp-5* RNAi the QL.d neuron migrates anteriorly same as the QR.d neuron (instead of migrating slightly posteriorly). Nevertheless, *rbbp-5* knock-down in wild type animals carrying only the *muIs32* transgene did not infuence the penetrance of the QL phenotype at all. Yet it would be worth to examine the influence of a real *rbbp-5* knock-out on Wnt signalling. It could also be very interesting to test *rbbp-5* for regulation of other morphologically symmetric cell pairs and find out whether it acts also in other left/right cell fate decisions.

In order to sum up the most important information discussed above, I would like to present a possible model of SEL-5, PPK-1 and CSNK-1 involvement in endocytosis within Wnt signalling (Fig. 22). In brief, PPK-1 (as the only PI(4)P 5-kinase in *C. elegans* (Weinkove et al., 2008)) would phosphorylate PI(4)P at the plasma membrane which would lead to enrichment of $PI(4,5)P_2$. The adaptor protein AP-2 is known to bind $PI(4,5)P_2$ and is essential for endocytosis of Wls (Pan et al., 2008; Port et al., 2008). Binding of AP-2 to $PI(4,5)P_2$ is required for early events in formation of coated vesicles stimulated by AP-2 (Jost et al., 1998). Simultaneously or after binding to $PI(4,5)P_2$, SEL-5 would phosphorylate μ2 subunit of AP-2 and thus support the affinity between AP-2 and cargo proteins destined for internalization. The role of CSNK-1 in this model would be as a negative regulator of PPK-1 (Panbianco et al., 2008). It was previously described that overexpression of PPK-1 leads to increase of $PI(4,5)P_2$ level (Weinkove et al., 2008) which could disrupt the integrity of $PI(4,5)P_2$ signalling or negatively influence binding of some target proteins. Therefore the idea is that CSNK-1 would be responsible for or at least take part in maintaining a homeostasis of $PI(4,5)P_2$ and other phosphoinositides at the plasma membrane through regulation of PPK-1 activity.

Finally, I would like to stress out that not all the information on which the model at Fig. 22 is based are results of my own experiments; the model summarizes my results, published data of other research groups and my own ideas and hypotheses based on information about the mammalian homologs of *C. elegans* proteins.

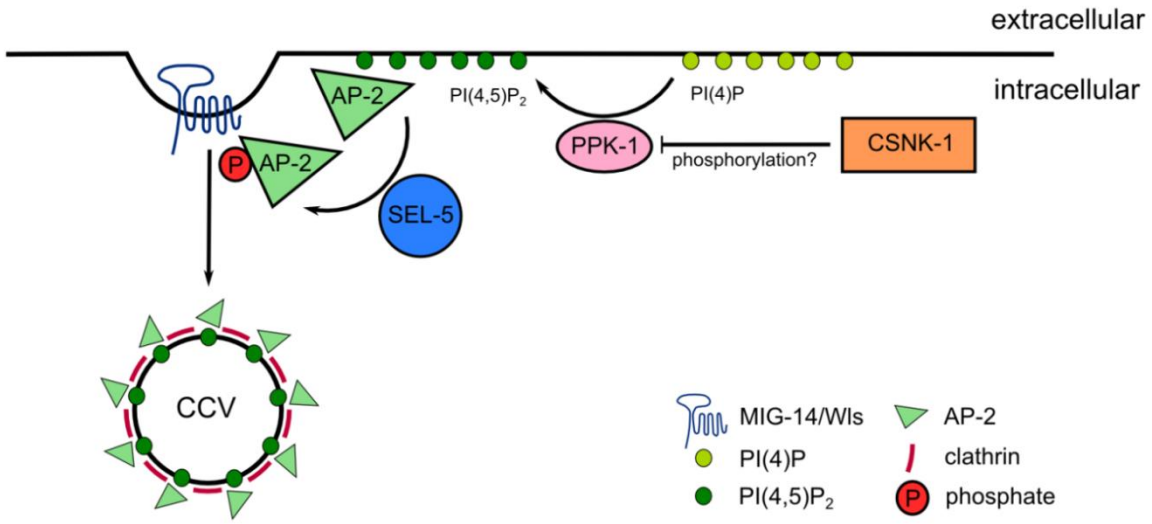


Fig. 22: **Model of SEL-5, PPK-1 and CSNK-1 function in endocytosis in Wnt producing cell.**

This model was designed based on information published about *C. elegans* genes/proteins or their mammalian homologs. The model shows a possible involvement of SEL-5, PPK-1 and CSNK-1 in endocytosis within Wnt signalling.

7. Conclusion

Within this diploma project, I searched for protein and genetic interactors of the *C. elegans* myotubularin-related lipid phosphatase MTM-6 in order to better understand its role in regulation of Wnt signalling. Using the Yeast Two-Hybrid Assay I tested a possible model of regulation of MTM-6 activity through interaction with PI3 kinase and Rab GTPases. I was not able to confirm the proposed model, partially maybe due to technical limitations of the Y2H technique. I only confirmed an already known interaction between MTM-6 and MTM-9. I further searched for novel binding partners of MTM-6 by screening the *C. elegans* Y2H cDNA library but unfortunately I was not succesful in recovering any positive hits .

Using the QL.d migration test as a read-out of Wnt signalling activity, I tested genetic interactions between *mtm-6* and several selected genes. Through the RNAi experiments I have discovered some strong genetic enhancers and suppressors of *mtm-6*. Although these genes are most likely not direct regulators of mtm-6, it apears that among the tested genes there are some very interesting putative regulators of Wnt signalling that would be certainly worth to explore in greater depth. To prepare grounds for future work I presented a possible model of involvement of some of the tested genes (namely *sel-5*, *ppk-1* and *csnk-1*) in endocytic processes in Wnt producing cell. This model could now be experimentally tested.

8. Literature

Bänziger, C., Soldini, D., Schütt, C., Zipperlen, P., Hausmann, G., Basler, K., 2006. Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. *Cell* 125, 509–522.

Bartscherer, K., Pelte, N., Ingelfinger, D., Boutros, M., 2006. Secretion of Wnt Ligands Requires Evi, a Conserved Transmembrane Protein. *Cell* 125, 523–533.

Belenkaya, T.Y., Wu, Y., Tang, X., Zhou, B., Cheng, L., Sharma, Y.V., Yan, D., Selva, E.M., Lin, X., 2008. The retromer complex influences Wnt secretion by recycling wntless from endosomes to the trans-Golgi network. *Dev Cell* 14, 120–131.

Berge, D. ten, Kurek, D., Blauwkamp, T., Koole, W., Maas, A., Eroglu, E., Siu, R.K., Nusse, R., 2011. Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells. *Nat Cell Biol* 13, 1070–1075.

Bonifacino, J.S., Glick, B.S., 2004. The mechanisms of vesicle budding and fusion. *Cell* 116, 153–166.

Bonifacino, J.S., Hurley, J.H., 2008. Retromer. *Curr Opin Cell Biol* 20, 427–436.

Brenner, S., 1974. The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71–94.

Burda, P., Padilla, S.M., Sarkar, S., Emr, S.D., 2002. Retromer function in endosome-to-Golgi retrograde transport is regulated by the yeast Vps34 PtdIns 3-kinase. *J Cell Sci* 115, 3889–3900.

Byrne, A.B., Weirauch, M.T., Wong, V., Koeva, M., Dixon, S.J., Stuart, J.M., Roy, P.J., 2007. A global analysis of genetic interactions in *Caenorhabditis elegans*. *J Biol* 6, 8.

Cao, C., Laporte, J., Backer, J.M., Wandinger-Ness, A., Stein, M.-P., 2007. Myotubularin lipid phosphatase binds the hVPS15/hVPS34 lipid kinase complex on endosomes. *Traffic* 8, 1052–1067.

Cao, C., Backer, J.M., Laporte, J., Bedrick, E.J., Wandinger-Ness, A., 2008. Sequential actions of myotubularin lipid phosphatases regulate endosomal PI(3)P and growth factor receptor trafficking. *Mol Biol Cell* 19, 3334–3346.

Carlton, J., Bujny, M., Peter, B.J., Oorschot, V.M.J., Rutherford, A., Mellor, H., Klumperman, J., McMahon, H.T., Cullen, P.J., 2004. Sorting nexin-1 mediates tubular endosome-to-TGN transport through coincidence sensing of high- curvature membranes and 3-phosphoinositides. *Curr Biol* 14, 1791–1800.

Clevers, H., 2006. Wnt/beta-catenin signaling in development and disease. *Cell* 127, 469–480.

Collins, B.M., 2008. The structure and function of the retromer protein complex. *Traffic* 9, 1811–1822.

Conner, S.D., Schmid, S.L., 2002. Identification of an adaptor-associated kinase, AAK1, as a regulator of clathrin-mediated endocytosis. *J Cell Biol* 156, 921–929.

Costa, M., Raich, W., Agbunag, C., Leung, B., Hardin, J., Priess, J.R., 1998. A putative catenin-cadherin system mediates morphogenesis of the *Caenorhabditis elegans* embryo. *J Cell Biol* 141, 297–308.

Coutts, A.S., MacKenzie, E., Griffith, E., Black, D.M., 2003. TES is a novel focal adhesion protein with a role in cell spreading. *J Cell Sci* 116, 897–906.

Cozier, G.E., Carlton, J., McGregor, A.H., Gleeson, P.A., Teasdale, R.D., Mellor, H., Cullen, P.J., 2002. The phox homology (PX) domain-dependent, 3-phosphoinositide-mediated association of sorting nexin-1 with an early sorting endosomal compartment is required for its ability to regulate epidermal growth factor receptor degradation. *J Biol Chem* 277, 48730–48736.

Dang, H., Li, Z., Skolnik, E.Y., Fares, H., 2004. Disease-related myotubularins function in endocytic traffic in *Caenorhabditis elegans*. *Mol Biol Cell* 15, 189–196.

Davidson, G., Wu, W., Shen, J., Bilic, J., Fenger, U., Stannek, P., Glinka, A., Niehrs, C., 2005. Casein kinase 1 γ couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* 438, 867–872.

Davy, A., Bello, P., Thierry-Mieg, N., Vaglio, P., Hitti, J., Doucette-Stamm, L., Thierry-Mieg, D., Reboul, J., Boulton, S., Walhout, A.J., Coux, O., Vidal, M., 2001. A protein-protein interaction map of the *Caenorhabditis elegans* 26S proteasome. *EMBO Rep* 2, 821–828.

Del Valle-Pérez, B., Arqués, O., Vinyoles, M., De Herreros, A.G., Duñach, M., 2011. Coordinated action of CK1 isoforms in canonical Wnt signaling. *Mol Cell Biol* 31, 2877–2888.

Doerks, T., Strauss, M., Brendel, M., Bork, P., 2000. GRAM, a novel domain in glucosyltransferases, myotubularins and other putative membrane-associated proteins. *Trends Biochem Sci* 25, 483–485.

Eisenmann, D.M., Maloof, J.N., Simske, J.S., Kenyon, C., Kim, S.K., 1998. The beta-catenin homolog BAR-1 and LET-60 Ras coordinately regulate the Hox gene lin-39 during *Caenorhabditis elegans* vulval development. *Development* 125, 3667–3680.

Fares, H., Greenwald, I., 1999. SEL-5, a serine/threonine kinase that facilitates lin-12 activity in *Caenorhabditis elegans*. *Genetics* 153, 1641–1654.

Franch-Marro, X., Wendler, F., Guidato, S., Griffith, J., Baena-Lopez, A., Itasaki, N., Maurice, M.M., Vincent, J.-P., 2008. Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex. *Nat Cell Biol* 10, 170–177.

Gasnereau, I., Herr, P., Chia, P.Z.C., Basler, K., Gleeson, P.A., 2011. Identification of an endocytosis motif in an intracellular loop of Wntless protein, essential for its recycling and the control of Wnt protein signaling. *J Biol Chem* 286, 43324–43333.

Goodman, R.M., Thombre, S., Firtina, Z., Gray, D., Betts, D., Roebuck, J., Spana, E.P., Selva, E.M., 2006. Sprinter: a novel transmembrane protein required for Wg secretion and signaling. *Development* 133, 4901–4911.

Greco, V., Hannus, M., Eaton, S., 2001. Argosomes: a potential vehicle for the spread of morphogens through epithelia. *Cell* 106, 633–645.

Gross, J.C., Chaudhary, V., Bartscherer, K., Boutros, M., 2012. Active Wnt proteins are secreted on exosomes. *Nat Cell Biol* 14, 1036–1045.

Harris, J., Honigberg, L., Robinson, N., Kenyon, C., 1996. Neuronal cell migration in *C. elegans*: regulation of Hox gene expression and cell position. *Development* 122, 3117–3131.

Harterink, M., Port, F., Lorenowicz, M.J., McGough, I.J., Silhankova, M., Betist, M.C., Van Weering, J.R.T., Van Heesbeen, R.G.H.P., Middelkoop, T.C., Basler, K., Cullen, P.J., Korswagen, H.C., 2011. A SNX3-dependent retromer pathway mediates retrograde transport of the Wnt sorting receptor Wntless and is required for Wnt secretion. *Nat Cell Biol* 13, 914–923.

Hodgkinson, J.L., Peters, C., Kuznetsov, S.A., Steffen, W., 2005. Three-dimensional reconstruction of the dynactin complex by single-particle image analysis. *Proc Natl Acad Sci USA* 102, 3667–3672.

Hofmann, K., 2000. A superfamily of membrane-bound O-acyltransferases with implications for wnt signaling. *Trends Biochem Sci* 25, 111–112.

Hunter, C.P., Harris, J.M., Maloof, J.N., Kenyon, C., 1999. Hox gene expression in a single *Caenorhabditis elegans* cell is regulated by a caudal homolog and intercellular signals that inhibit wnt signaling. *Development* 126, 805–814.

Ch'ng, Q., Williams, L., Lie, Y.S., Sym, M., Whangbo, J., Kenyon, C., 2003. Identification of Genes That Regulate a Left-Right Asymmetric Neuronal Migration in *Caenorhabditis elegans*. *Genetics* 164, 1355–1367.

Chen, M.-H., Li, Y.-J., Kawakami, T., Xu, S.-M., Chuang, P.-T., 2004. Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. *Genes Dev* 18, 641–659.

Chevray, P.M., Nathans, D., 1992. Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. *Proc Natl Acad Sci USA* 89, 5789–5793.

Jost, M., Simpson, F., Kavran, J.M., Lemmon, M.A., Schmid, S.L., 1998. Phosphatidylinositol-4,5-bisphosphate is required for endocytic coated vesicle formation. *Curr Biol* 8, 1399–1402.

Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K., Perrimon, N., 1996. The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. *Genes Dev* 10, 3116–3128.

Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D.P., Zipperlen, P., Ahringer, J., 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421, 231–237.

Kardon, J.R., Vale, R.D., 2009. Regulators of the cytoplasmic dynein motor. *Nat Rev Mol Cell Biol* 10, 854–865.

Kidd, A.R., 3rd, Miskowski, J.A., Siegfried, K.R., Sawa, H., Kimble, J., 2005. A beta-catenin identified by functional rather than sequence criteria and its role in Wnt/MAPK signaling. *Cell* 121, 761–772.

Kim, S.-A., Vacratsis, P.O., Firestein, R., Cleary, M.L., Dixon, J.E., 2003. Regulation of myotubularin-related (MTMR)2 phosphatidylinositol phosphatase by MTMR5, a catalytically inactive phosphatase. *Proc Natl Acad Sci USA* 100, 4492–4497.

Komekado, H., Yamamoto, H., Chiba, T., Kikuchi, A., 2007. Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a. *Genes Cells* 12, 521–534.

Kubota, Y., Sano, M., Goda, S., Suzuki, N., Nishiwaki, K., 2006. The conserved oligomeric Golgi complex acts in organ morphogenesis via glycosylation of an ADAM protease in *C. elegans*. *Development* 133, 263–273.

Kühl, S.J., Kühl, M., 2012. On the role of Wnt/β-catenin signaling in stem cells. *Biochem Biophys Acta*

Kurayoshi, M., Yamamoto, H., Izumi, S., Kikuchi, A., 2007. Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. *Biochem J* 402, 515–523.

Lachner, M., O'Sullivan, R.J., Jenuwein, T., 2003. An epigenetic road map for histone lysine methylation. *J Cell Sci* 116, 2117–2124.

Laporte, J., Blondeau, F., Buj-Bello, A., Tentler, D., Kretz, C., Dahl, N., Mandel, J.L., 1998. Characterization of the myotubularin dual specificity phosphatase gene family from yeast to human. *Hum Mol Genet* 7, 1703–1712.

Laporte, J., Blondeau, F., Buj-Bello, A., Mandel, J.L., 2001. The myotubularin family: from genetic disease to phosphoinositide metabolism. *Trends Genet* 17, 221–228.

Laporte, J., Bedez, F., Bolino, A., Mandel, J.-L., 2003. Myotubularins, a large disease-associated family of cooperating catalytically active and inactive phosphoinositides phosphatases. *Hum Mol Genet* 12 Spec No 2, R285–292.

Lauermann, V., 1991. Ethanol improves the transformation efficiency of intact yeast cells. *Curr Genet* 20, 1–3.

Li, T., Kelly, W.G., 2011. A Role for Set1/MLL-Related Components in Epigenetic Regulation of the *Caenorhabditis elegans* Germ Line. *PLoS Genet* 7.

Lin, X., 2004. Functions of heparan sulfate proteoglycans in cell signaling during development. *Development* 131, 6009–6021.

Lisa-Santamaría, P., Jiménez, A., Revuelta, J.L., 2012. The protein factor-arrest 11 (Far11) is essential for the toxicity of human caspase-10 in yeast and participates in the regulation of autophagy and the DNA damage signaling. *J Biol Chem* 287, 29636–29647.

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G.H., Tan, Y., Zhang, Z., Lin, X., He, X., 2002. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* 108, 837–847.

Logan, C.Y., Nusse, R., 2004. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 20, 781–810.

MacDonald, B.T., Tamai, K., He, X., 2009. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 17, 9–26.

Maloof, J.N., Whangbo, J., Harris, J.M., Jongeward, G.D., Kenyon, C., 1999. A Wnt signaling pathway controls hox gene expression and neuroblast migration in *C. elegans*. *Development* 126, 37–49.

Miller, J.R., 2002. The Wnts. *Genome Biol* 3, REVIEWS3001.

Miller, V.J., Ungar, D., 2012. Re'COG'nition at the Golgi. *Traffic* 13, 891–897.

Murray, J.T., Panaretou, C., Stenmark, H., Miaczynska, M., Backer, J.M., 2002. Role of Rab5 in the recruitment of hVps34/p150 to the early endosome. *Traffic* 3, 416–427.

Nakao, F., Hudson, M.L., Suzuki, M., Peckler, Z., Kurokawa, R., Liu, Z., Gengyo-Ando, K., Nukazuka, A., Fujii, T., Suto, F., Shibata, Y., Shioi, G., Fujisawa, H., Mitani, S., Chisholm, A.D., Takagi, S., 2007. The PLEXIN PLX-2 and the ephrin EFN-4 have distinct roles in MAB-20/Semaphorin 2A signaling in *Caenorhabditis elegans* morphogenesis. *Genetics* 176, 1591–1607.

O'Rourke, S.M., Dorfman, M.D., Carter, J.C., Bowerman, B., 2007. Dynein modifiers in *C. elegans*: light chains suppress conditional heavy chain mutants. *PLoS Genet* 3, e128.

Olusanya, O., Andrews, P.D., Swedlow, J.R., Smythe, E., 2001. Phosphorylation of threonine 156 of the mu2 subunit of the AP2 complex is essential for endocytosis in vitro and in vivo. *Curr Biol* 11, 896–900.

Pan, C.-L., Baum, P.D., Gu, M., Jorgensen, E.M., Clark, S.G., Garriga, G., 2008. *C. elegans* AP-2 and retromer control Wnt signaling by regulating mig-14/Wntless. *Dev Cell* 14, 132–139.

Panáková, D., Sprong, H., Marois, E., Thiele, C., Eaton, S., 2005. Lipoprotein particles are required for Hedgehog and Wingless signalling. *Nature* 435, 58–65.

Panbianco, C., Weinkove, D., Zanin, E., Jones, D., Divecha, N., Gotta, M., Ahringer, J., 2008. A Casein Kinase 1 and PAR Proteins Regulate Asymmetry of a PIP2 Synthesis Enzyme for Asymmetric Spindle Positioning. *Dev Cell* 15, 198–208.

Patton, A., Knuth, S., Schaheen, B., Dang, H., Greenwald, I., Fares, H., 2005. Endocytosis function of a ligand-gated ion channel homolog in *Caenorhabditis elegans*. *Curr Biol* 15, 1045–1050.

Pfeffer, S.R., 2007. Unsolved mysteries in membrane traffic. *Annu Rev Biochem* 76, 629–645.

Pollard, T.D., Cooper, J.A., 2009. Actin, a central player in cell shape and movement. *Science* 326, 1208–1212.

Port, F., Kuster, M., Herr, P., Furger, E., Bänziger, C., Hausmann, G., Basler, K., 2008. Wingless secretion promotes and requires retromer-dependent cycling of Wntless. *Nat Cell Biol* 10, 178–185.

Port, F., Basler, K., 2010. Wnt trafficking: new insights into Wnt maturation, secretion and spreading. *Traffic* 11, 1265–1271.

Reinke, S.N., Hu, X., Sykes, B.D., Lemire, B.D., 2010. *Caenorhabditis elegans* diet significantly affects metabolic profile, mitochondrial DNA levels, lifespan and brood size. *Mol Genet Metab* 100, 274–282.

Ricotta, D., Conner, S.D., Schmid, S.L., Von Figura, K., Honing, S., 2002. Phosphorylation of the AP2 mu subunit by AAK1 mediates high affinity binding to membrane protein sorting signals. *J Cell Biol* 156, 791–795.

Rocheleau, C.E., Downs, W.D., Lin, R., Wittmann, C., Bei, Y., Cha, Y.H., Ali, M., Priess, J.R., Mello, C.C., 1997. Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. *Cell* 90, 707–716.

Sarin, S., O'Meara, M.M., Flowers, E.B., Antonio, C., Poole, R.J., Didiano, D., Johnston, R.J., Jr, Chang, S., Narula, S., Hobert, O., 2007. Genetic screens for *Caenorhabditis elegans* mutants defective in left/right asymmetric neuronal fate specification. *Genetics* 176, 2109–2130.

Seaman, M.N., McCaffery, J.M., Emr, S.D., 1998. A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *J Cell Biol* 142, 665–681.

Seaman, M.N.J., 2005. Recycle your receptors with retromer. *Trends Cell Biol* 15, 68–75.

Schafer, D.A., Gill, S.R., Cooper, J.A., Heuser, J.E., Schroer, T.A., 1994. Ultrastructural analysis of the dynactin complex: an actin-related protein is a component of a filament that resembles F-actin. *J Cell Biol* 126, 403–412.

Schaletzky, J., Dove, S.K., Short, B., Lorenzo, O., Clague, M.J., Barr, F.A., 2003. Phosphatidylinositol-5-phosphate activation and conserved substrate specificity of the myotubularin phosphatidylinositol 3-phosphatases. *Curr Biol* 13, 504–509.

Silhankova, M., Korswagen, H.C., 2007. Migration of neuronal cells along the anterior-posterior body axis of *C. elegans*: Wnts are in control. *Curr Opin Genet Dev* 17, 320–325.

Silhankova, M., Port, F., Harterink, M., Basler, K., Korswagen, H.C., 2010. Wnt signalling requires MTM-6 and MTM-9 myotubularin lipid-phosphatase function in Wnt-producing cells. *EMBO J* 29, 4094–4105.

Stein, M.-P., Feng, Y., Cooper, K.L., Welford, A.M., Wandinger-Ness, A., 2003. Human VPS34 and p150 are Rab7 interacting partners. *Traffic* 4, 754–771.

Takada, R., Satomi, Y., Kurata, T., Ueno, N., Norioka, S., Kondoh, H., Takao, T., Takada, S., 2006. Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. *Dev Cell* 11, 791–801.

Tanaka, K., Kitagawa, Y., Kadowaki, T., 2002. *Drosophila* segment polarity gene product porcupine stimulates the posttranslational N-glycosylation of wingless in the endoplasmic reticulum. *J Biol Chem* 277, 12816–12823.

Tanneberger, K., Pfister, A.S., Brauburger, K., Schneikert, J., Hadjihannas, M.V., Kriz, V., Schulte, G., Bryja, V., Behrens, J., 2011. Amer1/WTX couples Wnt-induced formation of PtdIns(4,5)P2 to LRP6 phosphorylation. *EMBO J* 30, 1433–1443.

Tax, F.E., Thomas, J.H., Ferguson, E.L., Horvitz, H.R., 1997. Identification and characterization of genes that interact with lin-12 in *Caenorhabditis elegans*. *Genetics* 147, 1675–1695.

Taylor, G.S., Maehama, T., Dixon, J.E., 2000. Myotubularin, a protein tyrosine phosphatase mutated in myotubular myopathy, dephosphorylates the lipid second messenger, phosphatidylinositol 3-phosphate. *Proc Natl Acad Sci USA* 97, 8910–8915.

Thorpe, C.J., Schlesinger, A., Carter, J.C., Bowerman, B., 1997. Wnt signaling polarizes an early *C. elegans* blastomere to distinguish endoderm from mesoderm. *Cell* 90, 695–705.

Van Amerongen, R., Nusse, R., 2009. Towards an integrated view of Wnt signaling in development. *Development* 136, 3205–3214.

Waddle, J.A., Cooper, J.A., Waterston, R.H., 1993. The alpha and beta subunits of nematode actin capping protein function in yeast. *Mol Biol Cell* 4, 907–917.

Wassmer, T., Attar, N., Harterink, M., Van Weering, J.R.T., Traer, C.J., Oakley, J., Goud, B., Stephens, D.J., Verkade, P., Korswagen, H.C., Cullen, P.J., 2009. The retromer coat complex coordinates endosomal sorting and dynein-mediated transport, with carrier recognition by the trans-Golgi network. *Dev Cell* 17, 110–122.

Weinkove, D., Bastiani, M., Chessa, T.A.M., Joshi, D., Hauth, L., Cooke, F.T., Divecha, N., Schuske, K., 2008. Overexpression of PPK-1, the *Caenorhabditis elegans* Type I PIP kinase, inhibits growth cone collapse in the developing nervous system and causes axonal degeneration in adults. *Dev Biol* 313, 384–397.

Whangbo, J., Kenyon, C., 1999. A Wnt signaling system that specifies two patterns of cell migration in *C. elegans*. *Mol Cell* 4, 851–858.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., Nusse, R., 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423, 448–452.

Willert, K., Nusse, R., 2012. Wnt proteins. Cold Spring Harb Perspect Biol 4, a007864.

Wishart, M.J., Taylor‡, G.S., Slama, J.T., Dixon, J.E., 2001. PTEN and myotubularin phosphoinositide phosphatases: bringing bioinformatics to the lab bench. *Curr Opin Cell Biol* 13, 172–181.

Xu, X., Guo, H., Wycuff, D.L., Lee, M., 2007. Role of phosphatidylinositol-4-phosphate 5' kinase (ppk-1) in ovulation of *Caenorhabditis elegans*. *Exp Cell Res* 313, 2465–2475.

Xue, Y., Fares, H., Grant, B., Li, Z., Rose, A.M., Clark, S.G., Skolnik, E.Y., 2003. Genetic Analysis of the Myotubularin Family of Phosphatases in *Caenorhabditis elegans. J Biol Chem* 278, 34380 –34386.

Yang, P.-T., Lorenowicz, M.J., Silhankova, M., Coudreuse, D.Y.M., Betist, M.C., Korswagen, H.C., 2008. Wnt signaling requires retromer-dependent recycling of MIG-14/Wntless in Wnt-producing cells. *Dev Cell* 14, 140–147.

Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J., He, X., 2005. A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* 438, 873–877.

Zhong, Q., Lazar, C.S., Tronchère, H., Sato, T., Meerloo, T., Yeo, M., Songyang, Z., Emr, S.D., Gill, G.N., 2002. Endosomal localization and function of sorting nexin 1. *Proc Natl Acad Sci USA* 99, 6767–6772.

Zou, J., Zhang, C., Marjanovic, J., Kisseleva, M.V., Majerus, P.W., Wilson, M.P., 2012. Myotubularin-related protein (MTMR)9 determines the enzymatic activity, substrate specificity, and role in autophagy of MTMR8. *Proc Natl Acad Sci USA* 109, 9539–9544.